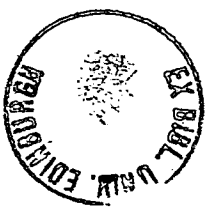
DETECTION OF MYCOPARASITES IN SOIL AND THEIR EFFECTS ON OTHER FUNGI

Deborah F. C. Mulligan

A Thesis presented for the
Degree of Doctor of Philosophy
University of Edinburgh
1993



DECLARATION

I declare that this thesis has been composed by myself, and that all the work herein is my own.

Deborah F. C. Mulligan

ACKNOWLEDGEMENTS

I would like to express my gratitude to Dr. Jim Deacon for his supervision during the last three years, especially in the last few months throughout the preparation of this thesis. I would also like to thank him for giving me the chance to experience his "Formula 1" driving. "*All the best*" Jim.

I would like to thank the Natural and Environmental Research Council for the studentship to undertake this work and for the conference in Canada; and the Institute of Cell and Molecular Biology, University of Edinburgh for the provision of research facilities. Also, Dr. Maurice Gallacher for his happy disposition.

I must also acknowledge the people that have kept me almost sane during the last three years: Eirian Jones (who has had to survive me from the beginning to end of this time, and I her!) and the others who have been in the lab at one time or another; Lorraine, Ulrike, Lisa, Steve and Stan (I still don't find the "eppendorf" joke funny), Alison, Rosie, Kithsiri, Paul, Ade (that boy needs a haircut!) and Dave. Also, Tony and Sally for supplying a home for the needy, Lynne, the people 'upstairs' who commonly refer to us as "Microbods" - Andy and Richard, my flatmates Jane and Tricia (and Kevin), and everybody else who has helped me or been there in the last three years. Now that I am leaving will the tea/coffee rota ever get organised? Will you ever have such interesting conversations about food? Will the Burn's Supper ever be the same?!

Finally, I would like to thank Gary but most of all I would like to acknowledge my family, especially my mum (yes I'm getting a 'real' job now!) and dad (Tuesday nights won't be the same), and my sisters Michelle and Lynsey, for their love, support and encouragement. This thesis I dedicate to them.

ABSTRACT

Soil samples were incubated on agar precolonised by different 'host' fungi for detection of presumptive mycoparasites. In a survey of 34 British soils, only *Pythium oligandrum, Gliocladium roseum* group, *Trichoderma* spp. and *Papulaspora* sp. were detected routinely, from 19, 34, 30 and 27 soils respectively; all soils contained more than one mycoparasite, 3 soils had two detectable types, 20 contained three types and 11 contained all four detectable types. The choice of host fungus strongly influenced the efficiency of detection of mycoparasites: *Fusarium culmorum* was best for *P. oligandrum*, *Rhizoctonia solani* for *Trichoderma* spp., and *Botrytis cinerea* for *Papulaspora* sp., but *G. roseum* (and *G. atrum* and *G. fimbriatum*) grew well from soil on colonies of *Trichoderma aureoviride, R. solani* or *B. cinerea*. The incidence of detection in replicate samples indicated that a range of host-colonised agar plates are needed to determine the spectrum of presumptive mycoparasites in a soil. Competition between these mycoparasites affected the success of detection because incorporation of metalaxyl into *B. cinerea*-colonised agar plates prevented growth by *P. oligandrum* and enhanced the growth of *Papulaspora* sp. from soil samples. Baiting of soils only partly increased the efficiencies of detection: for *Trichoderma* spp. when soil was baited with cellulose film precolonised by *Rhizoctonia oryzae*, and for *Papulaspora* sp. when the bait was cellulose film or mature, dried wheat leaves, precolonised by *Humicola grisea*.

Agar precolonised by *F. culmorum* was used to detect apparent changes in soil populations of *P. oligandrum*, using a most probable number method based on serial dilution of soil samples with sand. The addition of dried, mature wheat flag leaves or fresh, green grass leaves to soil enhanced the detectable populations for at least 280 days, although the effect of grass leaves was found only after a second supplement at 150 days. A metalaxyl-tolerant population of *P. oligandrum*, added to soil as oospores, was still detectable after 240 days

when soil samples were placed on *F. culmorum*-colonised agar containing metalaxyl.

In vitro studies in liquid culture, on agar and on cellulose film showed that *Papulaspora* sp. was non-cellulolytic, could not utilise nitrate and required vitamins, especially biotin and thiamine, for optimum growth. But there was no evidence that it parasitised other fungi or produced antibiotics. Instead, it seems to overgrow some (but not all) other fungi by sequestering low levels of nutrients and tolerating their metabolic by-products. The precolonised plate technique seems to select for these features rather than mycoparasitism *per se*.

In a detailed study of inter-hyphal interactions on films of water agar, recorded by videomicroscopy, *P. oligandrum* rapidly parasitised hyphae of *H. grisea* and a *Fusarium* sp. isolated from cellulose buried in soil. It also parasitised *R. oryzae*, which had been isolated in a similar manner from soil, but this host showed evidence of resistance: damage was confined to single host compartments, the host often regrew into damaged compartments from the delimiting septa, and several attempted penetration events seemed to be halted by papilla-like structures. The differences in host resistance were also evident in dual-membered culture on cellulosic substrata and in liquid media. Hyphal homogenates of one of the two tested isolates of *R. oryzae* poorly supported growth of *P. oligandrum*, indicative of the presence of growth inhibitors in the homogenates.

The findings are discussed in relation to the possible roles of mycoparasites in regulating the activities of other fungi in soil and in relation to their exploitation as potential biocontrol agents of plant pathogens.

TABLE OF CONTENTS

1. INTRODUCTION

1.1 Introduction

The natural soil ecosystem is complex, containing a diverse range of microorganisms which are affected by each other, and by an array of physical, chemical and nutritional factors. The interactions between microorganisms stabilise the environment and maintain the equilibrium of the soil. However this balance can be altered if the environmental conditions change, to favour the proliferation of unfavourable fungi such as fungal plant pathogens, or favourable ones such as mycorrhizal fungi. In modern cropping systems present-day constraints, especially on the use of chemicals in response to public awareness of possible ecological and health hazards (Bartle, 1991), coupled with an increase in resistance of pathogens to various chemicals, has led to a dramatic increase of interest in alternative management systems for controlling soil-borne pathogens. This includes the application of biological control.

More generally, there is a need to understand the microbial processes in soil, including the interactions of microorganisms, because these impinge on the maintenance of soil structure, recycling of nutrients, detoxification of xenobiotics and, perhaps in the future, containment of genetically modified organisms (Faull, 1988). Biological control (biocontrol) of plant pathogens in soil is only one facet of this topic, but a useful model one to study.

Biocontrol can be defined as the reduction of the propagule numbers of the pathogen, or of the disease-producing ability of those propagules, to a tolerable or economically acceptable level, due to the activities of biologically active organisms such as microbes (Papavizas & Lumsden, 1980). The requirements of a good biocontrol agent are that it is active in an environment conducive for the pathogen, that it is compatible with other agents, survives agricultural introduction, colonises the appropriate substratum or plant part and efficiently

suppresses the pathogen (Baker & Scher, 1987). It must also be able to survive in the long term, months rather than weeks, and endure changing environmental conditions such as moisture and nutrition (Faull, 1988).

Mycoparasites, i.e. fungi which parasitise other fungi, have been identified as having great potential to be exploited for biocontrol purposes (Lumsden, 1980; Baker, 1987; Whipps *et al.*, 1988; Deacon, 1991; Deacon & Berry, 1992). However, apart from limited glasshouse trials they have enjoyed little commercial success in this field despite much research (Adams, 1990). The problem seems to be that *in vitro* work and glasshouse and small-scale field experiments are inappropriate - their results cannot be extrapolated to the field on a commercial basis. In other words, the control agent does not work as effectively on a large scale as in model systems, is influenced by factors not previously considered, or it could work but the amounts of inoculum required for control on a large scale are economically impractical. Biocontrol agents are influenced profoundly by changes in environmental conditions. Also, the introduction of new microorganisms into an environment that they are not ecologically suited for prevents their establishment and thus effective control against pathogens. Allen *et al.* (1985) presented the following questions that should be asked in any biocontrol system.

1. Are the population interactions density dependent?
2. Is there a natural equilibrium and how stable is it?
3. Can the populations be altered to the needed levels?
4. What is the dispersion pattern of the species?
5. What are the environmental factors influencing the organisms and their interactions?

The relative neglect of mycoparasitic events in soil as opposed to *in vitro* was emphasized by Lumsden (1980) who realised that more work is required on the ecological aspects of mycoparasitism before mycoparasites can be manipulated

for effective biocontrol, and also before the extensive knowledge of interactions *in vitro* can be related to events in natural soils. In terms of the application of mycoparasitism for biocontrol, studies have to focus on basic questions such as, how significant is mycoparasitism as a mechanism of regulating the populations of other fungi in soil, and what potential is there for exploiting or manipulating this phenomenon?

Mycoparasitism has been reviewed in general (Lumsden, 1980; Papavizas & Lumsden, 1980; Baker, 1987; Burge, 1988; Lewis *et al.*, 1989; Deacon, 1991), so this section will not elaborate on all aspects of mycoparasitism *in vitro* and *in vivo*; instead it will focus primarily on the detection of mycoparasites and their activities in soil, with the purpose of relating the knowledge to achieve effective biocontrol. Thus, the following issues will be discussed, with particular reference to mycoparasitic *Pythium* spp. because one of these - *P. oligandrum* Drechsler - was a principal focus of study in this thesis.

(1) The types of mycoparasites, and the modes of action they exhibit in antagonising other fungi.
(2) The methods used to observe mycoparasitism directly in soil. This is necessary to determine if interactions found *in vitro* parallel those found *in vivo* because the effect of the soil environment might influence the extent of mycoparasitism.
(3) The techniques used to isolate and detect mycoparasites in soil, in order to assess their distribution and the effects of treatments on their population levels, with consideration of attempts to improve the efficiency of these methods.
(4) The effects of various environmental factors on populations of the mycoparasites and the mycohosts as assessed by isolation and detection methods.
(5) How the above relate to successful biocontrol, using suppressive soils as examples of the ability to exploit a natural soil phenomenon, and reports on biocontrol of plant diseases by inoculation of seeds with *P. oligandrum*.

1.2 Types of mycoparasites and their potential as biocontrol agents

Examples of mycoparasitism exist in all major groups of fungi (Lumsden, 1980). In essence, mycoparasitism involves the direct feeding of one fungus on another, that is, the parasitism of the mycohost by the mycoparasite. This mode of nutrition can be divided into two categories - biotrophy and necrotrophy - as recognised by Barnett & Binder (1973).

Biotrophic mycoparasites feed from the living host structures to obtain nutrients, while conferring no benefit in return. In some cases this type of relationship might have led to loss of ability in the mycoparasite to synthesise a certain nutrient and thus the fungus is dependent on the host for supply of nutrient(s). Moreover, it is not simply enough that the host fungus can supply the nutrients but that the mycoparasite also has appropriate means of obtaining them from the host.

The parasitising fungus, upon contact, can obtain nutrients through nutrient-absorbing cells ('buffer' cells), which contact the side of the hyphae with no penetration of the host; alternatively, the mycoparasite can form haustoria, which develop from a penetration peg inside the host, but without penetration of the host cell membrane; in yet other cases the mycoparasite can develop as a protoplast within the host hyphae, resulting in growth throughout the hyphae. The biotrophic relationship does not usually harm the host, although the host cytoplasm may disintegrate at a later stage in the association. This has been discussed in detail by Jeffries (1985).

Among examples of biotrophic mycoparasites are *Piptocephalis unispora* Benjamin and *Tieghemiomyces californica* Benjamin, which produce appressoria on contact with their hosts, *Cokeromyces recurvatus* Poitras and *Choanephora cucurbitarum* Berk. & Rav. respectively, and subsequently form penetration pegs and haustoria (Jeffries & Young, 1975, 1976; Jeffries, 1985).

Necrotrophic mycoparasites typically kill their hosts and feed on the dead host structures. The host cell may be destroyed by lysis, with the lysate serving as the food source, or the cytoplasm may coagulate before or upon penetration by the mycoparasite and then the parasite gains nutrients by growing through the host. Examples of necrotrophic mycoparasites include several *Pythium* spp. such as *P. oligandrum* and *P. acanthicum* Drechsler (Hoch & Fuller, 1977; Vesely, 1978; Lewis *et al.*, 1989; Laing & Deacon, 1990; Lodha & Webster, 1990), *Trichoderma* spp. (Boosalis, 1956; Dennis & Webster, 1971a,b,c; Chet, 1987) and *Gliocladium* spp. (Huang, 1978, Papavizas, 1985). Some mycoparasites - for example, *Trichoderma* spp. - produce powerful antibiotics and may operate by antibiosis to exclude or replace fungi that compete for similar nutrient sources, in which case they might not be considered true parasites but presumptive mycoparasites (Deacon & Berry, 1992). This term was used for fungi that overgrow other fungi on agar plates, or coil round their hyphae or produce antibiotics, but for which the precise role of parasitism (ie. nutrient acquisition from the fungal host) remains unknown.

Most biotrophic mycoparasites have limited value as applied biocontrol agents because they are difficult to grow in culture without their hosts (i.e. axenically), unless the nutrient that the host supplies is added to culture media. Even then the mycoparasite may grow slowly or be unable to utilise the major nutrients because of other, as yet undefined, factors. For example, it was believed that *Tieghemiomyces parasiticus* Benjamin was not able to metabolise glucose axenically, then it was found that the fungus could take up glucose if glycerol was added to the culture media (Jeffries, 1985). This carbon source appeared to increase the permeability of the membrane of the fungus, facilitating the uptake of glucose.

The necrotrophic mycoparasites are considered to be the best potential biocontrol agents, so research in relation to biocontrol has concentrated most

on these fungi. Their destructive modes of action make them strong antagonists. However, there is little information as to whether mycoparasitism actually occurs in soil because the most aggressive mycoparasites often have other attributes such as antibiosis or high competitive abilities that could equally confer success in the capture of nutrient sources. It is of course possible that these fungi use more than one mechanism to overcome other fungi. Indeed, lysis of host hyphae by parasites might be less common than is imagined, as hyphae have the ability to undergo autolysis in response to stress, as demonstrated by Lockwood (1960), Lloyd & Lockwood (1966) and Ko & Lockwood (1970).

1.2.1 The mycoparasitic Pythium species

Although there are many mycoparasites, those most commonly encountered and studied in soils are the mycoparasitic *Pythium* spp., *Gliocladium* spp., *Trichoderma* spp., *Coniothyrium minitans* Campbell and *Sporidesmium sclerotivorum* Uecker. The mycoparasitic *Pythium* spp. have received special attention in recent years, in terms of both their mode of action and their occurrence in soils (Drechsler, 1943; Deacon, 1976; Hoch & Fuller, 1977; Deacon & Henry, 1978; Al-Hamdani & Cooke, 1983; Elad *et al.*, 1985; Foley & Deacon, 1985, 1986a; Laing & Deacon, 1990, 1991; Ribeiro & Butler, 1992; White *et al.*, 1992). They have also been tested for, or implicated in, biocontrol of several pathogens (Vesely, 1977; Al-Hamdani *et al.*, 1983; Vesely & Hejdanek, 1984; Martin & Hancock, 1986, 1987; Paulitz & Baker, 1987a,b; Walther & Gindrat, 1987a; Lewis *et al.*, 1989; McQuilken *et al.*, 1990; Bradshaw-Smith *et al.*, 1991; Whipps & Lumsden, 1991; McQuilken *et al.*, 1992). So a case study of the mycoparasitic *Pythium* species is appropriate to illustrate the current status and directions of research on mycoparasites as potential biocontrol agents.

Mycoparasitic *Pythium* spp. are commonly found in soils, especially in those that also support the growth of plant-pathogenic *Pythium* spp. (Plaats-Niterink, 1975;

Deacon, 1976; Foley & Deacon, 1986b; Martin & Hancock, 1986). *P. oligandrum* was commonly isolated from British soils when these were plated on agar precolonised by a potential host fungus. It was obtained from 29% of a total 164 soil samples (Foley & Deacon, 1985). Using a more general method for isolating *Pythium* spp. from Dutch soils, Plaats-Niterink (1975) found that *P. oligandrum* was the second most common *Pythium* sp. - it represented 22% of all soil isolates of *Pythium* - whereas *P. sylvaticum* Campbell & Hendrix was the most common, found in 37% of samples. Six mycoparasitic *Pythium* spp. have been identified to date: *P. acanthicum, P. acanthophoron* Sideris, *P. mycoparasiticum* Deacon, Laing & Berry, *P. nunn* Lifshitz, Stanghellini & Baker, *P. oligandrum,* and *P. periplocum* Drechsler (Hoch & Fuller, 1977; Lifshitz *et al.*, 1984a; Foley & Deacon, 1986b; Lodha & Webster, 1990; Deacon, Laing & Berry, 1991; Ribeiro & Butler, 1992). Of these, *P. acanthophoron* has rarely been isolated, and until recently there was no information on its mycoparasitic mode of action (Lodha & Webster, 1990). Also, *P. nunn* has, to date, been isolated from only one soil, at a site (Nunn) in Colorado, U.S.A. (Lifshitz *et al.*, 1984a,c).

Foley & Deacon (1986b) investigated the physiological differences between mycoparasitic and plant-pathogenic *Pythium* spp. They found that four mycoparasitic species - *P. acanthicum, P. oligandrum, P. periplocum* and an unnamed species (later named *P. mycoparasiticum*) - required, in order to grow, an organic nitrogen source, thiamine (or its pyrimidine moiety) and sterols (for reproduction). Some mycoparasitic *Pythium* spp. can also use mannitol as the sole carbon source - a relatively unusual feature of *Pythium* spp. in general (Foley & Deacon, 1986b). However other workers have shown that the utilisation of mannitol only occurs in the presence of other nutrients such as calcium and cholesterol (Child *et al.*, 1969), or is dependent on the strain of the mycoparasite (E. E. Jones, pers. comm.). Also, Laing & Deacon (1990) demonstrated that *P. nunn* can use nitrate as the sole nitrogen source, unlike the other mycoparasitic pythia used for comparison (*P. oligandrum* and *P.*

mycoparasiticum) which required organic nitrogen for growth. It thus seems that the mycoparasitic *Pythium* spp. are not as similar in their nutrition as was first thought by Foley & Deacon (1986b), although most of them differ in nitrogen nutrition for the majority of plant-pathogenic *Pythium* spp.

P. oligandrum and *P. acanthicum* are aggressive mycoparasites and have wide host ranges (Haskins, 1963; Hoch & Fuller, 1977; Laing & Deacon, 1991). They are similar to *P. periplocum* in that they have echinulate oogonia, are physiologically alike and they parasitise host fungi with a similar mode of action. *P. nunn* and *P. mycoparasiticum* have more recently been isolated from soil; they grow more slowly than the other mycoparasitic *Pythium* spp. and have smooth-walled oogonia (Laing & Deacon, 1991; Lifshitz *et al.*, 1984a). The sixth mycoparasitic *Pythium* species, *P. acanthophoron*, has been isolated and studied from Indian soils and from a site at Dawlish, Devon (Lodha & Webster, 1990). It was obtained by plating soil onto agar inoculated with *Fusarium solani* (Martius) Saccardo. Ridings *et al.* (1969) showed *P. acanthophoron* to be self-sufficient for thiamine but it required organic nitrogen and sterols.

The parasitic modes of action of *P. nunn, P. oligandrum* and *P. mycoparasiticum* on a range of host fungi were investigated by Laing & Deacon (1991) using videomicrography. *P. oligandrum* was used as representative of the *Pythium* spp. with spiny oogonia while the other two represented the smooth-walled mycoparasites. All three mycoparasites were found to have similar modes of action against the host fungi tested: susceptible hosts stopped growth shortly after contact with the parasite, which caused lysis at the point of contact or coagulation/vacuolation of the host contents. Penetration occurred in just less than 50% of interactions. However Lifshitz *et al.* (1984a) discovered that the mode of attack depended on the host. Against *P. ultimum* Trow and *P. vexans* de Bary, the hyphae of *P. nunn* coiled around the host and caused lysis, while with *P. aphanidermatum* (Edson) Fitzpatrick and *Rhizoctonia solani* Kuhn the parasite formed appressorium-like structures. Coiling has also been observed

with *P. oligandrum* on *R. solani,* and appressorium formation was reported prior to host penetration (Walther & Gindrat, 1987b). Coiling is a common phenomenon of hyphal interactions involving mycoparasites or presumptive mycoparasites (Lifshitz *et al.*, 1984a; Inbar & Chet, 1992; Berry *et al.*, 1993). But there is debate as to whether it is a sign of resistance or vulnerability of the host fungus. Deacon (1976) believed that coiling by *P. oligandrum* was a sign of host resistance, because it occurred only on hyphae of hosts that were least affected by *P. oligandrum* in dual culture systems. Laing & Deacon (1991) ranked various host fungi in order of decreasing susceptibility to attack by *P. nunn*, in studies of interhyphal interactions on films of water agar. For example, *Fusarium oxysporum* Schlecht. was among the most susceptible and phytopathogenic *Pythium* spp. were among the least susceptible. This ranking order was broadly compatible with studies on the effects of *P. nunn* on fungal growth on cellulosic substrata (Laing & Deacon, 1990). In contrast, Elad *et al.* (1985) reported the opposite order of susceptibility to parasitism by *P. nunn*: other *Pythium* spp. were most susceptible whereas *F. oxysporum* was resistant. Laing & Deacon (1991) argued that this was because Elad *et al.* (1985) probably had used hyphal coiling as a criterion of susceptibility (or host range) - the criteria were not stated in their work.

It is notable that the earliest reports of mycoparasitism by *P. oligandrum*, *P. acanthicum* and *P. periplocum* were on hyphae of other *Pythium* spp. When Drechsler (1943) first isolated and described these mycoparasites he noted and illustrated many instances of hyphal coiling. Hoch & Fuller (1977) also observed coiling of *P. acanthicum* round the hyphae of other fungi. But in all such cases there was said to be little or no evidence of hyphal penetration from the coils. Most recently, Berry *et al.* (1993) resolved this point. They found that penetration of other *Pythium* spp. by *P. oligandrum* occurred after very extensive coiling of the mycoparasite, i.e. evidence of long-delayed penetration and host disruption, unlike the rapid (4 - 6 min) post-contact disruption of susceptible host fungi like *F. oxysporum*. Moreover, not all of the tested *Pythium* spp. were

susceptible to attack by *P. oligandrum*. In particular, *P. aphanidermatum* coiled round *P. oligandrum* and even disrupted the mycoparasite. So this study showed that even the distinction between mycoparasitic and plant-pathogenic *Pythium* spp. can be blurred: a phytopathogen could coil round, and penetrate, a mycoparasite on water agar. Similar evidence of non-specificity of parasitism is seen in the ability of the nematode-trapping fungus *Arthrobotrys oligospora* Fres. to form coils round, and damage, hyphae of other fungi such as *R. solani* (Persson *et al.*, 1990).

In a different context, the widely reported ability of *Trichoderma* spp. to coil round hyphae of other fungi was investigated by Dennis & Webster (1971c). These workers concluded that direct hyphal interactions were less important than antibiosis as a mode of antagonism by *Trichoderma* spp., and that coiling might represent a means of establishing close contact so that low levels of antibiotics could be more effective in antagonising other fungi. Whether or not hyphal coiling plays a significant role in mycoparasitism, it is evidence of a recognition event between a mycoparasite and other fungi. Barak & Chet (1990) studied such recognition by *Trichoderma* spp. and implicated lectin - sugar interactions in this phenomenon. Inbar & Chet (1992) recently coated nylon threads with concanavalin A or *Sclerotium rolfsii* lectin and found that hyphae of *Trichoderma harzianum* would coil round the threads. Related kinds of evidence were obtained by Elad *et al.* (1985) for *P. nunn*: *F. oxysporum* (resistant) was found to become susceptible to mycoparasitism when the host hyphae were treated with trypsin and/or potassium hydroxide, these treatments being thought to partially or completely remove a surface polysaccharide on the host hyphae that had blocked recognition of underlying host wall components by *P. nunn*.

Pythium oligandrum, with its widespread occurrence in soil, its wide host range and its aggressive mode of action, is seemingly an ideal fungus to develop for biocontrol (Deacon, 1976; Deacon & Henry, 1978; Al-Hamdani & Cooke, 1983;

Lutchmeah & Cooke, 1984; Vesely & Hejdanek, 1984; Foley & Deacon, 1986a; Bradshaw-Smith *et al.*, 1991). It can obtain its nutritional requirements from a host fungus, to the host's disadvantage. Tribe (1961) found it growing as a secondary colonizer on cellulose film in soil despite its lack of cellulolytic activity. It will grow on cellulose in association with several cellulolytic species of fungi in laboratory culture and produce spiny oogonia for which it needs sterols that must be supplied by the cellulolytic fungus. If the host is susceptible then *P. oligandrum* attacks the host and cellulolysis is reduced (Deacon, 1976; Lutchmeah & Cooke, 1983; Foley & Deacon, 1986a). In such systems *P. oligandrum* can be demonstrated to depend on other fungi for its organic carbon, nitrogen, vitamins and sterols (Foley & Deacon, 1986a) even though it grows readily on common labatory media - it is by no means an obligate parasite.

1.3 Techniques used for the observation of mycoparasitism directly in soil

Laing & Deacon (1990, 1991) conducted extensive work on mycoparasitic mechanisms of *Pythium oligandrum, P. nunn* and *P. mycoparasiticum*. They found these mycoparasites to differ significantly in their aggressiveness and parasitism towards a range of host fungi on films of water agar when assessed by means of video microscopy. The various morphological events reported in that study in conditions of nutrient deficiency such as might occur in soil could provide a starting point for seeking evidence of parasitism in soil. However the conditions in soil would be infinitely more complex and potentially variable than *in vitro*.

Clear evidence of mycoparasitism in soil is most easily obtained for biotrophic fungi because these will almost certainly proliferate only when they have an appropriate host fungus to infect. Hunter *et al.* (1977) examined the effects of various parameters on parasitism by *Syncephalis californica* Hunter & Butler on *Rhizopus oryzae* Went & Prinsen Geerligs, both *in vitro* and in soil. Discs cut from mycelial mats of *R. oryzae* were placed in soil to observe parasitism.

Parasitism was considered to occur when, upon infection of the host, large swellings developed within its hyphae, and, in turn, the number of host sporangia that formed decreased significantly. It was concluded that *S. californica* was an aggressive biotrophic mycoparasite, effective in parasitising *R. oryzae* under a wide range of natural conditions.

Sneh *et al.* (1977) also demonstrated parasitism in soil - in this case involving the intracellular mycoparasite *Hyphochytrium catenoides* Karling among others. The results showed that oospores of *Phytophthora megasperma* (Drechsler) var. *sojae* Hildb. become infected and subsequently contain resting spores of the parasite when buried in soil. The method of this study involved burial of spore-coated membrane filters in soil, covered with nylon net for ease of recovery. After appropriate incubation, the filters were recovered, placed on the lids of Petri dishes filled with crushed ice, and cooled water agar was poured onto the membranes to facilitate sectioning and subsequent microscopical examination. By using water agar as an adhering surface for the oospores from the membrane filter, the use of chemicals to make the filter transparent could be avoided; chemical treatment can distort or kill the fungal propagules. Sneh *et al.* (1977) reported that this method allowed observation of propagules from soil without obstruction by particles of soil.

Mycoparasitism was shown to occur on solid supports in soil in both of the studies above - Sneh *et al.* (1977) utilised polycarbonate membrane filters and Hunter *et al.* (1977) used disks cut from mycelial mats of *Rhizopus oryzae.* These direct methods of observation are among many that have been tested, not just to demonstrate mycoparasitism but also to study germination and hyphal lysis of introduced fungal structures (Adams, 1967; Lumsden, 1981).

The use of nylon fabric as a solid support was favoured by Lumsden (1981) as nylon net is biologically inert and discontinuous so allowing free movement of gases and water, and it also allowed roots and soil animals to come into close

contact with fungal propagules applied to the fabric. He used it to study the behaviour of oospores of *Pythium aphanidermatum* in soil and also to examine fungal structures, such as oospores, sclerotia and conidia, of a range of other soil fungi after retrieval from the soil. Lumsden (1981) found that the frequency of recovery of added oospores of *P. aphanidermatum* from soil was over 90%, and similar results were obtained for other fungi. He concluded that the method provided a suitable, adaptable and dependable means for studying the ecology of soilborne pathogens. The technique was later employed by Keinath *et al.* (1991) to investigate parasitism of *Verticillium dahliae* Kleb. by *Gliocladium roseum* Bainier in soil.

Other methods that have been used to retrieve fungi from soil and thereby to study microbial interactions involve the use of agar (Chesters, 1948), membrane filters (Adams, 1967), cellophane (Tribe, 1961), glass slides coated in agar (Johnson & Arroyo, 1983), glass fibre (Tsao, 1970) and glass fibre tape (Barton, 1958). In no case is the technique wholly natural or free of possible interpretational problems, especially when the supporting material is biodegradable; nevertheless the use of solid supports is normally required for effective recovery of fungal structures that are added to soil, especially when the structures can lose viability and thus not be recoverable by methods based on viable counts.

Soil smearing has also been employed to examine fungal propagules in soil (Tsao, 1970; Stanghellini & Burr, 1973; Ayers & Lumsden, 1977). The method involves suspending soil in a small amount of water on a microscope slide, and smearing it across the surface. Fungal propagules can be observed but the method causes disruption of the natural position of the propagules in soil and thus is not applicable if the undisturbed structures have to be examined.

Another approach to studying fungi and their activities in soil is the use of vital fluorochromes - dyes which fluoresce when excited by ultra-violet or other

short-wave radiation. They are absorbed by cell organelles or bind to specific residues inside or on the cell, and are non-toxic at appropriately low levels (Butt *et al.*, 1989). The advantage of using them to observe fungi and their interactions in soil is that fluorochromes enable the cells to be visualised against opaque backgrounds, using incident fluorescence microscopy.

Tsao (1970) used Calcofluor White M2R New to label mycelia and various sporing structures of *Phytophthora* spp. in order to investigate the saprophytic behaviour of this fungus in soil. Labelled propagules were introduced into soil and retrieved at time intervals via a soil-smear technique. The fluorescent brightener was found to be easily taken up by the fungus, was very stable once bound and was transported into new growth. Tsao found that the method was applicable for detection of germinating spores and formation of new mycelia, zoospores, sporangia and other structures. He concluded that it was a suitable procedure with a diversity of applications for studying microbial activity in soil.

Others have also studied the activities of *Phytophthora* spp. in soil using calcofluor (Mehrotra, 1972), fluorescent antibody stains (Malajczuk *et al.*, 1978; Cohen & Lockwood, 1981) and a range of other dyes (Cohen, 1984). In the latter case fluorescein diacetate and tetrazolium bromide were considered to be the most successful of eight dyes tested because they indicated viability of the stained cells.

Calcofluor White M2R New has also been used to compare germination of *Fusarium oxysporum* in suppressive and conducive soils; Scher & Baker (1983) observed the fungus to germinate less in suppressive soils. Similarly Soderstrom (1977) used fluorescein diacetate (FDA) to view metabolically active hyphae in soil. FDA does not fluoresce until it is cleaved by esterase, an enzyme active in living hyphae, and so only viable hyphae will show fluoresence.

In vitro, Calcofluor White M2R New has been used to study hyphal interactions between the mycoparasite *Pythiun nunn* and fungal plant pathogens (Elad *et al.*, 1985). In this case the work was done in laboratory media but an interesting observation was that hyphae of susceptible fungi showed most intense fluorescence at points where *P. nunn* attacked them, and this was attributed to the localised lysis (enzymatic dissolution) of host cell walls, exposing more ß-sites on the linked wall polymers that calcofluor is reported to bind to (Elad *et al.*, 1985). It should be possible to extend such work to studying interactions in soil, especially since observation of the germination of fungal spores and formation of other structures in soil has been shown to be possible (Tsao, 1970).

A wide range of fluorochromes is now available for staining fungal walls or specific internal components (Butt *et al.*, 1989; A. Stewart, in press). Some of them have been found to be translocated into new fungal growth on agar when loaded into hyphal fragments or spores. Examples are given in Table 1. - from A. Stewart (pers. comm.). They might also be used in soil.

1.4 Methods of detecting and assessing distribution of mycoparasites in soil

Mycoparasitism may be shown to occur in soil but reliable and efficient isolation and detection methods are also crucial in order to assess the distribution of the mycoparasites, and to measure changes in the populations when environmental factors are varied.

1.4.1 Isolation and detection of mycoparasites from soil

Most techniques used for the isolation and detection of mycoparasites from soil involve species-selective media (Mircetich, 1971; Papavizas & Lumsden, 1982; Dick & Ali-Shtayeh, 1986; Park *et al.*, 1992; White *et al.*, 1993). Detection and isolation is found to be improved further by employing baiting or enrichment. In some instances mycoparasites are not detected in soil at all unless baiting or enrichment is used. For example, baiting with mycelia of *R. solani* is found to be

Table 1. Reported staining specificities and spectral properties of fluorochromes (from A. Stewart, pers. comm.).

Stain*	Specificity	Absorption	Emission	Colour
Cellufluor	B glycans	340-400	400-440	blue
Nile Red	neutral lipids	450-500	520-560	yellow/gold
FDA	esterases	490-510	515-535	green
CFDA	esterases	490-510	515-535	green
CMFDA	glutathione-S -transferase	490-510	515-535	green
CMAC	glutathione-S -transferase	354	469	blue
DiI	membranes	550	565	red
DiO	membranes	484	501	green

* FDA= fluorescein diacetate; CFDA = carboxyfluorescein diacetate; CMFDA = chloromethylfluorescein diacetate; CMAC = aminochloromethyl coumarin; DiI = $DiIC_{18}(3)$; DiO = $DiOC_{18}(3)$

consistently the best method of detecting *Verticillium biguttatum* Gams & van Zaayen in potato fields (Boogert & Jager, 1983).

Foley & Deacon (1985) used a direct plating method, without baiting, whereby soil was placed on potato-dextrose agar precolonised by *Phialophora* sp. to isolate *Pythium oligandrum* and other necrotrophic mycoparasites from soil. Deacon & Henry (1978) had earlier found that this method detected mycoparasitic *Pythium* in nine out of sixteen soils, and the mycoparasites could subsequently be isolated easily into pure culture because they grew faster than *Phialophora*. *Gliocladium* spp., *Trichoderma* spp., *Papulaspora* sp. and an *Acremonium* stage were also observed on the precolonised plates. Foley & Deacon (1985) then extended this work to compare direct plating and the use of virgin agar medium (Schmitthenner's (1962) basal synthetic medium) containing selective antimicrobial components. All combinations of supplements in this medium were found to be inferior to the use of precolonised plates for detection of *P. oligandrum* and *P. mycoparasiticum*. Using *Phialophora*-precolonised plates, they detected mycoparasites of one type or another in 84% of samples of soil and other natural materials - water samples, organic matter, etc. - from around Britian. The results also revealed the occurrence of different mycoparasites according to the characteristics of the soil samples or vegetation that the soils supported. This method appears to be a highly selective technique to detect and isolate mycoparasites, especially *P. oligandrum*, and to determine their distributions. Lodha & Webster (1990) detected and isolated *P. acanthophoron* in a similar way using agar inoculated with *Fusarium solani*; Boogert & Jager (1983) similarly isolated *Verticillium biguttatum* by plating onto agar precolonised by *Rhizoctonia solani*. Both of these mycoparasites are rarely isolated by other methods, if at all.

Other techniques have also been tested for the isolation of pythia from soils. Barton (1958), for example, used live seedlings as baits for plant pathogenic *Pythium* spp. and assessed their activities in various soils. Ribeiro & Butler

(1992) buried sclerotia of *Sclerotinia sclerotiorum* (Lib.) de Bary in soil to isolate mycoparasitic pythia. Sandys-Winsch *et al.* (1993) used baiting and precolonised agar to assess the world distribution of the mycoparasite *Coniothyrium minitans* - a sclerotial mycoparasite. For more than 380 soil samples Sandys-Winsch *et al.* (1993) found that plating of soil crumbs on PDA precolonised by the host fungus *Sclerotinia sclerotiorum* yielded only 0.2% positive samples, whereas baiting of soils with sclerotia then dissection of these and plating onto PDA with antibacterial antibiotics yielded 10.2% positive samples. Such values can be compared with the detection of mycoparasitic *Pythium* spp. in 29% of British soil samples which were placed as crumbs on agar precolonised by *Phialophora* sp. (Foley & Deacon, 1985). Although these two studies are not directly equivalent, it seems that a very common mycoparasite of broad host range, such as *P. oligandrum*, can be detected readily without baiting, whereas more host-specific mycoparasites like *Coniothyrium minitans* and *Verticillium biguttatum* may require baiting in order to assess their occurrence and distribution.

Baiting has also been used to isolate other mycoparasites. Huber *et al* (1966) studied the mechanisms of biocontrol in a bean root rot soil; they used the "plate profile technique" (Andersen & Huber, 1965) for the isolation of microorganisms active in biocontrol of *Fusarium* spp. and *Rhizoctonia solani.* The technique involved placing 'profile' plates in the soil two months after the beans *(Phaseolus vulgaris)* have been planted. These plates were made from plastic and contained small holes that had been filled with agar and covered in tape, which was punctured with a pin before the plates were placed in soil. Adams & Ayers (1981) also used baiting with sclerotia to isolate *Sporidesmium sclerotivorum* which invades the sclerotia of *Sclerotinia minor.*

Enrichment is employed to augment the populations of plant pathogens that are hosts to mycoparasites in soil thus increasing the mycoparasite populations and making detection and isolation easier. It has been used successfully for the isolation of *Verticillium biguttatum* from soil (Boogert & Jager, 1983). In this

case the addition of live mycelia of *Rhizoctonia* to soil enriched for populations of the mycoparasite. Similarly, Liu & Baker (1980) isolated *Trichoderma harzianum* from buried hyphal mats of *R. solani*.

Not only do baiting techniques and enrichment increase the frequency of detection and isolation of mycoparasites, but these methods have also been employed for other purposes, such as to facilitate observation of parasitism in soil. For example, Ayers & Lumsden (1977) studied the mycoparasitism of *Pythium* spp. by *Hyphochytrium catenoides* by enriching the soil with 500 000 oospores of *Pythium* per gram of soil. This high amount of oospores increased the opportunities for infection and epidemic spread of the mycoparasite, and allowed easier microscopic observation of parasitism.

Enrichment has also been used to change conducive soils to suppressive soils. This was shown by Liu & Baker (1980), who added *Trichoderma harzianum* to conducive soils containing *Rhizoctonia solani*, at the level that *Trichoderma* was found in suppressive soils, and found that suppressiveness of *R. solani* was achieved. Enrichment of soil with fungi is discussed later in more detail within the subject of soil suppressiveness. However the subject of soil suppressiveness highlights the need to have reliable methods not only for assessing the distributions of mycoparasites but also for measuring the populations of mycoparasites and the mycohosts in soil, before and after various treatments.

1.4.2 Measurement of populations of mycoparasites and other fungi in soil

Assessment of populations allows us to determine the effect of treatments on fungi and their interactions. Several methods have been employed for this, depending on the fungi (Adams, 1979).

The most common method, undoubtedly, is the use of species-specific isolation media, used with dilution plating and/or the Most Probable Number method. Populations are determined by counting characteristic structures or examining

mycelial growth of the fungi. Many different selective media have been investigated and used for population estimates of plant pathogenic and mycoparasitic pythia (Mircetich, 1971; Martin & Hancock, 1986; Paulitz & Baker, 1987a; White *et al.*, 1993). In addition, media have been developed for other mycoparasites such as *Gliocladium* spp. (Park *et al.*, 1992) and *Trichoderma* spp. (Papavizas & Lumsden, 1982).

A slightly different approach was utilised by Len & Dick (1986) who used a soil fractionation technique to measure the population densities of *Pythium* and *Aplanopsis* spp. This procedure consisted of diluting sieved soil, mixing it with cornmeal agar and then adding hempseed to small blocks of the agar to favour the oomycetes and record their incidence. The results were comparable with previous studies which employed other methods (Dick, 1966; Dick & Ali-shtayeh, 1986).

Other techniques, for example for plant pathogens, include using a dilution series of soil to infect plants and using the incidence of infection to calculate the Most Probable Number of propagules (Maloy & Alexander, 1958). Alternatively, the population of *P. oligandrum* was detected by Foley & Deacon (1985) who diluted dry sieved soil samples with dry sterile sand in a twofold dilution series, then added equal aliquots to agar plates precolonised by *Phialophora*. The development of the spiny oogonia of *Pythium oligandrum* on these plates indicated a positive identification. The method was validated by using suspensions of known numbers of oospores of *P. oligandrum* and was found to have a detection efficiency of 48-60%. Taking into account the percentage of oospores that germinated in pure culture, owing to the constitutive dormancy of part of the oospore population, the efficiency of detection of germinable oospores in soil was actually 71-88%.

Germinability must be taken into account when estimating populations. Maloy & Alexander (1958) studied two plant pathogens, *Fusarium solani* and

Thielaviopsis basicola (Berk. & Broome) Ferraris, and commented that a direct microscopic count, a plate count and a MPN method gives values for different factors - the total number of propagative units, the number of viable units and an estimate of the infective population, respectively. Estimates of the numbers of propagules per gram of soil can be difficult to interpret unless the nature of the propagules is known (Burr & Stanghellini, 1973). Nevertheless, for fungi, the propagules of which usually germinate readily in appropriate conditions, an estimate of population density can be used to follow seasonal variation and the effects of experimental treatments on components of the fungal community.

A different approach was used by Pearson *et al.* (1991) who devised a model for short-term fluctuations of *Pythium* propagules in soil. This model describes the time-dependent process of zoospore production and provides a tool by which the changes in the population of zoospores can be predicted when selective conditions are applied. They suggested that the model could be used to compare the difference in population structures of pathogens and saprophytes.

Indeed, the use of models and statistical treatments to evaluate populations is becoming increasingly common. Eastburn & Butler (1988a,b) used multiple logistic regression analysis to assess the factors influencing the distribution of *Trichoderma harzianum* Rifai in soil, Rouse & Baker (1978) used an inoculum density-disease incidence (ID-DI) curve to determine the effect of organic amendments on *Rhizoctonia* pre-emergence damping-off of radish and Fravel *et al.* (1992) studied the effects of *Sporidesmium sclerotivorum* as a biocontrol agent against *Sclerotinia minor* Jagger using disease progress curves.

1.4.3 The use of marked fungal strains

If a successful isolation method is obtained for a particular fungus then the technique can be taken advantage of by labelling the fungus with a marker in order to assess the effect of a mycoparasite that is added to soil. This involves mutating the fungus, by chemicals, ultra violet light or recombinant genetic

methods, and selecting for resistance to fungicides or antibiotics, etc. The activity of this marked strain can then be followed in soil because it can be identified separately from the indigenous population by modifying the selective isolation technique to select for mutated strains.

Stack & Millar (1985) produced metalaxyl-tolerant strains of *Phytophthora megasperma*, which did not differ in morphology and growth from the wild type, and traced their activities and survival in soil. This allowed, using selective media, easy recovery of the fungus from soil and separation from the wild indigenous type, and also eliminated interference from *Pythium* spp. due to incorporation of the fungicide into the selective plates.

It should also be noted that mutation has been used for other fungi, such as *Trichoderma* spp., to develop tolerance to fungicides in order to improve their rhizosphere competence (Ahmad & Baker, 1987a, 1987b, 1988a, 1988b) and their activity as biocontrol agents (Papavizas *et al.*, 1982; Zhou & Reeleder, 1990; Graeme-Cook & Faull, 1991). The common finding in these reports is that selection for fungicide tolerance sometimes resulted in a coincidental increase in fitness or activity even in the absence of the fungicide. The reason has not been explained but it indicates that caution is necessary in using mutants for population studies - the mutants may not necessary behave similarly to wild-type indigenous strains of a fungus.

1.5 Ecology of mycoparasites: factors affecting their activities in soil

Development of successful methods for isolation and detection of mycoparasites and their hosts from soil allows the effects of the environment on the fungal populations to be assessed. Populations of plant pathogens and their antagonists, and the interactions between them, are influenced by internal and external factors. The former include the inherent variability between strains of the organisms, whereas the latter include abiotic and biotic factors, such as nutrient availability, pH, temperature, moisture availability, aeration, soil

mineralogy and the activities of other microorganisms. Evidence relating to some of these external factors in mycoparasitism is discussed below.

1.5.1 Nutrition

In *in vitro* research Whipps (1987a) found significant differences in hyphal interactions between mycoparasites and their fungal hosts when tested on different agar media. The most obvious differences related to the size of zones of inhibition as a result of the production of diffusible inhibitors by the mycoparasites. Such zones were larger on the nutritionally richer media but it has yet to be demonstrated that antibiosis is part of a true parasitic process involving nutrient transfer from one fungus to another (Deacon & Berry, 1992).

Host fungi can also differ in resistance depending on their nutritional status or history. For example, Merriman (1976) found that sclerotia of the pathogen *Sclerotinia sclerotiorum* from infected lettuce supported a higher incidence of infection from *Fusarium* spp., *Mucor* spp. and *Trichoderma* spp. than did sclerotia formed on sterilised potato tissue. Boosalis (1956) had earlier found that parasitism of *Rhizoctonia solani* by *Penicillium vermiculatum* Pitt (= *Talaromyces flavus*) was markedly dependent on the carbohydrate component and the C:N ratio of the medium on which the interactions occurred.

In vivo, various organic amendments have been added to soil in order to study how fungi are influenced by nutrients. Rouse & Baker (1978) supplemented soils with cellulose and chitin to study the effect on inoculum potential of *Rhizoctonia*. The two substrates were found to have no significant effect on the inoculum level of the fungus, in comparison to the control. In contrast, Stanghellini & Burr (1973) added nutrient solutions or bean seed exudate to soil to investigate the effect on germination of oospores of *Pythium aphanidermatum*. The nutrients activated germination, but in the absence of a solid substrate to colonise, the fungus could not form survival structures and so could not persist. This also has relevance for control of the pathogen. Martin &

Hancock (1986) found that *P. oligandrum* competed saprophytically with *P. ultimum* for colonisation of cotton leaf debris, but its survival was favoured in soils with high levels of chloride because it is more tolerant of chloride than the plant pathogen.

1.5.2 Temperature

Temperature affects the severity of mycoparasitism. Boosalis (1956) demonstrated parasitism of *Rhizoctonia solani* by *Talaromyces flavus* and *Trichoderma* spp. in soil at a temperature of 28°C but not at 18°C. Similarly, Lifshitz *et al.* (1984b) showed that at 26°C, but not at 19°C, a periodic supplement of dried ground bean leaf to a sandy loam decreased propagule densities of a plant pathogenic *Pythium* sp. while population of the mycoparasite *Pythium nunn* increased.

Boogert & Saat (1991) studied temperature in relation to biocontrol of *R. solani* by the mycoparasite *Verticillium biguttatum*. The mycoparasite requires higher temperatures for growth than the host, growing at a minimum temperature of 10 - 13°C; this is within the range that *R. solani* infects potatoes. However, it was found that strains of *V. biguttatum* selected for their ability to grow well at these low temperatures were not the most suitable for biocontrol of the pathogen. Boogert & Saat (1991) concluded that selection of strains of the mycoparasite on the basis of ability to grow at low temperatures was not a suitable criterion for selecting this biocontrol agent.

Other work has investigated soil solarisation as a method of controlling disease, with or without the addition of antagonistic fungi (Olsen & Baker, 1968; Ristaino *et al.*, 1991). The soil is heated to temperatures high enough to kill, or lower the viability of, the pathogen and this allows proliferation of the more heat-tolerant antagonists; alternatively, the antagonist is added to the soil after solarisation, so that it colonises the soil with little competition from the reduced and weakened microflora and thus prevents re-establishment of the pathogen.

1.5.3 pH

The soil pH can influence other components of the soil such as solubility of minerals and ionization of weak acids, which in turn affects the nutritional status of mycoparasites and their hosts (Paulitz & Baker, 1987b). More directly, mycoparasites have been shown to differ in pH optima. For example, *Pythium* spp. in general seldom grow at much below pH 4, or above pH 8. In a survey of the occurrence of different mycoparasites in soil, Foley & Deacon (1985) detected mycoparasitic *Pythium* spp. only in soils with a pH range of 4.5 - 7.0, and most often at pH 6.0, while Lewis *et al.* (1989) recorded the pH range for growth of *P. oligandrum* in laboratory cultures as being between pH 3.0 and pH 8.0, with an optimum of pH 6.0 - 7.0. Hence, the probable reason why *P. oligandrum* does not parasitise mycelia of *Sclerotinia sclerotiorum in vitro* (Whipps, 1987b) is that this potential host generates oxalic acid which lowers the pH of the media on which it grows.

1.5.4 Moisture

Saturated soil can differ from drier soil in microbial status or activity. For example, chytridiaceous mycoparasites are more common in wetter soil, as they depend on the free water for zoospore activity (Lumsden, 1980). Differences between genera of fungi have also been observed. Len & Dick (1986) measured populations of *Pythium* spp. and *Aplanopsis* spp. in soils with dissimilar moisture contents; their results revealed that the amount of *Pythium* "H.S. group" in drier soils was less than that of *A. spinosa*, although this was not the case in wetter soils. Also Sneh *et al.* (1977) discovered that changes in soil moisture over time resulted in a succession of fungi parasitising oospores of *Phytophthora megasperma* var *sojae, P. cactorum* (Lebert & Cohn) Schroeter, *Pythium* spp. and *Aphanomyces euteiches* Drechsler.

The aforementioned abiotic parameters have been extensively investigated to elucidate how they influence soil mycoparasites and their populations. Eastburn & Butler (1988a,b) conducted a study with *Trichoderma harzianum* and the

factors affecting its population density in natural soil. They used multiple logistic regression to analyse 55 factors including the presence of 42 individual fungi, 9 abiotic components and the total populations of bacteria, fungi and actinomycetes. They found that the abiotic components were not generally significant within the model, but several of the biotic factors exerted an influence on the distribution of *Trichoderma*. In other words, the most significant factors in a site where the abiotic environment is not notably limiting are those involving competition, antagonism and synergism.

However, other researchers have identified important effects of abiotic factors. Paulitz & Baker (1987b) examined the effects of the soil temperature, pH, matric potential and organic amendments on *Pythium nunn*, in relation to its activity in biocontrol of *Pythium*-incited damping-off of cucumbers. When the pathogen (*P. ultimum*) and the antagonist (*P. nunn*) were added together to soil (Nunn sandy loam), inoculum density of the pathogen and disease incidence were significantly lower at 26°C (but not 22 or 17°C), at pH 6.7 (but not pH 5 or 6), and at matric potentials above saturation, in comparison to disease incidence when only *P. ultimum* was present. Paulitz & Baker (1987b) also found that when organic amendments such as bean or cotton leaves or alfalfa or wheat straw were added to soil, there was an increase in inoculum level of *P. ultimum* and of disease incidence caused by *P. ultimum* only if no antagonist (*P. nunn*) was added to the soil. If *P. nunn* was also added to the soil then the organic amendments had no effect on the inoculum level of *P. ultimum*.

Boosalis (1956) also tested the effects of temperature and organic amendments on parasitism of *Rhizoctonia solani* by two mycoparasites, *Talaromyces flavus* and *Trichoderma* sp. Investigating the effects of temperatures of 18 and 28°C and of green manure addition (soybean stems and leaves), Boosalis found that the lower temperature did not lead to noticeable amounts of parasitism in amended or unamended soil, whereas the higher temperature did so. In the latter case parasitism was more abundant in green manure-amended than in

unamended soils (18% and 8% parasitism of the *Rhizoctonia* hyphae, respectively). But it was also discovered that at 28°C, after eight weeks in the soil, the numbers of unparasitised hyphae that were viable were only 20 and 40% of the total, in amended and unamended soil respectively. This led Boosalis to the conclusion that other parameters affected the hyphae more than did actual parasitism.

Hunter *et al.* (1977) investigated the effects of environmental factors on parasitism of *Rhizopus oryzae* by *Syncephalis californica*. In addition to nutrition and temperature, the effects of soil moisture and gas composition were also studied. Parasitism was observed (by characteristic swellings of the hyphae of *Rhizopus*) *in vitro* and *in vivo* at oxygen concentrations of 1 - 21%, carbon dioxide concentrations of 0.03 - 5% and moisture levels below saturation. Temperature, pH and nutrition were only examined *in vitro*; infection of the pathogen was found to occur most at 21 - 33°C, pH 5.0 - 6.5 and a carbon to nitrogen level of 10:1. Apparently *S. californica* can operate as a mycoparasite under diverse conditions; this is reflected in its presence in a wide range of soils.

Likewise, Adams (1987) investigated the survival of sclerotia of *Sclerotinia minor* and *Sclerotium cepivorum* Berk., and of macroconidia of the mycoparasite *Sporidesmium sclerotivorum*, at different soil temperature, moisture and depth. He found that at lower depths in the soil (2 - 14 cm), where the temperatures were lower and the moisture levels were higher, there was more incidence of parasitism of the sclerotia of the pathogens by *Sporidesmium sclerotivorum* than there was near the soil surface.

Other workers have shown effects of nutrition (Tribe, 1966; Rouse & Baker, 1978; Lifshitz *et al.*, 1986), pH (Lifshitz *et al.*, 1984b; Martin & Hancock, 1986), temperature (Trutmann & Keane, 1990) and moisture (Cook, 1988) on various host-mycoparasite interactions.

1.5.5 The influence of population levels on interactions between fungi

One other factor to consider in studying the ecology of mycoparasites is the relative levels of a mycohost and mycoparasite. As well as the effects of environmental factors, these can greatly influence the success in control of one fungus by another in attemps to develop a biocontrol system.

Boogert & Velvis (1992) demonstrated that increases in the population of the mycoparasite *V. biguttatum* were largely influenced by the population density of the host, *Rhizoctonia solani,* but also were affected by the temperature of the soil and the soil type. Thus when the population density of the host increased on the underground parts of the potato plants then the amount of the mycoparasite was also enhanced, with a simultaneous increase in the proportion of the hyphae of *R. solani* that was parasitised, resulting in decline of the pathogen. Population density-dependent mortality was believed to exist because increases in the population of *R. solani* corresponded to increases in the degree of parasitism. Sometimes, though, as observed by Jager & Velvis (1989), decreases in the population of *R. solani* in potato soils did not correspond to an increase in parasitism of its hyphae, or population of *V. biguttatum*, or any other mycoparasite, so other factors apart from mycoparasitism appeared to influence the populations of the fungi. Nevertheless, it was concluded that growth and proliferation of *V. biguttatum* was largely controlled by the presence of the host.

Similarly Whipps *et al.* (1992) discovered that the mycoparasites *Coniothyrium minitans* and *Pythium oligandrum* were only effective as biocontrol agents against their plant pathogenic hosts, *Sclerotinia sclerotiorum* and damping-off pathogens respectively, when the inoculum potential of the host was low. In soil with high levels of the pathogens the mycoparasites were less effective, because sufficient pathogen inoculum remained to cause crop disease.

These studies emphasise the neccessity of understanding the ecology and the interactions of both the antagonist and the pathogen, and therefore the need for more appropriate research in soils.

1.6 Exploitation of soil suppressiveness to achieve effective biocontrol

Soil-borne organisms exist in a biologically buffered ecosytem in dynamic equilibrium, which is influenced by their physical environment (Martin & Hancock, 1986). If the equilibrium changes to favour fungi antagonistic to plant pathogens then disease incidence can be reduced and the soil is said to become suppressive. This phenomonen has been exploited to elucidate the factors involved in maintaining suppression in soils and to use the knowledge in attempts to convert conducive soils to suppressive soils.

Martin & Hancock (1986) reported that a soil suppressive to *P. ultimum* was associated with a high chloride content and also with a high incidence of *P. oligandrum*. They could reproduce this suppressiveness by supplementing conducive soils with chloride ions, resulting in *P. ultimum* being displaced by *P. oligandrum* during competitive saprophytic colonisation of crop residues. Laboratory studies showed that *P. ultimum* could not compete saprophytically with *P. oligandrum* at high chloride concentrations. This suppressiveness was inoculum density dependent, such that it could be overcome by a high enough population of *P. ultimum*.

In addition, Paulitz & Baker (1987b) discovered that the action of *P. nunn* in suppression of other *Pythium* spp. was dependent on the presence of organic substrates. This was supported by the demonstration that the population densities of *P. nunn* added to the soil did not affect those of *P. ultimum* in the absence of organic amendments.

The control of other plant pathogenic pythia via soil suppressiveness has been studied in the field (Kao & Ko, 1983, 1986a, 1986b; Lumsden *et al.*, 1987) and in

container media (Chen *et al.,* 1987; Chen *et al.*, 1988; Hadar & Mandelbaum, 1992). Kao & Ko (1983, 1986a, 1986b) extensively investigated suppression of cucumber damping-off caused by *Pythium splendens* Braun. They attributed the inhibition of germination of the sporangia of the fungus, and therefore the reduction in disease incidence, to the high level of calcium and microorganisms in the soils. Conducive soils could be made suppressive with amendments of calcium or alfalfa meal, which increase the populations of soil microorganisms.

The research of the aforementioned workers showed that suppression to *Pythium*-related diseases was not always due to any one factor, but soil type, method of cultivation of the plants, biological activity and content of minerals and organic matter all contributed to suppressiveness.

Schippers (1992) also reviewed soil suppressiveness, to other diseases, and highlighted three types of disease suppressive soils, all influenced by factors such as competition for carbon and/or iron, antibiosis and induced resistance. Also, Fahima & Henis (1990) studied *Trichoderma hamatum* (Bonorden) Bainier and *Talaromyces flavus* as biocontrol agents of *Rhizoctonia solani* and *Verticillium dahliae,* and demonstrated that both the host plant and the pathogen were required to instigate suppressiveness.

1.7 Use of P. oligandrum as a biocontrol agent

As discussed in section 1.2.1 *P. oligandrum* has been widely investigated as a potential biocontrol agent of seedling diseases caused by *P. ultimum, Rhizoctonia solani, Phoma betae* and *Mycocentrospora acerina* (Vesely, 1977; Al-Hamdani *et al.*, 1983; Vesely & Hejdanek, 1984; Martin & Hancock, 1986, 1987; Walther & Gindrat, 1987a; McQuilken *et al.*, 1990; Bradshaw-Smith *et al.*, 1991; Whipps & Lumsden, 1991; McQuilken *et al.*, 1992a).

Appropriate and efficient methods are required in the use of *P. oligandrum* to achieve control of plant diseases; methods of applying the fungus to soil have to

be considered. Manipulation of the soils to enhance the population of *P. oligandrum*, and/or addition of oospore biomass have been used to achieve soil suppressiveness to *Pythium ultimum*. Alternatively, seed coating with oospores of *P. oligandrum* has been employed for control of pre-and post-emergence damping-off of cress or sugar beet seeds. Oospores of *P. oligandrum* are suitable propagules to use in applications to soils or seeds due to their ability to withstand storage and drying (Lutchmeah & Cooke, 1985).

Knowledge of the nutrition and physiology of *P. oligandrum* has been investigated in order to determine the nutrition and other external conditions that influence both the production of oospore biomass, and the germinability of the oospores. For example, McQuilken *et al.* (1992b) determined the optimum temperature (20 - 35°C), pH (6.0 - 7.5) and carbon:nitrogen ratio (5-30:1), and tested the best carbon and nitrogen source for optimal production of oospore biomass. Some of these conditions, for example pH, were found to relate to those found in soils which support natural populations of *P. oligandrum* (Foley & Deacon, 1985). Walther & Gindrat (1987a) also recognised the need to evaluate oospore germination and the breaking of constitutive dormancy, in order to improve the efficiency of oospore inocula; they found that pre-treatment of the oospores with water and myo-inositol increased their germinability. Potentially germinable oospores can then be applied to seeds.

The most commonly used methods of application of oospores to seeds are pelleting and film coating (Al-Hamdani *et al.*, 1983; Vesely & Hejdanek, 1984; Lutchmeah & Cooke, 1985; McQuilken *et al.*, 1990, 1992a). For example, Al-Hamdani *et al.* (1983) used a carboxymethyl cellulose-based seed coating to incorporate *P. oligandrum* oospores onto seeds. Germination of the oospores occurred in naturally infested soil and in artificially infested sand, and resulted in mycelia of the fungus colonising the seed and preventing infection from mycelia of *P. ultimum*. Al-Hamdani *et al.* (1983) obtained decreases in the incidence of damping-off, using seeds coated with oospores of *P. oligandrum*

which were then placed in soil or sand. In addition they also found that the oospores survived during storage of seeds for 10 - 20 weeks.

Coating of seeds with films incorporating oospores was compared to the use of seed pelleting by McQuilken *et al.* (1990). In general, pelleting of cress seeds was more successful in preventing damping-off than was film-coating. Vesely & Hejdanek (1984) found that a powder formulation of *P. oligandrum* oospores applied to seeds was more effective than a liquid film.

In many cases control of damping-off by seed-applied oospores of *P. oligandrum* was equivalent to that obtained using fungicides (Martin & Hancock, 1987; McQuilken *et al.*, 1990) but this control was more variable. Many factors may contribute to this variability; for example, the technique used to apply the oospore inocula, or the inoculum density of the pathogen. So it is important to utilise the *in vitro* knowledge of nutrition, mode of action and germination when considering the application of oospores to seeds.

To date, only one commercial preparation of *P. oligandrum* - "Polygandron" - has been produced, by Vyzkummy Ustav Rostlinne Vyroby, Czechoslovakia. Several strains of the fungus have been used but the results of trials on sugar beets have been variable (Whipps & Lumsden, 1991).

1.8 Conclusion and aims

The work reviewed above suggests that mycoparasitism does occur in soil but will always be a potentially difficult phenomonen to investigate in natural soils. Despite this, it was noted that appropriate isolation and detection methods for mycoparasites allow their distributions to be assessed and the effects of varying environmental conditions on their populations to be determined. Thus, even if mycoparasitism cannot be directly shown in soil, successful biocontrol can still be achieved by understanding how the populations of the mycoparasites can be

manipulated, whether they reduce the inoculum density of the pathogen to below disease level by competition, mycoparasitism or antibiosis.

The overall objective of the work in this thesis was to understand the activities of mycoparasites in relation to other fungi, both in soil and *in vitro*. Within this overall objective, the following specific points were addressed.

(1) Development and optimisation of a technique to determine the mycoparasitic spectra of soils.

(2) Study of the population level of *P. oligandrum* in soil, as influenced by organic amendments, and use of a fungicide-tolerant strain to follow the activity of *P. oligandrum* from introduced inoculum.

(3) Study of the effect of *P. oligandrum* on common saprophytic soil fungi, in contrast to the predominance of previous work on plant-pathogenic fungi. It was hoped that this would form a basis for understanding the role of *P. oligandrum* as a regulator of the activities of some other soil fungi.

2. MATERIALS AND METHODS

2.1 Culture media

Potato Dextrose Agar (PDA): potato dextrose agar (Oxoid), 39g; distilled water, 1l.

Potato Dextrose Broth (PDB): potato extract (Difco), 4g; glucose, 20g; distilled water, 1l.

Purified Water Agar (PWA): Bacto-agar (Difco), 20g; distilled water, 1l.

Water Agar (WA): agar (Oxoid No. 3), 20g; distilled water, 1l.

Molasses Broth (MB): cane molasses, 30g; distilled water, 1l.

Mineral Nutrient Broth A (MNA): KH_2PO_4, 1.23g; K_2HPO_4, 0.17g; KCl, 0.5g; $MgSO_4.7H_2O$, 0.5g; $FeCl_3.7H_2O$, 1mg; $ZnSO_4.7H_2O$, 0.9mg; $MnSO_4.4H_2O$, 0.4mg; distilled water, 1l.

Mineral Nutrient Broth B (MNB): $NaNO_3$, 5g l^{-1}; KH_2PO_4, 1g; KCl, 1g; $MgSO_4.7H_2O$, 0.5g; yeast extract, 0.5g; distilled water, 1l.

Cellulose Mineral Nutrient Agar (CMN): Mineral Nutrient broth (MNB) supplemented with powdered cellulose (20g l^{-1}) and agar (Oxoid No. 3, 20g l^{-1}).

All culture media were sterilised by autoclaving at 15 psi for 15 min at 121°C.

2.2 Fungal cultures

The fungi used during this study are listed in Table 2.1. Some were obtained from culture collections and others were isolated from soil during the course of

Table 2.1. Fungal cultures.

Fungus	Isolate code (where appropriate)	Source
ASCOMYCOTINA		
Chaetomium globosum Kunze	-	ICMB*
Gaeumannomyces graminis (Sacc.) Arx & Olivier var. *tritici* Walker	GGT21	Wheat, Lothians
Sclerotinia sclerotiorum (Lib.) de Bary	-	Hog-weed, East Lothian, ICMB
BASIDIOMYCOTINA		
Rhizoctonia oryzae (Saw.) Mordue	J336	R. Burton & J. Coley -Smith, University of Hull
Rhizoctonia oryzae	S1,23	Garden soil (11), Edinburgh
Rhizoctonia solani Kuhn	GM1	AG 5 tester strain
DEUTEROMYCOTINA		
Botrytis cinerea Sardina	BC1	Grapes, ICMB
Fusarium culmorum (W.G.Sm.) Sacc.	CD9	Wheat, ICMB
Fusarium oxysporum Schlecht f. sp. *lycopersici*	-	Tomatoes, ICMB
Fusarium sp.	S3,23	Garden soil (11), Edinburgh
Gliocladium roseum Bainier	G11	Garden soil (11), Edinburgh
Humicola grisea Traaen	A14	Garden soil (11), Edinburgh

Table 2.1 cont.

Fungus	Isolate code (where appropriate)	Source
DEUTEROMYCOTINA CONT.		
Papulaspora sp. Preuss	B1,4	Cereal soil (4), Liberton
Phialophora sp. (lobed hyphopodia)	IMI187786 (ATCC22834)	Maize, Essex, ICMB
Trichoderma aureoviride Rifai	TA1	ICMB
MASTIGOMYCOTINA		
Pythium oligandrum Drechsler	B4,11	Garden soil (11), Edinburgh
Pythium aphanidermatum (Edson) Fitzpatrick	CBS 634.70	Tomatoes, Israel
Phytophthora cinnamomi Rands	C9	Kiwi fruit, A. Stewart, New Zealand
ZYGOMYCOTINA		
Mucor hiemalis Wehmer	M4-	ICMB
Zygorhynchus moelleri Vuill	-	from A. Brown, Belfast

* = Fungus from culture collection at ICMB, Edinburgh University.

this work. Fungal cultures were maintained on PDA plates and sub-cultured onto fresh plates once the colony margin reached the edge of the Petri dish; this time varied according to the growth rate of the fungus. Agar blocks were cut from the leading edge of the colony and inoculated at the edge of fresh plates, which were then incubated at 25°C. The fungi were also stored as agar blocks under distilled water in McCartney bottles at room temperature (about 20°C) and at 4°C, or on PDA slopes at 4°C.

2.3 Soils

The soils shown in Table 2.2 were used in the precolonised plate method (see section 2.4.1), and soil 11 was used throughout the rest of this work. Most of the soils were collected from areas in Scotland; the majority of them were loams or clay-loams, but soil 13 was a silty loam and soils 22, 24, 25, 29 and 33 were woodland humus. Each soil sample was bulked from usually 2 or 3 subsamples from the top 10cm of the soil profile, then air-dried, sieved (2mm mesh), mixed thoroughly and stored for up to 1 week in a polyethylene bag. Soil 11 was collected as required. The soil pH was measured for all the soils in a soil:water slurry (2:1, v/v).

2.4 Methods

All experiments were carried out at 25°C, unless otherwise stated.

2.4.1 Precolonised agar plates

Plates of PDA (9cm, each containing *ca* 15g agar) were inoculated centrally with different fungi and incubated for various times, until the colony margin just reached the edge of the agar. Then each plate was cut into six equal sectors, which were placed separately in washed plastic Petri dishes, recycled from experiments that did not involve fungi. A sample of soil (0.4ml, *ca* 0.4g) was placed on the middle of each host sector. The sectors were incubated at 20°C and examined (x100 magnification) after 7, 14 and 21 days. Presumptive mycoparasites were detected by their sporulation or other structures on the host

Table 2.2. The soils used for the detection of mycoparasites.

Soil	Cropping	Location	pH
1.	Arable (oil seed rape)	Buil, Midlothian	6.45
2.	Garden (herbaceous)	Penicuik, Midlothian	6.75
3.	Arable (cereal)	Burdiehouse, Midlothian	7.70
4.	Arable (cereal)	Liberton, Midlothian	7.55
5.	Arable (cereal)	Lasswade, Midlothian	6.16
6.	Arable (cereal)	Inchinan, Renfrewshire	6.03
7.	Arable (cereal)	Paisley, Renfrewshire	6.46
8.	Arable (cereal)	Bathgate, W. Lothian	6.58
9.	Arable (grass)	Bathgate, W. Lothian	6.96
10.	Garden (herbaceous)	Bathgate, W. Lothian	5.07
11.	Garden (herbaceous)	Edinburgh	6.00
12.	Arable (potatoes)	Longside, Aberdeen	6.58
13.	Arable (peas)	York, Yorkshire	5.69
14.	Arable (cereal)	York, Yorkshire	7.72
15.	Arable (potatoes)	York, Yorkshire	7.12
16.	Arable (oil seed rape)	York, Yorkshire	6.69
17.	Arable (cereal)	York, Yorkshire	7.59
18.	Arable (cereal)	York, Yorkshire	6.04
19.	Arable (cereal)	Roslin, Midlothian	5.98
20.	Woodland (deciduous)	Liberton, Midlothian	4.83
21.	Woodland (deciduous)	Penicuik, Midlothian	5.05
22.	Woodland (deciduous)	Bathgate, W. Lothian	3.72
23.	Woodland (deciduous)	Roslin, Midlothian	6.68
24.	Woodland (deciduous)	Edinburgh	4.26
25.	Garden (herbaceous)	Edinburgh	4.85
26.	Parkland trees	Edinburgh	5.65
27.	Arable (strawberries)	Penicuik, Midlothian	5.61
28.	Arable (strawberries)	Penicuik, Midlothian	6.17
29.	Woodland (deciduous)	Dumbarton, Dumbartonshire	4.11
30.	Woodland (deciduous)	Luss, Dumbartonshire	4.37
31.	Woodland (deciduous)	Crianlarich, Perthshire	5.06
32.	Woodland (deciduous)	Lochgilphead, Perthshire	5.31
33.	Woodland (coniferous)	Callander, Perthshire	3.65
34.	Woodland (deciduous)	Aberfoyle, Dumbartonshire	4.61

colony. Identifications were confirmed with pure cultures, obtained by subculturing the mycoparasites from representative host sectors onto plates of PDA.

2.4.2 Isolation of cellulolytic fungi from soil

Plastic boxes with lids (120 x 120 x 13 mm, supplied as bio-assay dishes by Nunc (Gibco/BRL)) received a bottom layer of 50g air-dried soil no. 11, then rectangles of nylon gauze (160 x 115 mm) were laid on top of the soil in each box, with the edges hanging over. A further 90g soil was then added and flattened to provide an even surface. The soil was saturated to 50% of field capacity with distilled water and strips of cellulose film (1 x 3 cm) (supplied as "Rayophane" PU 525, British Sidac Ltd, St. Helens, Merseyside), which had been autoclaved in distilled water and allowed to drain, were then added to the soil. These were inserted between the bottom layer of soil and the nylon gauze by carefully lifting the nylon gauze away with the top layer of soil, randomly placing the strips of cellulose film on the bottom layer of soil and then positioning the gauze with the top layer of soil back into the box. The boxes were incubated at 20°C for 3 to 7 days, then the gauze and overlying soil were lifted away and the cellulose strips were retrieved, cut into segments (1 x 1 cm) and placed on plates of CMN. Any soil particles adhering to the surface of the film were gently removed using fine forceps. The plates were incubated at 20°C and potential cellulolytic fungi were isolated as they appeared. The cellulolytic activity of each isolated fungus was tested by using the penetrometer method described in section 2.4.8.1.

2.4.3 Detection of mycoparasites from host-colonised substrata that had been buried in soil

Strips of cellulose film (1 x 2 cm) or, in some experiments, segments of wheat flag leaves (1 x 2 cm) were autoclaved in distilled water, drained, placed on PDA and inoculated at the edge with various cellulolytic fungi that had been isolated from soil no. 11 (section 2.4.2). After colonisation of the strips (2-5 days

incubation depending on the fungus), 1 x 1 cm segments were cut and buried in soil with uninoculated (control) strips, as described in section 2.4.2. There was 12 pieces of cellulose film, or 9 pieces of wheat leaves randomly placed into each box. The boxes were incubated at 20°C for 5 days and then the strips were retrieved and placed on precolonised agar sectors of fungi to detect mycoparasites (section 2.4.1).

2.4.4 Determination of the sensitivity of P. oligandrum to metalaxyl

PDA plates containing different concentrations of the fungicide metalaxyl (supplied as Metalaxyl Technical (Bx 909099) from Ciba-Geigy Agrochemicals, Cambridge) were centrally inoculated with discs of *P. oligandrum* (5mm diam.), incubated for 24 h and then the diameters of colonies were measured along two marked lines running through the inoculum disc at right angles. From these measurements the estimated dose (ED_{50}) of metalaxyl required to reduce the growth rate by half was calculated.

2.4.5 Production of metalaxyl-tolerant P. oligandrum

P. oligandrum was inoculated centrally onto plates of PDA and incubated until growth had reached the edge (usually 2 days). The plates were then exposed to ultraviolet irradiation (long wave, 366nm) for 60 seconds, and then several small agar blocks were removed and transferred to fresh PDA plates supplemented with metalaxyl (20μg ml^{-1}). Once any fungal hyphae had grown out from the inoculum, agar blocks were taken from the margin of these colonies and inoculated onto a series of PDA plates of increasing concentrations of metalaxyl. This procedure was repeated until *P. oligandrum* was growing on PDA supplemented with metalaxyl at 50μg ml^{-1}, at a rate similar to the wild type on PDA only (approximately 56.5 ± 0.7 mm $(24h)^{-1}$).

2.4.6 Production of oospores of P. oligandrum

To produce an oospore suspension, PDA discs (7mm) of *P. oligandrum* were inoculated into 200ml medical flats containing 8 ml of molasses broth. The discs

were placed at the mid point of one of the broad sides of each flat and then the flats were incubated for 7 weeks on their broad sides. After this time the liquid from each bottle was decanted off and the mycelial mat was removed. The agar plug was separated from the mycelium and the latter was spread out and dried overnight in a sterile Petri dish in a laminar flow cabinet. The dried mycelium was resuspended in 1ml of sterile distilled water in a centrifuge tube, homogenised for 60 seconds at 20,500 rpm with an Ultra Turrax T25 blender, then centrifuged at 1534**g** for 15 seconds. The pellet was washed in sterile distilled water, the centrifugation and washing procedure repeated twice, and then the pellet was suspended in sterile distilled water. Oospores were counted and used immediately after preparation.

2.4.7 Use of the Most Probable Number method to determine changes in the population of P. oligandrum in soil

Plastic boxes (120 x 120 x 13 mm, as before) were prepared containing 140g of air-dried soil no. 11, with or without a 1% supplement of wheat or grass leaves. The fresh green grass (*Dactylis glomerata* L. (cocksfoot)) and the dried, mature wheat flag leaves were cut into small pieces (2 x 3 mm) and then added to the dry soil and thoroughly mixed in to obtain random distribution. The soil was adjusted to 50% saturation with distilled water and lids were placed on the dishes which were then incubated in polyethelyene bags sealed tight at 20°C. In this way approximately 1g of water was lost through evaporation per week and so the saturation level was maintained by regular watering.

Samples of soil were taken at intervals with a cork borer (7mm diam.) - the borer was depressed into the soil, removed and the soil stuck in the tube was freed by pushing the soil out using the handle of an inoculating loop. Subsamples of soil were taken from 6 - 8 randomly located positions within each box to provide a total *ca* 3.5g of soil at each sampling time. The sample from each box were air-dried for approximately 4 hours and then crushed to a coarse powder. Then, from each sample, 1.4g was removed and thoroughly mixed with

1.4g of sand (sieved through a 2mm mesh) in a small bottle. From this mixture 1.4g was removed and mixed with sand, the process being repeated in a series of 2-fold dilutions down to 1 part soil in 256 parts of sand. The soil-sand mixtures were mixed thoroughly each time by agitation with a whirlimixer for 5 - 10 seconds, and then by rotating the bottle by hand for a further 20 seconds. For each soil-sand mixture at each dilution level, 0.35g samples were placed onto precolonised sectors of *Fusarium culmorum* (as described in section 2.4.1) and incubated at 20°C. Presence or absence of *Pythium oligandrum* on these plates was assessed at 7 and 14 days. The boxes of soil were sampled at 0, 10, 20, 40 and 80 days, and every 40 days thereafter.

In some cases the boxes were re-treated with the same grass or wheat supplement, as originally, at 150 days. In these cases sampling was done at 160 days, and every 40 days thereafter. This re-treatment involved removing the soil from each box, mixing it with the supplement, and then gently re-packing the soil into the box, but without drying the soil.

In a variation of this method, a water-based suspension of oospores from a metalaxyl-tolerant strain of *P. oligandrum* was used to bring the soils to 50% saturation. The oospores were produced as described previously (section 2.4.6). This provided an estimated 4.23×10^3 oospores g^{-1} air-dried soil. Other boxes were watered as normal, without the oospore supplement. For the boxes containing the added oospores, 7g of soil was removed from each box at each sampling and treated as before, except that half of the soil-sand mixture at each dilution was placed on agar plates precolonised by *F. culmorum* and half onto similar plates that contained metalaxyl incorporated into the agar ($50\mu g\ ml^{-1}$) and were colonised by *F. culmorum*. Any growth of *P. oligandrum* that developed on these latter plates was assumed to represent the fungicide-resistant strain of *P. oligandrum* added initially. This was confirmed regularly by sub-culturing colonies onto fresh PDA plates with metalaxyl ($50\mu g\ ml^{-1}$), and further confirmed by the fact that *P. oligandrum* never grew from soil placed on

plates of *Fusarium* with metalaxyl if a metalaxyl-tolerant mutant of *P. oligandrum* had not previously been added to the soil.

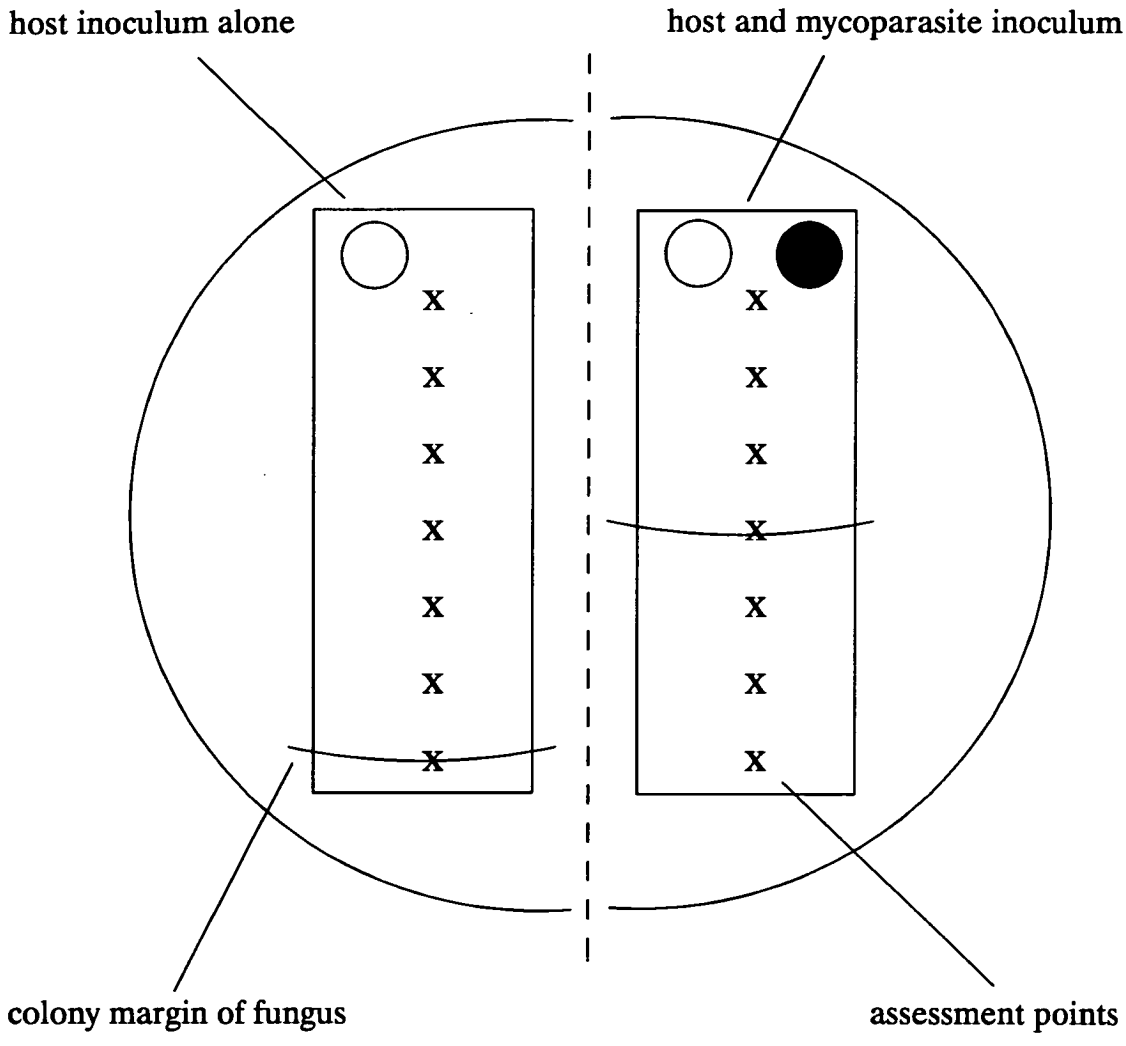
2.4.8 Interactions with cellulolytic fungi in media containing different cellulose sources

2.4.8.1 Interactions on cellulose film

The method of Laing & Deacon (1990), for juxtaposed inocula, was used to determine the effect that *P. oligandrum* had on the cellulolytic activity of a range of cellulolytic fungi. Strips of cellulose (2 x 6 cm) were autoclaved in distilled water, drained and then laid on plates of MNB supplemented with agar (Oxoid No. 3) (20g l^{-1}). Cellulose was the main carbon source. The test fungi were inoculated as 5mm PDA discs in one corner of the cellulose strips, and then 24 h later some of the strips were inoculated at the same end with *P. oligandrum* (Fig. 2.1). The plates were incubated for 8 days and then the strength of the cellulose film was assessed with a needle penetrometer. This comprised the base of a small (5cm diam) plastic petri-dish glued to the wooden handle of a dissecting needle, the shaft of which was supported by a plastic tube. The needle tip was slowly lowered on to the cellulose film. If the film was not punctured the needle was raised and weights were added at 5g intervals until the tip of the needle punctured the film. The needle was raised between adding the weights, and then lowered to a point immediately adjacent to the previous point that had been tested, to avoid progressive weakening of the cellulose film.

Assessments were made, as shown in Fig. 2.1, at 7 points along the length of each strip, from the front of the host inoculum to its colony margin. Control (uninoculated) strips required a weight of approximately 65g at each point to puncture them; any reduction of this on the inoculated strips was indicative of the degree of cellulolysis. Comparisons were made between cellulose strips inoculated with potential host fungi alone, potential mycoparasites alone, and paired combinations as in Fig. 2.1.

Fig. 2.1. Diagrammatic representation of inoculation of cellulolytic fungi on cellulose strips, with or without juxtaposed inoculum of the mycoparasite, on mineral nutrient agar, with one treatment only per petri dish.



Each plate containing a cellulolytic fungus with *P. oligandrum* was paired with a plate with the former fungus alone. The points were marked at the same distance in order to compare the reduction in growth rate and decrease in cellulolytic activity.

2.4.8.2 Interactions on filter papers

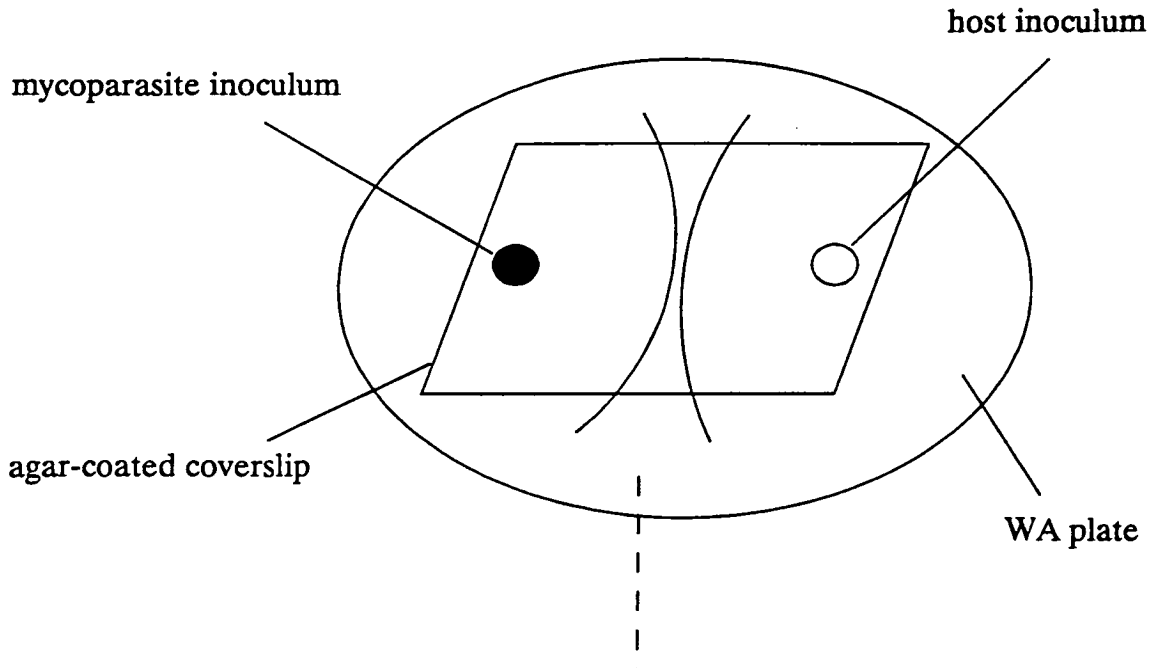
The method described below was similar to that used by Deacon (1976) to determine the cellulolytic ability of *Pythium*, when filter paper was the sole carbon source. Wads of ten air-dried filter papers (Whatman No. 3; 7.0 cm diam.) were weighed accurately and placed in 250ml flasks. To each flask 20ml of mineral nutrient solution was added, then the flasks were plugged with foam bungs and these were covered in aluminium foil and autoclaved. The mineral solution consisted of $NaNO_3$ (5g), K_2HPO_4 (1.0g), $MgSO_4.7H_2O$ (0.5g), $FeCl_3.7H_2O$ (1mg), $ZnSO_4.7H_2O$ (0.9mg), $MnSO_4.4H_2O$ (0.4mg), thiamine (100μg), biotin (10μg) and distilled water (1l), and was adjusted to pH 6.0.

The top of each filter paper wad was inoculated at the edge with a PDA disc (7mm) of a host fungus alone or mycoparasite alone. Other flasks received discs of both a host and a mycoparasite, side by side. Flasks were incubated for up to 8 weeks, and replicates were sampled every 2 weeks. At sampling the flask contents were removed, dried overnight in an oven at 80°C and weighed. After taking into account the weight of the mineral nutrients, the agar block and the loss in weight of control filter wads during oven drying, the weight loss of the oven-dried filter paper wads, due to the activities of the fungi, was calculated by subtracting their weight from the original dry weight of each filter paper wad.

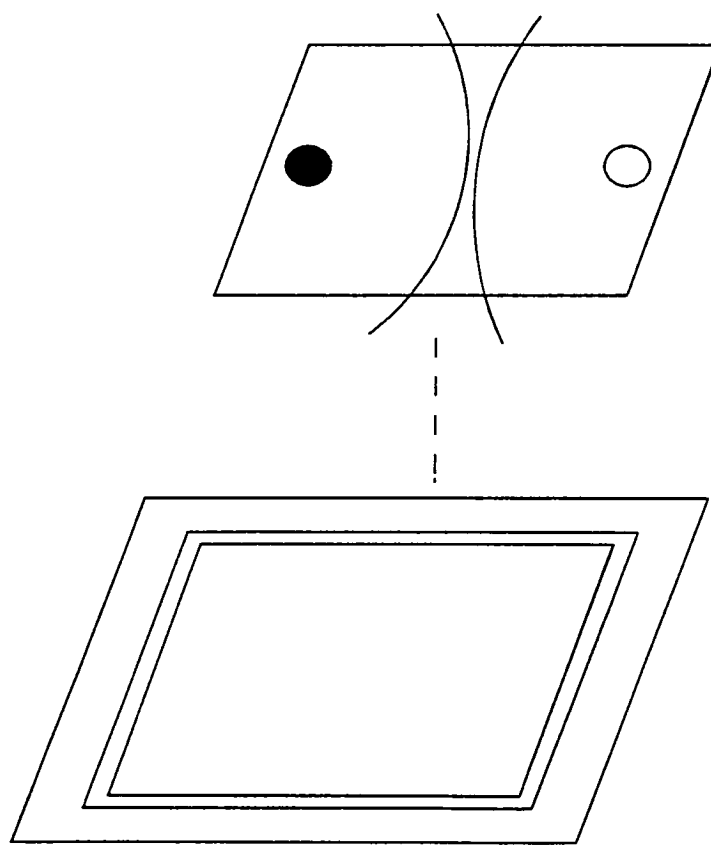
2.4.9 Video Microscopy

Inter-hyphal interactions of host fungi and mycoparasites were studied using video microscopy (Laing & Deacon, 1991). Sterile glass coverslips (38 x 38 mm) were immersed in molten purified water agar (PWA), excess agar was drained off such that only a thin layer coated the coverslip, and then the coverslips were laid flat on the surface of WA plates. Agar discs (5mm) of host fungi and mycoparasites were inoculated onto the agar films on the coverslips at different distances apart. The plates were incubated overnight then the coverslips were removed from the agar plate and inverted onto observation chambers coated in vaseline to seal the coverslips (Fig. 2.2). The chambers were made from large

Fig. 2.2. Diagrammatic representation of the procedure used to obtain interacting colonies for video microscopy.



Fungal colonies grown on a PWA-coated coverslip, placed on a WA plate, until the margins are just touching; then the coverslip is removed and inverted onto an observation chamber.



microscope slides to which were glued lengths of glass capillary tubing, *ca* 1 mm in diameter. The slides were placed under a microscope and interactions between individual host and mycoparasite hyphae at the colony margins were observed using a x70 oil-immersion objective.

The interactions were recorded, with or without time-lapse recording, using a video camera attached to a Leitz Orthoplan microscope. The equipment comprised a Panasonic S-VHS F15 colour video camera, Panasonic S-VHS AG-6720 video recorder with time lapse facility and a Panasonic S-VHS BT-M1420PY colour video monitor. Recordings were made on Master Broadcast S-VHS videotapes and photographs were taken with a Mitsubushi video copy processor.

2.4.10 Demonstration of a possible inhibitor in hyphae of R. oryzae

Medical flats containing PDB were inoculated with various host fungi as described in section 2.4.6, and incubated for 10 days. The contents of each flat were then aseptically removed and the mycelial mat was washed three times with sterile distilled water. The mycelium was then freeze-dried using liquid nitrogen and ground to a fine powder in a coffee grinder for 120 sec. This powder was transferred to a centrifuge tube and allowed to thaw, resulting in *ca* 25ml of resuspended mycelium homogenate for each fungus. The tube contents were filtered through a double layer of muslin and then through a filter paper circle (Whatman, 9cm). The filtrate was filter-sterilised using a millipore filter (pore size 0.45μm) and then 3.5ml aliquots were dispensed into small (5cm diam) empty plastic Petri dishes. The dishes were inoculated with 5mm WA discs of *P. oligandrum* and incubated for 2 days. The diameters of the colonies in the different media were then measured.

2.4.11 Production of Bulbils by Papulaspora

To produce a bulbil suspension from *Papulaspora* the fungus was allowed to colonise a PDA plate, which was then washed with sterile distilled water and the

mycelium and bulbils were scraped off into a centrifuge tube. The contents were held against a vortex mixer for 10 sec and then centrifuged for 10 min at 281**g**. The supernatant was decanted off, distilled water was added to the pellet which was then resuspended and centrifuged and washed as before. The new suspension was then filtered through a layer of muslin and the liquid containing the bulbils was centrifuged for 5 min at 1534**g**. The pellet was resuspended in water and the bulbils in the resulting suspension were counted.

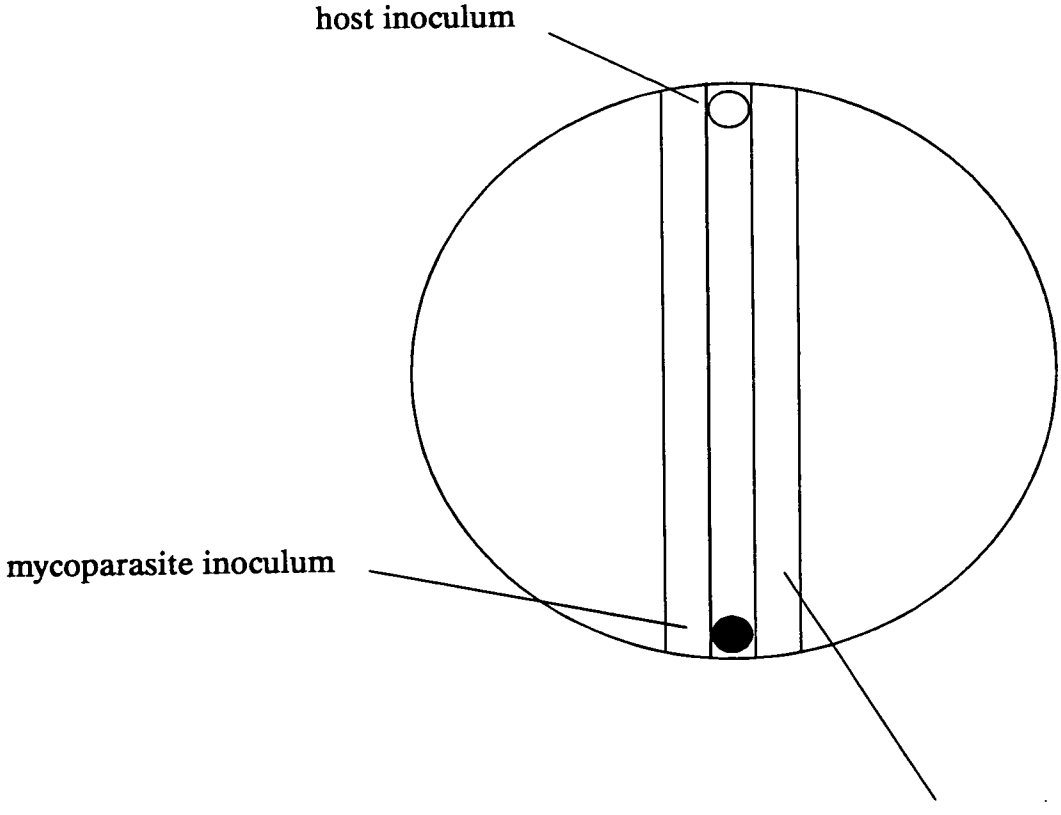
Suspensions were prepared with known bulbil concentrations and 50μl of each was pipetted onto replicate PDA sectors precolonised by various host fungi. The plates were incubated for up to 14 days and examined for growth of *Papulaspora* from the suspension across the fungal colonies.

2.4.12 Quantification of growth of Papulaspora across fungus-colonised plates

PDA discs (5mm) of host fungi were inoculated at the margins of PDA plates, and the plates were incubated until the fungi had just reached the other side of the plate. WA discs (5mm) of *Papulaspora* were then inoculated onto the young margin of the host colony. Four parallel lines (5mm apart) were marked on the base of each plate to mark three agar strips, the centre one including the two inoculum discs. The plates were incubated for 7 days, then the centre strip was cut out and removed. It was cut into blocks of 5mm and the successive blocks were placed onto discs (17mm) of PDA precolonised by *B.cinerea* (see Fig. 2.3, and Laing & Deacon, 1990). These circles were incubated for up to 14 days to allow *Papulaspora* to colonise the *Botrytis* discs and produce bulbils, an indication of the distance that *Papulaspora* had grown across the original precolonised agar plate. The second and third strips were removed from the original plates at 14 and 21 days and treated similarily.

2.4.13 Nutritional studies

Fig. 2.3. Procedure used to assess growth of a mycoparasite across host-colonised agar plates.



Papulaspora sp. MNA was used as the basal medium and was supplemented with various combinations of carbon, nitrogen or vitamin sources. Difco yeast nitrogen base without amino acids and ammonium sulphate contained no nitrogen or carbon sources and was used to supply vitamins, trace elements and salts in various quantities (Difco, 1984). The vitamins supplied in the yeast base included biotin ($2\mu g\ l^{-1}$) and thiamine ($400\mu g\ l^{-1}$).

The nutritional requirements of *Papulaspora* were studied in different media, as described below.

1. *Nitrogen requirements*. The basal medium, MNA, was supplemented with glucose ($20g\ l^{-1}$) and used as the control medium (no nitrogen or vitamin supplement), or other nutrients were also added to the medium, either (a) Difco yeast nitrogen base ($6.7g\ l^{-1}$) as a vitamin source; (b) sodium nitrate ($2g\ l^{-1}$) but no vitamin supplement; (c) sodium nitrate and yeast nitrogen base; (d) L-asparagine ($1.51g\ l^{-1}$) but no vitamin supplement; or (e) L-asparagine and yeast nitrogen base.
2. *Carbon requirements*: MNA was supplemented with L-asparagine and used as the control medium (no carbon or vitamin supplement), or additional nutrients were added, either (a) yeast nitrogen base; (b) mannitol ($10g\ l^{-1}$) but no vitamin source; (c) mannitol and yeast nitrogen base; (d) trehalose ($10g\ l^{-1}$) but no vitamin source; (e) trehalose and yeast nitrogen base; (f) glucose ($10g\ l^{-1}$) but no vitamin source; and (g) glucose and yeast nitrogen base.
3. *Vitamin requirements*: MNA was supplemented with L-asparagine and glucose and used as the control medium (no vitamins), or the medium was supplemented with other nutrients, either (a) biotin only ($100\mu g\ l^{-1}$); (b) thiamine only ($1000\mu g\ l^{-1}$); (c) yeast nitrogen base only ($6.7g\ l^{-1}$); (d) biotin and thiamine; and (e) biotin, thiamine and yeast nitrogen base.

The basal media, and the media containing a carbon source and/or sodium nitrate, were dispensed in 8ml aliquots into 200ml medical flats and autoclaved.

The yeast nitrogen base, biotin, thiamine and asparagine were filter-sterilised and aseptically added to the autoclaved media. The medical flats were inoculated with WA discs (7mm) of *Papulaspora* at the mid point of one of the broad sides of each flat and laid on this side for incubation, such that the agar disc was partly submerged in the liquid. After 14 days the mycelium in each flat was collected under vacuum on weighed Whatman cellulose nitrate membrane filters (pore size 0.45μm) using a Buchner funnel, and washed with 50ml distilled water. The filters were then dried at 80°C overnight, reweighed and the weight gain due to the mycelial mat was calculated. Controls were treated similarily but using flats inoculated with uncolonised WA discs.

In a similar experiment the pH optimum of *Papulaspora* was determined by dispensing aliquots (7ml) of autoclaved buffered PDB into 150ml medical flats. The PDB broth was buffered with phosphate or citrate-phosphate buffer (0.2M) over a pH range 3.0 - 8.0. The medical flats were inoculated with *Papulaspora* and treated as before.

2.4.14 Production of non-volatile metabolites

PDA plates were overlaid with 75mm circles of autoclaved cellulose film then inoculated centrally with 5mm discs of *Papulaspora*, or one of the host fungi, cut from PDA plates. *Papulaspora* was allowed to grow for 4 days, and the host fungi for 1 day (*T. aureoviride*), 2 days (*R. solani*) or 3 days (*B. cinerea*), then the cellulose and fungus were removed. The plates previously inoculated with *Papulaspora* were then inoculated with each of the host fungi while the plates previously inoculated with the host fungus were inoculated with the same host, as controls. After 2 days (*T. aureoviride*), 3 days (*R. solani*) or 5 days (*B. cinerea*), the linear extent of growth by the host fungi was assessed (Dennis & Webster, 1971a).

2.4.15 Production of volatile compounds

PDA plates were inoculated centrally with agar discs (5mm) of *Papulaspora* and allowed to grow for 7 or 10 days. PDA plates were also inoculated with potential host fungi and allowed to grow for 1 day if side inoculated, and 2 days (*T. aureoviride*), 3 days (*R. solani*) or 5 days (*B. cinerea*) if centrally inoculated. The bases of the plates containing the host fungi (side inoculated) were then inverted onto the bases of plates containing *Papulaspora*, or to the same host fungus (centrally inoculated), and sealed to the base plate with Parafilm. The plates were incubated for 2 days (*T. aureoviride*), 3 days (*R. solani*) or 5 days (*B. cinerea*), then the linear extent of growth of host fungi was recorded (Dennis & Webster, 1971b).

2.4.16 Growth of Papulaspora in liquid culture with other fungi

Aliquots of MNA (8ml) supplemented with glucose (20g l^{-1}) and either $NaNO_3$ (3.67g l^{-1}) or L-asparagine (3.32g l^{-1}) were dispensed into 200ml medical flats and autoclaved. The bottles were inoculated with different host fungi (7mm diam. WA discs) as described in section 2.4.13, and incubated for 12 days on their broad sides. Then some of the bottles were inoculated with WA discs (7mm) of *Papulaspora*, at the margin of the host colony, and incubated for a further 7 days. The growth of *Papulaspora* across the host fungi was measured after incubation, from the *Papulaspora* disc to the furthest visible bulbil. The contents from other bottles were collected under vacuum on Whatman cellulose nitrate membrane filters (pore size 0.45μm), and the oven-dry weights of the mycelial mats were calculated. The liquid from these bottles was collected and pooled, and 8ml aliquots were dispensed into fresh 200 ml bottles which were autoclaved. The bottles were then inoculated with WA discs of *Papulaspora*, as described before, and incubated for 7 days. WA discs of *Papulaspora* were also inoculated into medical flats containing the original (uninoculated) medium. After incubation the oven-dry weight of *Papulaspora* growing in this medium was calculated.

3. DETECTION OF MYCOPARASITES IN SOIL

3.1 Introduction

Mycoparasites have the potential to be exploited as biocontrol agents but their activities in soil must be investigated and understood if they are to be successfully exploited. Numerous methods have been employed to study mycoparasitism in soil and to investigate the ecology of mycoparasites, as reviewed in section 1. These methods include observations directly, for example by the use of supporting material (Lumsden, 1981; Keinath *et al.*, 1991) or indirectly, for example by studying the effects of nutrition (organic amendments, etc.) on host and mycoparasite populations (Hunter *et al.*, 1977). Indirect methods have been used extensively to study the roles of mycoparasites as population regulators of fungi in the soil, and to investigate the effect of environmental factors on mycoparasites.

The ecology of many mycoparasites has been studied: the mycoparasitic *Pythium* spp. (Vaartaja, 1967; Plaats-Niterink, 1975; Deacon & Henry, 1978; Foley & Deacon, 1985; Lodha & Webster, 1990; Ribeiro & Butler, 1992), *Coniothyrium minitans* (Whipps *et al.*, 1992; Sandys-Winsch *et al.*, 1993), *Gliocladium* spp. (Keinath *et al.*, 1991), *Sporidesmium sclerotivorum* (Adams & Ayers, 1981; Adams *et al.*, 1984), *Trichoderma* spp. (Lifshitz *et al.*, 1986; Lewis & Papavizas, 1991) and *Verticillium biguttatum* (Boogert & Saat, 1991; Boogert & Velvis, 1992). Species-specific isolation media has been developed and commonly used in conjunction with dilution plating and most probable number methods to determine the populations of the mycoparasites (Papavizas, 1981; Park *et al.*, 1992; White *et al.*, 1992).

Baiting (section 1.4.1) has also been used to selectively isolate mycoparasites from soil and to assess their distributions. It can involve the addition of seeds, fungi or fungal structures such as sclerotia or oospores to soil (Huber *et al.*, 1966; Ayers & Lumsden, 1977; Boogert & Jager, 1983). Ribeiro & Butler (1992)

used sclerotia of *Sclerotinia sclerotiorum* to increase the isolation and detection of the mycoparasitic pythia - *P. acanthicum, P. oligandrum, P. periplocum*. The sclerotia were placed on soil for 48 h then transferred to selective media; results were better than those achieved with a dilution plate technique. Adams & Ayers (1981) also used sclerotia, but of *Sclerotinia minor*, to isolate *Sporidesmium sclerotivorum* from soil.

In most such studies the aim was to assess the population or distribution of individual mycoparasites although sometimes *Trichoderma* spp. and *Gliocladium virens* have been considered simultaneously (Beagle-Ristaino & Papavizas, 1985), as have different mycoparasitic and plant-pathogenic pythia (Flowers & Hendrix, 1969; Ali-Shtayeh *et al.*, 1986; Martin & Hancock, 1986). Very few reports concerned the mycoparasitic spectra of soils. Foley & Deacon (1985) did this, through a direct plating method designed to detect *P. oligandrum* but which also detected other mycoparasites (*Gliocladium* spp., *P. mycoparasiticum* - as *Pythium* SWO - and *Trichoderma viride*). The method involved placing soil samples on agar plates previously colonised by a host fungus (*Phialophora*), then recording the different mycoparasites that grew across the host colonies and formed characteristic structures. This study was the closest to developing a method to examine the mycoparasitic spectra of soils, but the use of only one host fungus limited the potential scope of the method.

The aim of work described in this section was to improve the precolonised plate method by using a wider range of fungal hosts and baiting in order to increase detection efficiency of the known mycoparasites. The method was also used as the basis of a Most Probable Number analysis of populations of *P. oligandrum* in soil so as to determine the effects of various soil treatments on the population of the mycoparasite.

3.2 Results

3.2.1 Detection of presumptive mycoparasites in soil using host-colonised agar

Precolonised plates were prepared as described in section 2.4.1, using seven host fungi: *Botrytis cinerea, Fusarium culmorum, Gaeumannomyces graminis, Phialophora* sp., *Pythium aphanidermatum, Rhizoctonia solani* and *Trichoderma aureoviride*. An initial experiment was done in which samples of 12 soils were placed on colonies of each fungus, using six replicate plates for each soil and each host. Thereafter, the number of host fungi was reduced to four - *B. cinerea, F. culmorum, R. solani* and *T. aureoviride*; sometimes the number of replicate plates was increased to 10 (soils 13 - 18), and a larger number of soils were used (total 34). In all cases the host sectors were randomised, so for any one soil they were usually from different colonies.

3.2.1.1 Presumptive mycoparasites detected

Four types of presumptive mycoparasites were detected throughout the study, as briefly described below.

1. *Pythium oligandrum*, identified by the features of its spiny-walled oogonia (Plaats-Niterink, 1981), was seen on the host sectors usually after 7 days, when it had completely covered the host sectors (Fig. 3.1, Table 3.1). Smooth-walled oogonia were sometimes observed on host sectors suggesting the presence of *P. mycoparasiticum* (Deacon, Laing & Berry, 1991). However only *P. oligandrum* could be isolated into pure culture from such plates, perhaps because it outgrew *P. mycoparasiticum* on PDA or because the smooth-walled oogonia were immature forms of *P. oligandrum*.

2. *Gliocladium* spp. formed slow-growing, sporulating colonies near the soil inoculum after 7 days, but more commonly after 14 - 21 days incubation (Fig. 3.2, Table 3.2). Most of the colonies observed (>500) were characteristic of *G. roseum* Bain. (Morquer *et al.*, 1963) as confirmed by isolation in pure culture. A few colonies however were identified as *G. atrum* Gilman & Abbot (total 23 occurrences), *G. catenulatum* Gilman & Abbot (9 occurrences) or *G. fimbriatum*

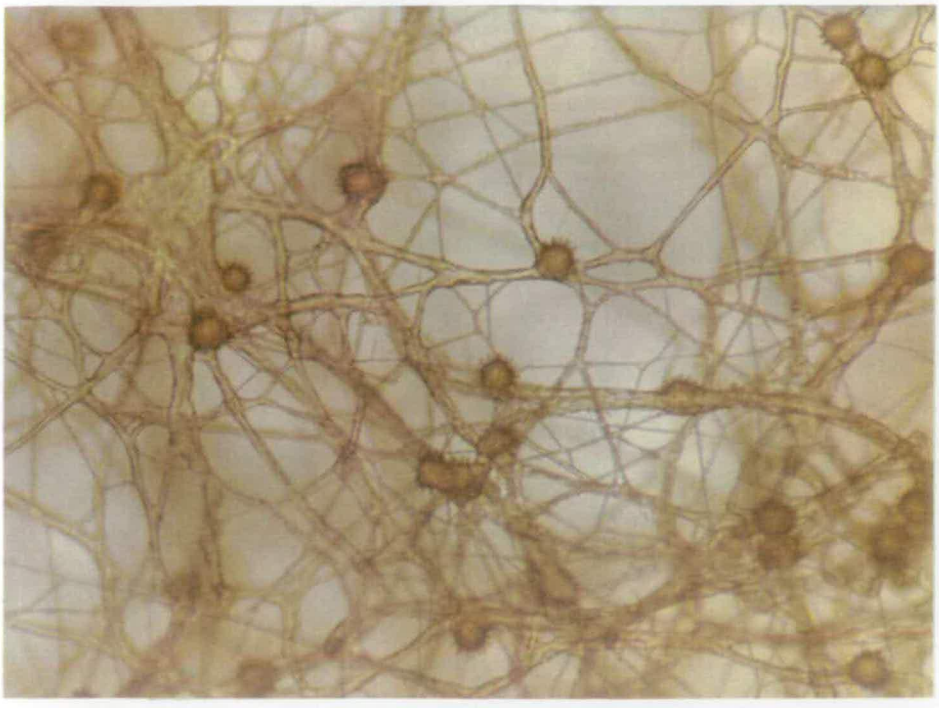


Fig. 3.1. Oospores of *P. oligandrum,* as seen on agar colonised by *Fusarium culmorum.*

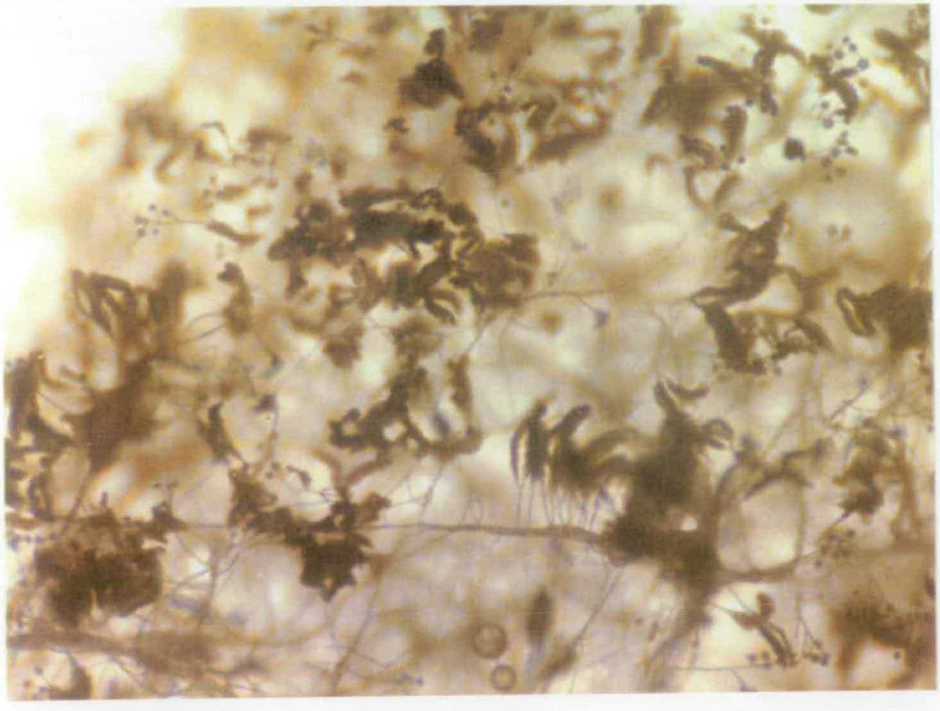


Fig. 3.2. Conidia of *Gliocladium* sp. as seen on agar colonised by *Botrytis cinerea.*

Table 3.1. Summary of occurrence of *P. oligandrum* in each of 34 soil samples as evidenced by microscopical observation of agar sectors precolonised by different host fungi and inoculated with soil.*

Soil+	Fungus used to precolonise agar				Total
	Botrytis cinerea	*Fusarium culmorum*	*Rhizoctonia solani*	*Trichoderma aureoviride*	
1	5	6	0	0	11
2	6	6	0	0	12
3	2	6	0	0	8
4	0	6	0	0	6
5	0	4	0	0	4
6	0	0	0	0	0
7	0	5	0	0	5
8	0	0	0	0	0
9	5	6	0	0	11
10	0	0	0	0	0
11	0	2	0	5	7
12	0	6	0	0	6
13	3	10	0	0	13
14	4	10	0	0	14
15	10	10	0	0	20
16	7	10	0	0	17
17	10	10	0	0	20
18	10	10	0	0	20
19	0	4	0	0	4
20	0	0	0	0	0
21	0	0	0	0	0
22	0	0	0	0	0
23	0	0	0	0	0
24	0	0	0	0	0
25	6	6	0	6	18
26	6	6	0	3	15
27	0	0	0	0	0
28	0	0	0	0	0
29	0	0	0	0	0
30	0	0	0	0	0
31	0	0	0	0	0
32	0	0	0	2	2
33	0	0	0	0	0
34	0	0	0	0	0
Total	74	123	0	16	**213**

* = Data are the numbers of replicate agar sectors (maximum 6, but 10 for soils numbered 13 - 18) on which *P. oligandrum* was detected.
+ = See Table 2.2.

Table 3.2. Summary of occurrence of *Gliocladium* spp. in each of 34 soil samples as evidenced by microscopical observation of agar sectors precolonised by different host fungi and inoculated with soil.*

Soil+	Fungus used to precolonise agar				Total
	Botrytis cinerea	*Fusarium culmorum*	*Rhizoctonia solani*	*Trichoderma aureoviride*	
1	3	1	6	6	16
2	3	0	6	6	15
3	4	0	4	6	14
4	6	0	3	5	14
5	6	6	6	6	24
6	6	6	6	6	24
7	6	6	6	6	24
8	6	6	6	6	24
9	5	0	6	6	17
10	6	6	6	6	24
11	5	2	3	1	11
12	0	0	6	6	12
13	10	8	9	10	37
14	8	7	9	9	33
15	0	0	7	3	10
16	6	1	7	7	21
17	5	0	7	2	14
18	0	2	10	4	16
19	6	5	5	3	19
20	3	6	0	3	12
21	4	3	3	0	10
22	3	6	1	1	11
23	6	6	3	5	20
24	3	6	3	5	17
25	4	0	6	6	16
26	2	1	6	5	14
27	6	5	4	4	19
28	4	1	3	6	14
29	6	0	0	0	6
30	6	6	6	6	24
31	4	3	4	0	11
32	6	6	5	6	23
33	5	1	0	0	6
34	5	6	6	6	23
Total	158	112	168	157	**595**

* = Data are the numbers of replicate agar sectors (maximum of 6, but 10 for soils numbered 13 - 18) on which *Gliocladium* spp. were detected.
+ = See Table 2.2.

Gilman & Abbot (3 occurrences), also confirmed by isolation into pure culture. These species were detected on different host sectors from *G. roseum*, but were relatively uncommon and always found in the same soils as *G. roseum*. Presumably their occurrence on different host sectors to those that supported growth of *G. roseum* reflected competition between these fungi for colonisation of the host colonies.

3. *Trichoderma* spp. were detected most often at 7 - 14 days by their characterisitic sporing structures (Rifai, 1969) on the soil surface or at the periphery of the host sectors (Fig. 3.3, Table 3.3). The isolates identified in pure culture were usually *T. harzianum* Rifai, but occasionally *T. hamatum* (Bonorden) Bainier or *T. koningii* Oudem.

4. *Papulaspora* sp. was detected as brown bulbils (Weresub & LeClair, 1971) across host sectors after 7 - 14 days (Fig. 3.4, Table 3.4). All pure cultures were identical in growth form and features of the bulbils and are represented by isolate IMI 357504, deposited at the International Mycological Institute.

3.2.1.2 Differential effects of host fungi

Initially for the first 12 soils, 7 different host fungi were used. It was found however that three of these hosts, *G. graminis, Phialophora* sp. and *P. aphanidermatum,* were no better than the others for detecting the presumptive mycoparasites. As shown in Table 3.5, *G. graminis* was effective for detecting only *Gliocladium*; *Phialophora* sp. for detecting only *Gliocladium* and *P. oligandrum*; and *P. aphanidermatum* for detecting only *Gliocladium* and *Trichoderma*. *Papulaspora* was not detected on any of these three hosts. In addition, the use of these three host fungi enabled none of the mycoparasites to be detected in soils from which they were not detected by using the other four hosts (compare Tables 3.1 - 3.4 with 3.5). These findings, together with the inconvenience of using *G. graminis* and *Phialophora* sp. owing to their relatively slow or erratic growth, and of using *P. aphanidermatum* owing to the collapse of its aerial mycelia led to the omission of these three hosts from the rest of the study.

Fig. 3.3. Conidiophores of *Trichoderma* sp. as seen on agar colonised by *Rhizoctonia solani.*

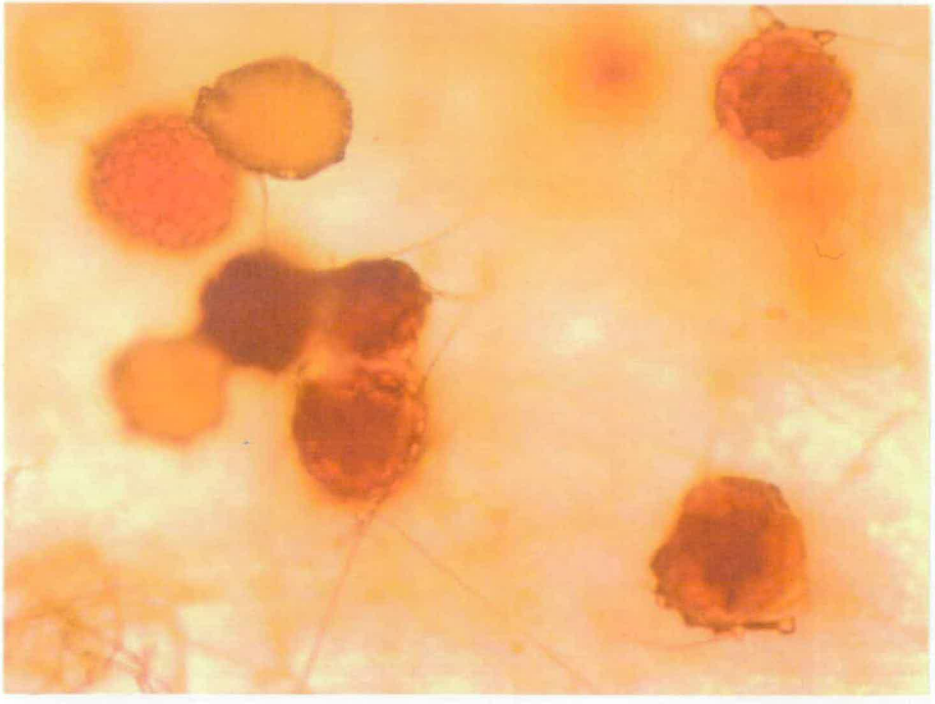


Fig. 3.4. Bulbils of *Papulaspora* sp. as seen on agar colonised by *Botrytis cinerea.*

Table 3.3. Summary of occurrence of *Trichoderma* spp. in each of 34 soil samples as evidenced by microscopical observation of agar sectors precolonised by different host fungi and inoculated with soil.*

Soil +	Fungus used to precolonise agar				Total
	Botrytis cinerea	*Fusarium culmorum*	*Rhizoctonia solani*	*Trichoderma aureoviride*	
1	2	0	6	0	8
2	0	0	1	0	1
3	2	0	0	0	2
4	0	0	2	0	2
5	0	0	1	0	1
6	1	0	6	0	7
7	0	0	1	0	1
8	0	0	1	0	1
9	0	0	4	0	4
10	6	0	6	0	12
11	1	0	2	0	3
12	1	0	1	0	2
13	0	0	0	0	0
14	0	0	0	0	0
15	0	0	4	0	4
16	0	0	0	0	0
17	0	0	0	0	0
18	0	0	3	0	3
19	2	0	0	0	2
20	6	2	1	0	9
21	3	0	1	0	4
22	5	0	2	0	7
23	0	0	2	0	2
24	6	3	4	0	13
25	0	0	6	0	6
26	0	0	2	0	2
27	3	0	3	0	6
28	2	0	1	0	3
29	6	1	6	0	13
30	6	0	6	0	12
31	2	0	3	0	5
32	2	1	4	0	7
33	6	0	6	0	12
34	4	0	6	0	10
Total	66	7	91	0	**164**

* = Data are the numbers of replicate agar sectors (maximum of 6, but 10 for soils numbered 13 - 18) on which *Trichoderma* spp. were detected.
+ = See Table 2.2.

Table 3.4. Summary of occurrence of *Papulaspora* sp. in each of 34 soil samples as evidenced by microscopical observation of agar sectors precolonised by different host fungi and inoculated with soil.*

Soil +	Fungus used to precolonise agar				Total
	Botrytis cinerea	*Fusarium culmorum*	*Rhizoctonia solani*	*Trichoderma aureoviride*	
1	0	0	0	1	1
2	0	0	1	2	3
3	2	0	0	2	4
4	5	0	0	0	5
5	3	0	0	3	6
6	0	0	0	0	0
7	1	0	0	0	1
8	2	0	0	0	2
9	0	0	0	0	0
10	1	0	0	0	1
11	6	0	1	1	8
12	1	0	0	0	1
13	6	0	0	0	6
14	2	0	0	0	2
15	1	0	1	0	2
16	2	0	0	0	2
17	0	0	0	0	0
18	0	0	0	0	0
19	6	0	0	2	8
20	1	0	0	6	7
21	3	0	0	5	8
22	3	0	0	0	3
23	3	0	0	4	7
24	0	0	0	0	0
25	0	0	0	0	0
26	0	0	0	0	0
27	1	0	0	4	5
28	5	0	0	3	8
29	2	0	0	1	3
30	0	0	0	1	1
31	6	0	0	6	12
32	6	0	0	5	11
33	2	0	0	2	4
34	5	0	0	5	10
Total	75	0	3	53	**131**

* = Data are the numbers of replicate agar sectors (maximum of 6, but 10 for soils numbered 13 - 18) on which *Papulaspora* sp. was detected.
+ = See Table 2.2.

Table 3.5. Summary of occurrence of mycoparasites in each of 12 soil samples as evidenced by microscopical observation of agar sectors precolonised by different host fungi and inoculated with soil.*

Soils	*Gaeumannomyces graminis* host				*Phialophora* sp. host				*Pythium aphanidermatum* host			
	Po	Glio	Trich	Pap	Po	Glio	Trich	Pap	Po	Glio	Trich	Pap
1	0	6	0	0	6	5	0	0	0	4	5	0
2	0	6	0	0	6	2	0	0	0	1	2	0
3	0	4	0	0	6	2	0	0	0	4	0	0
4	0	5	0	0	4	0	0	0	0	2	1	0
5	0	6	0	0	2	6	0	0	0	6	2	0
6	0	6	0	0	0	6	0	0	0	5	5	0
7	0	6	0	0	3	5	0	0	0	6	2	0
8	0	6	0	0	0	6	0	0	0	5	2	0
9	0	5	0	0	5	6	0	0	0	2	0	0
10	0	6	0	0	0	6	0	0	0	4	6	0
11	0	1	0	0	6	2	0	0	0	0	4	0
12	0	6	0	0	0	6	0	0	0	3	4	0
Total+	0	63	0	0	38	52	0	0	0	42	33	0

* = Data are numbers of fungus-colonised agar sectors (max. 6 for each host for each soil) on which the mycoparasites were detected; the mycoparasites were *P. oligandrum* (Po), *Gliocladium* spp. (Glio), *Trichoderma* spp. (Trich) and *Papulaspora* sp. (Pap).
+ = Maximum 72 per host fungus.

The four remaining hosts (*B. cinerea, F. culmorum, R. solani* and *T. aureoviride*) used for all 34 soils gave different patterns of detection of the presumptive mycoparasites. *P. oligandrum* was detected most often on sectors of *F. culmorum*, less frequently on *B. cinerea*, occasionally on *T. aureoviride* and never on *R. solani* (Table 3.1). *Gliocladium* spp. were detected on all four hosts but to a lesser degree on *F. culmorum* than on the other hosts (Table 3.2). *Trichoderma* spp. were detected on *B. cinerea* and *R. solani*, but infrequently on *F. culmorum* and never on *T. aureoviride* (Table 3.3). *Papulaspora* was detected on *B. cinerea* and *T. aureoviride*, rarely on *R. solani* and never on *F. culmorum* (Table 3.4).

In terms of the efficiency of detection of mycoparasites by single hosts, Table 3.1 shows that *P. oligandrum* was detected in the largest number of soils (18) by plating onto *F. culmorum*, whereas it was detected in only 12 soils plated onto *B. cinerea*. Also, in soils where it was detected on both host colonies it was often found on more replicate plates of *F. culmorum* than of *B. cinerea*. In only one soil (soil 32) was it detected on another host (*T. aureoviride*) when it was not detected on *F. culmorum*. So the findings indicate that *F. culmorum* could, with few exceptions, be used to detect *P. oligandrum* in most soils in which it could be detected at all by the precolonised plate method.

Gliocladium was not detected preferentially on any host (Table 3.2). It was detected in 31 soils using agar plates colonised with *B. cinerea* and *R. solani,* 30 soils using *T. aureoviride*, and 25 soils with *F. culmorum*. *Gliocladium* was only detected on plates of *F. culmorum* from a soil sample if it was detected on at least one other host. Moreover, *Gliocladium* was usually detected on more replicate plates of *B. cinerea, R. solani* and *T. aureoviride*, than on *F. culmorum*. For each soil sample placed on these fungi, *Gliocladium* was detected on at least one detector plate on each of at least two hosts, except with one soil (soil 29) from which *Gliocladium* was detected only on plates of *B. cinerea* and not on the other hosts. Thus, it seems that for best detection of *Gliocladium* at least two of the host fungi would have to be used.

Trichoderma was detected in 28 soils using plates of *R. solani*, and in 19 soils if *B. cinerea* was the host (Table 3.3); *Trichoderma* was also detected on more replicate plates of *R. solani* than of *B. cinerea*. If *R. solani* had been used on its own as the detector host it would have failed to detect *Trichoderma* in only 2 soils where it was known to occur (and was detected instead on plates of *B. cinerea*) and both these soils had low abundance of *Trichoderma*. Thus detection of *Trichoderma* in soil would require *R. solani*, as the best host of those used, for its detection in soil by the precolonised plate method.

As shown in Table 3.4, *Papulaspora* was detected most often on agar plates of *B. cinerea* (24 soils), and frequently on *T. aureoviride* (17 soils). There were only two instances in which *Papulaspora* was not detected in a soil by using *B. cinerea*, but in these cases was detected on agar colonised by *T. aureoviride*. Furthermore *Papulaspora*, when present, was observed on usually more replicate plates of *B. cinerea* than of any host fungus. Thus it would seem that for detection of *Papulaspora*, the best and most efficient host to use would be *B. cinerea*.

Sometimes more than one mycoparasite occurred on a single sector of a host; in this case any combination of two mycoparasites might be found, and occasionally three mycoparasites were detected on one host sector. The possible combination of the mycoparasites on any sector was obviously influenced by the susceptibility of the host fungus to overgrowth by the mycoparasite. *P. oligandrum* was usually the first mycoparasite to appear on any host (*ca* 7 days after inoculation), followed by *Papulaspora* or *Trichoderma* (7 - 14 days post-inoculation) and then *Gliocladium* (14 - 21 days post-inoculation).

As shown in Table 3.6, *Gliocladium*, which could be found on colonies of all four host fungi, was detected most often alone but also frequently in combination with the other mycoparasites. Sometimes it was present on agar

Table 3.6. Incidence of detection of mycoparasites in combinations with others, as evidenced by microscopical observation of single agar sectors precolonised by different host fungi and inoculated with soil.*

Combination of mycoparasites	Host fungus				
	B. cinerea	*F. culmorum*	*R. solani*	*T. aureoviride*	**Total**
Po only	44	89	0	6	139
Glio only	34	72	96	116	318
Trich only	10	1	19	0	30
Pap only	5	0	0	21	26
Po + Glio	26	34	0	9	69
Po + Trich	1	0	0	0	1
Po + Pap	1	0	0	0	1
Glio + Trich	32	6	70	0	108
Glio + Pap	46	0	1	31	78
Trich + Pap	4	0	1	0	5
Po + Glio + Trich	1	0	0	0	1
Po + Glio + Pap	1	0	0	1	2
Po + Trich + Pap	0	0	0	0	0
Glio + Trich + Pap	18	0	1	0	19

* = Data are numbers of host sectors on which mycoparasites or combinations of them were detected (maximum 228 for each host). The mycoparasites detected were Po = *P. oligandrum*; Glio = *Gliocladium* spp.; Trich = *Trichoderma* spp.; Pap = *Papulaspora* sp.

sectors with both *Trichoderma* and *Papulaspora*, but rarely in triple combinations involving *P. oligandrum*. A combination of *P. oligandrum*, *Trichoderma* and *Papulaspora* never occurred on any one host sector, although any combination of two of these mycoparasites was occasionally observed on individual host-colonised sectors. *P. oligandrum* was mainly detected alone on agar sectors, although it was also quite common in combination with *Gliocladium*, and only rarely with *Trichoderma* or *Papulaspora*. *Trichoderma* occurred much less often alone than in combination with *Gliocladium*; it was seldom detected with either *P. oligandrum* or *Papulaspora*, although it did occur in three-membered culture with *Gliocladium* and *Papulaspora*. *Papulaspora* also occurred much less often often alone than in combination with *Gliocladium*; it was rarely observed with *P. oligandrum* or *Trichoderma* unless in three-membered culture with *Gliocladium*.

3.2.1.3 Occurrence of mycoparasites in different soils

Gliocladium was detected in all 34 soils, *Trichoderma* in 30 soils, *Papulaspora* in 27 soils and *P. oligandrum* in 19 soils (Table 3.7). Moreover, all soils contained more than one mycoparasite: 3 soils had two detectable types, 20 contained three types and 11 contained all four detectable types. However these counts included several instances in which a mycoparasite was detected on only one of six (or ten) replicate host sectors. As an estimate of abundance of each of the mycoparasites in each soil, Table 3.7 shows (bold type) the cases in which a mycoparasite was detected on at least half of the replicate sectors of any one host. Judged by this criterion, one soil (soil no. 8) contained a single 'abundant' mycoparasite, 18 soils had two 'abundant' mycoparasites (most often a combination of *Gliocladium* with *P. oligandrum*, *Trichoderma* or *Papulaspora*), 15 soils had three types, and no soil had all four mycoparasites present in abundance. It was also observed that *Gliocladium* and *P. oligandrum* (in all but one soil) were 'abundant' in every soil in which they occurred, whereas *Trichoderma* and *Papulaspora* were 'abundant' in only about half the soils in

Table 3.7. Measure of abundance of presumptive mycoparasites in soil, as evidenced by detection on at least half of the replicate plates of host fungi.

Soil	Overall abundance*				Abundance when using best host+				
1	**Po**	**G**	**T**	Pap	**Po**	**G**	**T**		
2	**Po**	**G**	T	Pap	**Po**	**G**	T		
3	**Po**	**G**	T	Pap	**Po**	**G**		Pap	
4	**Po**	**G**	T	**Pap**	**Po**	**G**	T	**Pap**	nc
5	**Po**	**G**	T	**Pap**	**Po**	**G**	T	**Pap**	nc
6		**G**	**T**			**G**	**T**		nc
7	**Po**	**G**	T	Pap	**Po**	**G**	T	Pap	nc
8		**G**	T	Pap		**G**	T	Pap	nc
9	**Po**	**G**	**T**		**Po**	**G**	**T**		nc
10		**G**	**T**	Pap		**G**	**T**	Pap	nc
11	**Po**	**G**	T	**Pap**	Po	**G**	T	**Pap**	
12	**Po**	**G**	T	Pap	**Po**	G	T	Pap	nc
13	**Po**	**G**		**Pap**	**Po**	**G**		**Pap**	nc
14	**Po**	**G**		Pap	**Po**	**G**		Pap	nc
15	**Po**	**G**	T	Pap	**Po**	G	T	Pap	nc
16	**Po**	**G**		Pap	**Po**	**G**		Pap	nc
17	**Po**	**G**			**Po**	**G**			nc
18	**Po**	**G**	T		**Po**	G	T		nc
19	**Po**	**G**	T	**Pap**	**Po**	**G**		**Pap**	
20		**G**	**T**	**Pap**			T	Pap	
21		**G**	**T**	**Pap**		**G**	T	**Pap**	
22		**G**	**T**	**Pap**		G	T	**Pap**	
23		**G**	T	**Pap**		**G**	T	**Pap**	nc
24		**G**	**T**			**G**	**T**		nc
25	**Po**	**G**	**T**		**Po**	**G**	**T**		nc
26	**Po**	**G**	T		**Po**	G	T		nc
27		**G**	**T**	**Pap**		**G**	T	Pap	
28		**G**	**T**	**Pap**		**G**	T	**Pap**	nc
29		**G**	T	Pap			T	Pap	
30		**G**	T	Pap		**G**	T		
31		**G**	**T**	**Pap**		**G**	**T**	**Pap**	nc
32	Po	**G**	**T**	**Pap**		**G**	**T**	**Pap**	
33		**G**	**T**	Pap			T	Pap	
34		**G**	**T**	**Pap**		**G**	T	**Pap**	nc

* = Instances in which each mycoparasite was detected, and (bold symbols) instances in which mycoparasites were detected on at least half the replicate plates of any one host fungus.

+ = Equivalent results to those of "overall abundance" if only the findings for the best detector host for each mycoparasite are used.

nc = No change in estimate of abundance using the two different criteria.

which they were detected. Table 3.7 also compares the use of four hosts, in contrast to the use of the 'best' detector host, to measure abundance of mycoparasites in the soils. If abundance had been assessed by using the results from only the best detector host then only 21 of the 34 soils would have given equivalent results to those when all four hosts were used.

As a caveat it must be stated here that these assessments of abundance - and even presence - of mycoparasites are based solely on the results of the precolonised plate method. A potential problem with this method, as with all soil-plating methods, is that individual fungi might interfere with the detection of others by competition or antagonism on the detection plates.

Overall, as shown in Table 3.8, *Gliocladium* was detected on the largest proportion of all host sectors (65%) while the other three mycoparasites were detected to a lesser extent: *P. oligandrum* on 23% of all sectors, *Trichoderma* on 18%, and *Papulaspora* on 14%. However, with the exception of *Gliocladium*, the efficiency of detection at least doubled when the 'preferred host' of each mycoparasite was used to assess its occurrence. It is also shown, in the third column of Table 3.8, the percentage of host sectors bearing each mycoparasite when using only the results for the preferred host of each mycoparasite and only for those soils in which each mycoparasite was detected (on any host). From this it is seen that *P. oligandrum* was detected with very high efficiency on replicate host sectors in the soils where it was known to occur, *Gliocladium* somewhat less efficiently, and *Trichoderma* and *Papulaspora* even less so. But it is not known whether these results reflect only the efficiency of the method or whether they reflect also the relative numbers of propagules in soil.

3.2.2 Detection of Papulaspora in soils previously thought not to contain it

The experiments below were done as an attempt to increase the efficiency of detection of *Papulaspora* sp. and to investigate the limits of the basic precolonised plate method for detection of this fungus.

Table 3.8. Summary of percentage of subsamples of soil (= host sectors) containing the different mycoparasites.

Mycoparasite	Total % of subsamples containing mycoparasites		
	On all hosts	On Preferred host*	On preferred host, for soils known to contain the mycoparasites+
P. oligandrum	23	54	89
Gliocladium	65	74	74
Trichoderma	18	40	48
Papulaspora	14	33	42

* = Percentage positive subsamples on the host that gave the highest level of detection for a particular mycoparasite.

+ = Percentage positive subsamples on the host that gave the highest level of detection for a particular mycoparasite, for soils known to contain the mycoparasite (as evidenced by microscopical detection on any host).

3.2.2.1 Increasing the replication level

Agar sectors of *B. cinerea* were inoculated with soils 1, 2 and 6 as described in section 2.4.1, with 54 replicate host sectors per soil. Soils 1 and 2 were known to contain *Papulaspora* at low frequency, as evidenced by detection on plates of *T. aureoviride* (Table 3.4), whereas soil 6 did not contain any detectable *Papulaspora* when only 6 replicate samples were placed on sectors of each of four potential hosts. Moreover, none of these 3 soils had revealed the presence of *Papulaspora* when plated on sectors of *B. cinerea* - the best host for detection of *Papulaspora* overall (Table 3.4).

It was found when the level of replication was increased to 54 that *Papulaspora* was detected on 17 replicate sectors of *B. cinerea* for soil 6 and on one sector for each of soils 1 and 2.

3.2.2.2 Reducing competition from P. oligandrum to increase the probability of detecting Papulaspora

Agar plates with or without metalaxyl (50μg ml^{-1}) were colonised with *B. cinerea* or *F. culmorum,* then sectors of these plates were used for detection of *Papulaspora* from soil, as described in section 2.4.1. Six replicates were used per treatment, for each of 3 soils in which *P. oligandrum* had previously been detected (soils 1, 7 and 17) - see Table 3.1 - but *Papulaspora* sp. had not been detected (soil 17) or (soil 1 and 7) had been recorded infrequently - see Table 3.4.

As shown in Table 3.9, *Papulaspora* was detected on all replicate sectors of *B. cinerea*, for all soils, when the agar sectors contained metalaxyl, whereas *P. oligandrum* was never detected in these circumstances. But when metalaxyl was absent from the agar then *P. oligandrum* was detected often and *Papulaspora* rarely. *P. oligandrum* was also observed on sectors of *F. culmorum* in the absence of metalaxyl but not in its presence, while *Papulaspora* was never detected on the *Fusarium* sectors, even in the presence of metalaxyl.

Table 3.9. Incidence of detection (max. 6) of *Papulaspora* and *P. oligandrum* in three soils placed on host-colonised sectors, with or without metalaxyl.

Soil	Host	Metalaxyl	P. oligandrum	Papulaspora
1	*B. cinerea*	present	0	6
		absent	4	0
	F. culmorum	present	0	0
		absent	6	0
7	*B. cinerea*	present	0	6
		absent	0	3
	F. culmorum	present	0	0
		absent	4	0
17	*B. cinerea*	present	0	6
		absent	6	0
	F. culmorum	present	0	0
		absent	6	0

It seems, therefore, that the presence of *P. oligandrum* can inhibit growth of *Papulaspora* across agar sectors of *B. cinerea*, and that *F. culmorum* was not a suitable host for detection of *Papulaspora*.

3.2.3 Detection of mycoparasites on precolonised agar plates inoculated with host-colonised substrata that had been buried in soil

Host-colonised strips of cellulose film or segments of wheat flag leaves were prepared and treated as described in section 2.4.3; other pieces of cellulose film or wheat flag leaf were left uncolonised as controls. The host fungi used to colonise the substrata were *Rhizoctonia oryzae* and *Humicola grisea* and had been inoculated 2 and 5 days, respectively, before use. The substrata were buried in dishes of soil (soil no. 11) for 5 days then retrieved and placed on host sectors for detection of mycoparasites. The host sectors were *B. cinerea, F. culmorum, R. solani* and *T. aureoviride*. The agar colonised by *B. cinerea* contained metalaxyl at 50μg ml^{-1} to inhibit growth of *P. oligandrum* and increase the efficiency of detection of *Papulaspora*. Twenty replicate pieces of each substratum were used for each detector fungus. The presence of the presumptive mycoparasites on the agar sectors was recorded for up to 21 days incubation (section 2.4.1).

The choice of substratum, colonising fungus and detector fungus all influenced the frequency of detection of the presumptive mycoparasites, as shown in Tables 3.10 and 3.11. Chi-squared analysis was done on the data. However, *T. aureoviride* had been used only in the part of the experiment involving buried cellulose film, not wheat leaves, and was not more efficient than the others in detecting the mycoparasites so it was excluded from analysis of the data.

3.2.3.1 Effect of detector fungus

Statistical analysis (χ^2) of the total number of detections of each mycoparasite on each host for all (precolonising) treatments revealed that the detector fungus used had a highly significant effect ($P<0.01$) on the efficiency of detection of

Table 3.10. Incidence of detection (max. 20, or 60 total) of different mycoparasites from cellulose film buried in soil for 5 days; 20 replicates per treatment per host fungus.

Mycoparasite detected	Treatment of cellulose film +	Number of substratum pieces with detectable mycoparasite when plated on the following host sectors				
		B.cinerea	*F.culmorum*	*R.solani*	**Total**	*T. aureoviride*
P.oligandrum	*H. grisea*	0	9	0	9	0
	R. oryzae	0	3	0	3	0
	Control	0	4	0	4	0
Gliocladium spp.	*H. grisea*	2	1	1	4	2
	R. oryzae	2	1	2	5	0
	Control	2	0	1	3	2
Trichoderma spp.	*H. grisea*	0	0	0	0	0
	*R. oryzae**	8	0	20	28	0
	Control	1	0	7	8	0
Papulaspora sp.	*H. grisea**	17	0	0	17	6
	R. oryzae	5	0	0	5	5
	Control	0	1	0	1	1

+ = Cellulose film was uncolonised (control) or colonised by *Humicola grisea* or *Rhizoctonia oryzae* for 5 and 2 days respectively before burial in soil.

* = Denotes significant departure from expectation of similar detection of a mycoparasite from host-colonised or uncolonised substrata ($P < 0.01$), as evidenced by Chi-squared analysis.

Table 3.11. Incidence of detection (max. 20, or 60 total) of different mycoparasites from wheat leaves buried in soil for 5 days; 20 replicates per treatment per host fungus.

Mycoparasite detected	Treatment of wheat leaves+	Number of substratum pieces with detectable mycoparasite when plated on the following host sectors			
		B.cinerea	*F.culmorum*	*R.solani*	Total
P.oligandrum	*H. grisea*	0	6	0	6
	R. oryzae	0	0	0	0
	Control*	0	12	0	12
Gliocladium spp.	*H. grisea*	2	0	0	2
	R. oryzae	0	1	0	1
	Control	2	0	1	3
Trichoderma spp.	*H. grisea*	0	0	0	0
	R. oryzae	0	0	4	4
	Control	0	0	0	0
Papulaspora sp.	*H. grisea**	14	0	0	14
	R. oryzae	0	0	1	1
	Control	2	0	4	6

+ = Wheat leaves were uncolonised (control) or colonised by *Humicola grisea* or *Rhizoctonia oryzae* for 5 and 2 days respectively before burial in soil.
* = Denotes significant departure from expectation of similar detection of a mycoparasite from host-colonised or uncolonised substrata ($P < 0.01$), as evidenced by Chi-squared analysis.

different mycoparasites. The best host for detection of *P. oligandrum* was *F. culmorum,* for *Trichoderma* spp. it was *R. solani,* and for *Papulaspora* it was *B. cinerea.* There was no significantly preferable host for the detection of *Gliocladium,* but *B. cinerea* gave the higher level of detection of this fungus. These results agree with those in Tables 3.1 - 3.4.

3.2.3.2 Effect of buried substrata

The experiments with cellulose and wheat were done in identical conditions but sequentially rather than concurrently because of the amount of sampling involved, so comparisons were made with caution. Combining the result for all treatments of the cellulose film for all detector hosts for each mycoparasite, the most conspicious feature when compared to similar results for the wheat leaves, was that both *Gliocladium* and *Trichoderma* spp. were detected more often from buried cellulose film than from buried wheat leaf, whereas the incidence of detection of *P. oligandrum* or *Papulaspora* was similar for the two substrata (Tables 3.10 and 3.11).

3.2.3.3 Effect of prior colonisation of substrata

Analysis (χ^2) of the total number of detections of each mycoparasite on all hosts for each treatment of the cellulose film revealed differences in detection efficiency of the substratum. Prior colonisation of cellulose film by the two cellulolytic fungi, *H. grisea* and *R. oryzae,* markedly increased the colonisation of this substratum by some mycoparasites, relative to colonisation of uninoculated cellulose film (Table 3.10). For example, *Trichoderma* spp. were more often detected on cellulose film precolonised by *R. oryzae* than on control cellulose film or film precolonised by *H. grisea.* In contrast *Papulaspora* was more often detected on cellulose film precolonised by *H. grisea* than on control or *R. oryzae*-colonised film. There was no clear evidence that prior colonisation of the substrata influenced the subsequent colonisation by either *P. oligandrum* or *Gliocladium* spp., but any differences that might have occurred with *Gliocladium* spp. were not analysable because of the low number of total detections which

preclude Chi-squared analysis. Similar trends to these were seen when wheat leaves had been precolonised by *R. oryzae* (favouring *Trichoderma* spp.) or *H. grisea* (favouring *Papulaspora*). The incidence of detection of *Gliocladium* spp. on any of the wheat leaves was very low and so the results could not be analysed. *P. oligandrum*, however, was detected preferentially on wheat leaves that had not been colonised (Table 3.11).

3.2.4 Behaviour of a fungicide-tolerant mutant of P. oligandrum

The wild type strain of *P. oligandrum* began to show a significant reduction in extension growth across PDA plates at metalaxyl concentrations as low as 0.05μg ml^{-1}, and colony extension was effectively stopped at about 1μg ml^{-1}. From a plot of the percentage reduction of linear growth rate (expressed as probit) against logarithm of metalaxyl concentration (Fig. 3.5) the ED_{50} (estimated dose for 50% reduction of growth) was calculated as 0.20μg metalaxyl ml^{-1}.

However the fungicide-tolerant mutant of *P. oligandrum*, developed as described in section 2.4.5, grew at undiminished rate on PDA containing 50μg ml^{-1} metalaxyl. In a direct comparison the mutant strain grew at equivalent rates (colony diameter mm (24h)$^{-1}$) on PDA (58.9 ± 0.5 mm) and PDA containing 50μg ml^{-1} metalaxyl (55.0 ± 0.6 mm), and at similar rate to the wild type on PDA (56.5 ± 0.7 mm), whereas the wild type made no growth on PDA supplemented with metalaxyl.

The metalaxyl-tolerant mutant retained its tolerance for at least 2 years in culture, even when routinely sub-cultured on PDA in the absence of metalaxyl. The mutant also grew consistently well across PDA plates containing metalaxyl (50μg ml^{-1}) and previously colonised by *F. culmorum*, whereas the wild type strain never grew on these plates.

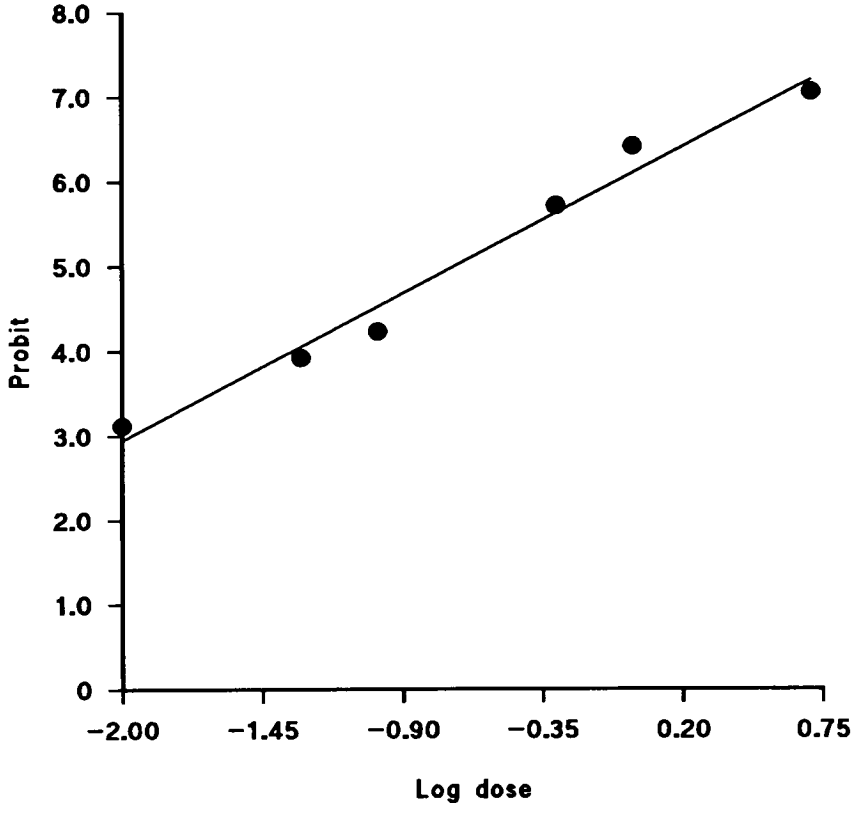


Fig. 3.5. Percentage reduction of linear growth rate (expressed as probit) of *P. oligandrum* against logarithm of metalaxyl concentration. The equation of the straight line was $y = 1.57x + 6.1$; $r = -0.99$, $P<0.001$. From this the ED_{50} (estimated dose for 50% reduction of growth) was calculated as 0.20μg ml^{-1}.

In videotaped inter-hyphal interactions (section 2.4.9), using *T. aureoviride* as a representative host fungus, the metalaxyl-tolerant mutant of *P. oligandrum* was as aggressive as the wild type: it caused host lysis and cytoplasmic coagulation as rapidly as the wild type and in all respects was indistinguishable from it. Thus it was concluded that the mutant showed stable fungicide-tolerance, evidently with no loss of fitness in terms of growth or mycoparasitic activity.

3.2.5 Use of the Most Probable Number method to determine changes in the population of P. oligandrum in soil

Organic supplements were added to soil and the effect on the population of *P. oligandrum* was monitored over time; similarily, oospores of a fungicide-tolerant mutant of *P. oligandrum* were added to soil and their survival was also recorded over time. The organic supplements were fresh green grass leaves and dried, mature, wheat flag leaves. These separate experiments were set up as described in section 2.4.7, with 4 replicate detector plates of *F. culmorum* per dilution level (two-fold dilution) for each of 3 replicate boxes of treated soil. The presence or absence of *P. oligandrum* on agar colonised by *F. culmorum* was recorded and the Most Probable Number was calculated from these results.

The Most Probable Number (MPN) method can be used to determine the density of the microorganisms present in a given sample when only their presence or absence can be determined because counts cannot be made. Use of the precolonised plate method was appropriate for determining the MPN because it is based on the principle of detecting the presence of *P. oligandrum* on host-colonised agar. The choice of replication and dilution level was made to be compatible with tables in Fisher & Yates (1963) so that MPN values could be read from the tables.

The calculation of the number of propagules present in the soil was based on determining the mean fertile level x. This is the total number of positive detector plates (number of plates of *F. culmorum* that supported oospores of *P.*

oligandrum) divided by the number of replicate plates (n) at each dilution. Then x is substituted into the equation:

$$\log y = x.\log a - K,$$

where y is the estimated number of propagules present in the soil sample, a is the dilution factor and K is a tabular entry from Table VIII of Fisher & Yates (1963) based on x. The SEM between any two samples was calculated as

$$0.55 \sqrt{(\log a/n_1 + \log a/n_2)}$$

and from this the LSD at 95% was calculated using probability tables. For performing statistical tests of significance it was necessary to use the mean fertile level (x) in preference to $\log y$ (Fisher & Yates, 1963). The use of the logarithm of x was actually employed in calculating significance between any two samples because it provides a nearly symmetrical distribution (Cochrane, 1951). A representative example of the calculation from some of the results obtained is shown in Fig. 3.6, and Appendices I and II show the tests of significance.

3.2.5.1 Changes in the populations of P. oligandrum in soil with different supplements

The effect of adding the organic supplements, wheat or grass, to soil on the population of *P. oligandrum* is shown in Fig. 3.7. In untreated soil (Control A) the population of *P. oligandrum* remained relatively constant over 280 days of sampling. When grass leaves were added to the soil initially, then a similar pattern was observed. However, when wheat leaves were added to soil, an early and progressive increase in the population resulted, so at 280 days the population of *P. oligandrum* in wheat-amended soil was still higher than that in the unamended soil, although, as shown in Appendix I, this was not a significant increase.

Other treatments involved re-supplemention of soil with grass or wheat at 150 days, shown as "2 additions" in Fig. 3.7, or the soil was left untreated (Control B). The second addition of wheat or grass caused an increase in the population

Fig. 3.6. A representative example of the calculation involved in determining (a) the infective units of *P. oligandrum* per gram of air dry soil, by the Most Probable Number method, and (b) the SEM of the sample and subsequent LSD.

(a)

Replicate	Dilutions									Total
	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	
1	4	4	4	4	4	1	1	0	1	23
2	4	4	4	4	3	2	3	0	0	24
3	4	4	4	4	3	3	1	2	0	25

This gives a mean of 24 positive detector plates.

Mean fertile level $x = 24/4 = 6$

Now,

$$\log y = x.\log a - K$$

If $x = 6$, and the dilution level is 9, then from Table VIII, in Fisher & Yates (1963), $K = 0.401$. The dilution factor is 2 ($=a$).

Therefore,

$$\log y = 6.\log 2 - 0.401$$

For each replicate dilution plate 0.35g of soil or soil/sand mixture was placed on the host, so at the lowest dilution level,

$$y = 25.41 \text{ per } 0.35\text{g of soil,}$$

giving 73 infective units of *P. oligandrum* g-1 air-dry soil.

(b)

$$\text{SEM} = 0.55 \sqrt{(\log a/n_1 + \log a/n_2)}$$

where $a = 2$ and $n_1, n_2 = 12$.

Therefore

$$\text{SEM} = 0.12$$

The Least Significant Difference (LSD) at 95% between samples, using $\log x$, was then calculated from probability tables as 0.26.

It should be noted that 3 replicate samples of soil were used in order to seek consistency of results in calculating the MPN. However, for statistical analysis the results were combined such that $n = 12$ instead of $n = 4$ at each dilution.

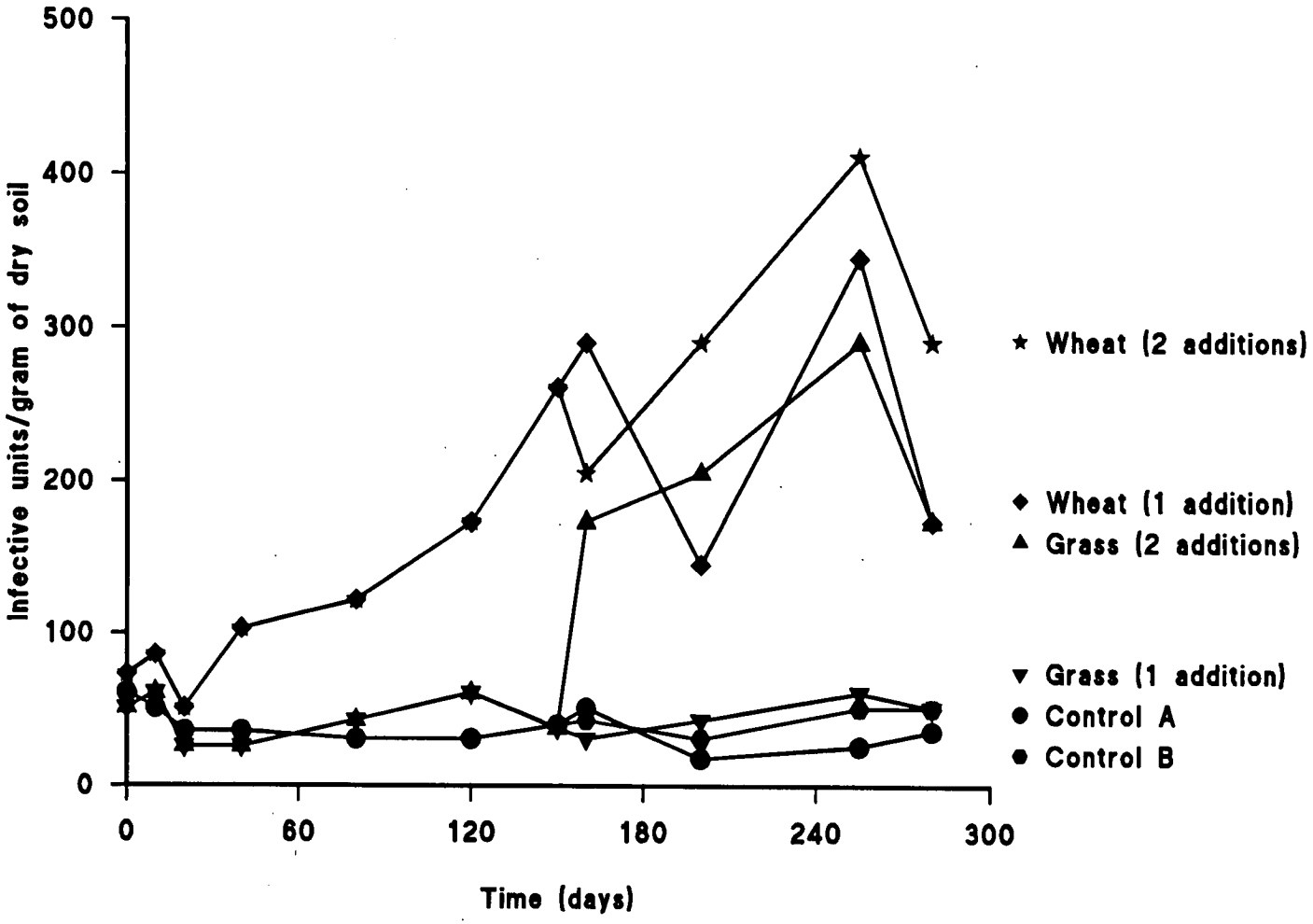


Fig 3.7. Changes in the detectable population of propagules of *P. oligandrum* in soil supplemented with 1 or 2 additions of grass or wheat, or left alone (control), as estimated by the MPN method.

of *P. oligandrum*. These increases were to significantly higher levels than the population in the control (Control B), but only at 200 days for both amendments, and also at 255 days for the wheat-amended soil only.

In general, the addition of wheat, and the repeated addition of wheat or grass, caused an increase in the population of *P. oligandrum* in soil.

3.2.5.2 Survival of oospores of P. oligandrum added to soil

The survival of oospores added to soil was determined as shown in Fig. 3.8. In control soil, with no added oospores, the detectable populations of *P. oligandrum* remained fairly constant over the whole 240 day duration of the experiment. In contrast, the total detectable population of *P. oligandrum* oospores in soil, amended with oospores of the metalaxyl-tolerant strain of *P. oligandrum*, was noticeably greater than in the control throughout the experiment. As would be expected, the detectable population of the metalaxyl-tolerant strain of *P. oligandrum* followed a similar trend to that of the total population (Fig. 3.8) but at a lower level. However these results were not significantly different from each other (Appendix II).

3.3 Discussion

Mycoparasites have commonly been detected in soil by selective isolation methods (section 1.4) but little work had focused on the spectrum of mycoparasites in soil. The precolonised agar method, used throughout this section, was particularly convenient for this because it was possible simultaneously to detect several potential mycoparasites on individual host fungal colonies. In this respect the work was based on the previous studies of Deacon & Henry (1978) and Foley & Deacon (1985) who first used *Phialophora*-precolonised agar for detection of mycoparasitic *Pythium* spp. in soil. Host-colonised agar has also been used by other workers to detect mycoparasites such as *P. acanthophoron* (Lodha & Webster, 1990) and *Verticillium biguttatum* (Boogert & Jager, 1983). The method, however, has a

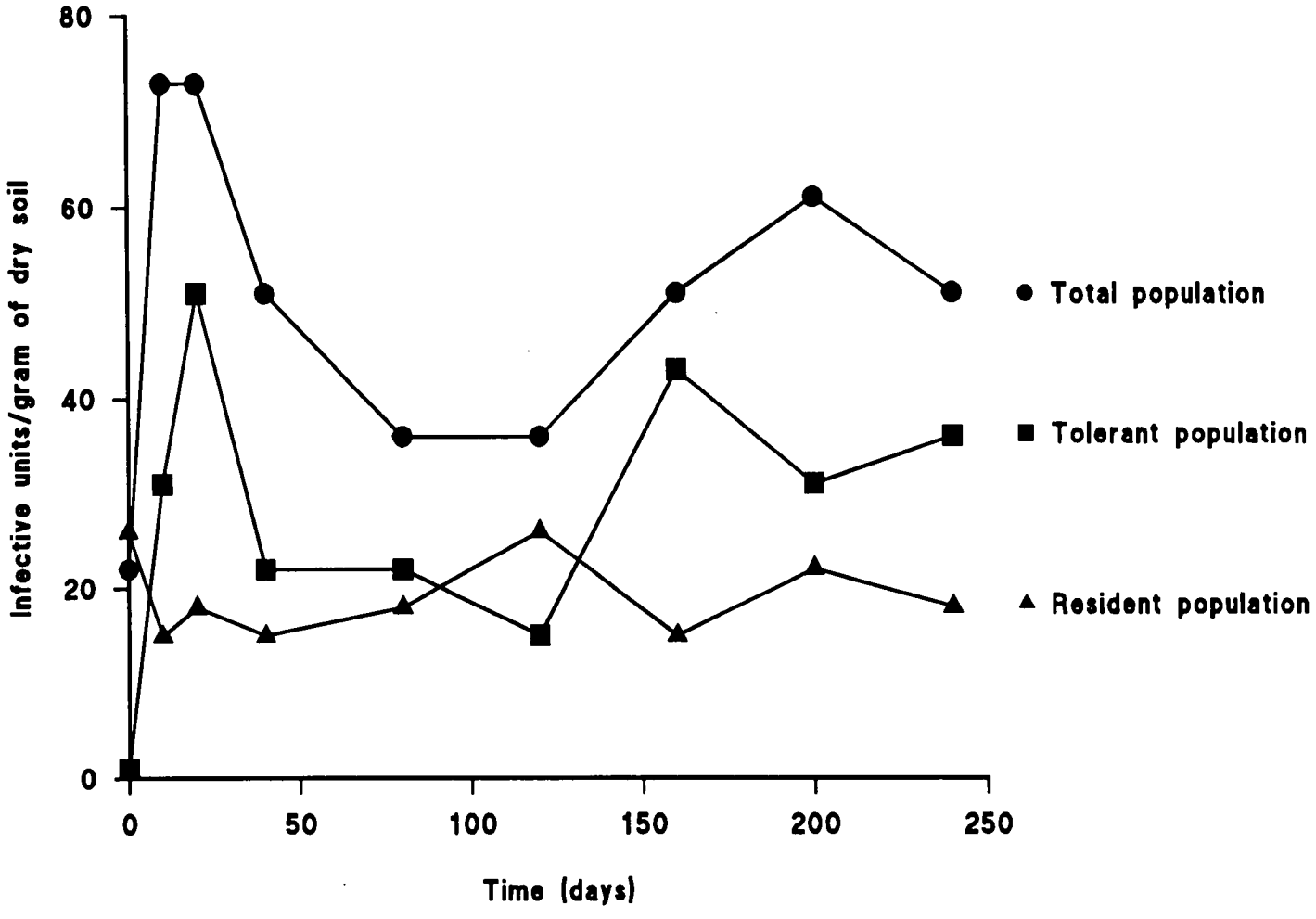


Fig 3.8. Changes in the detectable population of propagules of metalaxyl-tolerant, resident and total *P. oligandrum* in soil, as estimated by the MPN method.

longer history than this because it is based on earlier attempts to detect bacteriophage and the minute parasitic bacterium *Bdellovibrio bacteriovorus* Stolp & Starr when natural soils and water, or dilution series of these, are placed on bacterial lawns on agar (Stolp & Starr, 1963).

The method used here was simple and economical. Recycled (washed) Petri dishes and sectors of host colonies, rather than the whole colony, were used to achieve 6-fold economy on time and materials. Also, the host fungi were selected, based on an initial study of their suitability, for speed and consistency of growth on agar. The soils were air-dried to minimise the occurrence of bacteria, mites and nematodes on the precolonised agar sectors, so as to enable easy detection of the mycoparasites and facilitate their isolation into pure culture, if necessary, without the need for anti-bacterial supplements. In fact pure cultures of all the potential mycoparasites detected microscopically on the agar sectors could be obtained quite easily by subculture from the soil-free regions of the sectors onto PDA, followed by serial subculture once the mycoparasites began to grow out.

Initial studies (not shown) had attempted to use the precolonised plate method for detecting mycoparasites in fresh (undried) soil samples. This was possible in many cases, especially for *P. oligandrum*, but often the plates supported large numbers of nematodes after incubation for more than one week. The activities of these nematodes and of bacteria that grew in the spreading water film caused by the nematodes resulted in the collapse and lysis of much of the surface mycelium of host fungi, making the plates difficult to assess microscopically. Moreover, it seemed likely that detection of some mycoparasites would be reduced in such conditions - especially for those mycoparasites such as *Gliocladium* spp. that required the longer incubation times for detection. In the initial experiments in which direct comparisons were made between air-dried and fresh soils, the former yielded no fewer mycoparasites than the fresh soils. So, even if air-drying did reduce the viability of mycoparasite inoculum to some

degree (which is unknown), this was probably matched by a decrease in detection from fresh soils for the reasons given above. Two final considerations are relevant here. First, the use of air-dried soils ensured that, at least, all soil samples were treated similarly, irrespective of their initial moisture contents. Second, it was necessary to use air-dried soils for dilution series with sand and thus for estimation of population levels by the MPN method.

The use of a range of fungus-colonised agar plates to detect and isolate mycoparasites has not been investigated until now. Foley & Deacon (1985) used agar precolonised by *Phialophora* and detected primarily *P. oligandrum* in the 164 soil samples tested, and subsequently *Gliocladium* spp., *Trichoderma* spp. and *P. mycoparasiticum* (which was not detected in this study). *Papulaspora* was detected infrequently. This suggested that the use of more hosts would differentially select other mycoparasites, so this study employed more detector fungi and fewer soils to investigate the mycoparasitic spectra of soils. The soils used here were similar to those used by Foley & Deacon (1985) in that they were mainly from a range of disturbed sites, but also from some undisturbed areas.

Originally in this study seven host fungi were used but three of them were found to be no better than the others and grew erratically or presented other problems, so they were not used further (section 3.2.1.2). The remaining hosts - *B. cinerea, F. culmorum, T. aureoviride* and *R. solani* - grew rapidly and uniformly across PDA and the ability of the mycoparasites to overgrow them in pure culture was known (Laing & Deacon, 1990) so could be compared to the results from the soil detection study (section 3.2.1). *Papulaspora* was the only potential mycoparasite of those detected here that Laing & Deacon (1990) did not investigate for ability to overgrow different host fungi. But other studies (section 5.2.2) showed that, in pure culture, *Papulaspora* was able to overgrow plates of *B. cinerea* and *T. aureoviride*.

Several important features emerged from the work described in this section of the thesis, and they are discussed separately below. However, at this stage it should be noted that the mycoparasites detected in soil - *P. oligandrum*, *Gliocladium* spp., *Trichoderma* spp. and *Papulaspora* sp. - have been referred to as 'presumptive mycoparasites'. This is because, as shown later (section 5.2), *Papulaspora*, at least, had no detectable mycoparasitic activity. This raises the issue of why precolonised agar plates are able to select particular types of fungus from soil. It is true that *P. oligandrum* is an aggressive mycoparasite (Lewis *et al.*, 1989; Laing & Deacon, 1991) and might be selected for on this basis alone. Also, both *Gliocladium* and *Trichoderma* are reportedly mycoparasitic in so far as they antagonise and overgrow or displace other fungi in substrata by virtue of contact-mediated antagonism or production of diffusible antibiotics (Papavizas, 1985; Chet, 1987; Fravel, 1988). But the failure of *Papulaspora* to show any mycoparasitism or antagonism against other fungi in culture (section 5.2) suggests that a different interpretation is needed in this case. As discussed in section 5.3, it is possible that *Papulaspora* has the common properties of (a) being able to scavenge nutrients and (b) being tolerant of general metabolic by-products of other fungi.

No single host fungus was found to be suitable for detection of all the mycoparasites detected here (section 3.2.1.2). For example, *Papulaspora* was detected frequently on agar colonised by *B. cinerea*, and less so on other host fungi, while *P. oligandrum* was detected preferentially on agar plates of *F. culmorum*. Indeed the use of *B. cinerea* enabled *Papulaspora* to be detected from a large number of the soils tested, suggesting that it may be much more frequent in soils than previous records would indicate (Domsch, Gams & Anderson, 1980; Foley & Deacon, 1985). The best detector host fungus for detecting all the mycoparasites was *B. cinerea*; however if it had been used solely then it would have failed to detect mycoparasites in soils where they were recorded as abundant on other hosts. The ability of the presumptive mycoparasites to overgrow the host-colonised agar from soil was found to

correlate with results obtained *in vitro* for growth of the mycoparasites across the same host-colonised agar (Laing & Deacon, 1990).

Isolation and detection of the mycoparasites has seldom focused on the use of host fungi, and instead has focused on selective media components. *Gliocladium* spp. and *Trichoderma* spp. have been isolated and detected mostly using selective media; for example, Papavizas (1981) developed a medium, designated TME, containing a variety of antimicrobial agents which selected for *Trichoderma* spp. and eliminated other fungi and bacteria. This medium was improved by Papavizas & Lumsden (1982) for use with soils that contained high populations of Mucorales - previously these fungi had inhibited growth of *Trichoderma*. Media have also been developed for isolation and enumeration of *Gliocladium roseum* and *G. virens* (Park *et al.*, 1992), or for both *Trichoderma* and *Gliocladium* spp. (Smith *et al.*, 1990). Selective media have also been used commonly to detect and isolate pythia from soil (Vaartaja, 1968; Flowers & Hendrix, 1969; Hendrix & Campbell, 1970; White *et al.*, 1992). However the use of alternative or modified methods has been investigated recently, particularly for *P. oligandrum* (Foley & Deacon, 1985; Ribeiro & Butler, 1992). The precolonised plate method was used by Deacon (1978) and Foley & Deacon (1985), while Ribeiro & Butler (1992) used sclerotia of *Sclerotinia sclerotiorum* to select for *P. oligandrum* from soil, followed by the use of selective media to detect *P. oligandrum* in the sclerotia.

The use of the appropriate hosts increases the efficiency of detection of the different mycoparasites (Table 3.8) and this efficiency increases again when the hosts are used with soils known to contain the mycoparasites. Examination of the results showed that presumptive mycoparasites, with the exception of *P. oligandrum*, were not detected preferentially in soils with a particular pH or from soils of a particular type. *P. oligandrum* however was detected more often in disturbed soils, rarely in woodland habitats, and within the pH range 3.65 - 6.68. Previously, Foley & Deacon (1985) found *P. oligandrum* most frequently in

disturbed soils with a pH range of 5.5 - 6.5, and seldom in samples below pH 4.5. Such samples of low pH were usually from woodland. Obviously, pH could be a limiting factor for absence of *P. oligandrum* from certain soils, but it was also observed that the soil samples that *P. oligandrum* was not detected in were frequently from woodland/forest areas, suggesting that undisturbed sites were not favourable for growth of *P. oligandrum* (Table 2.2 and Table 3.1). Vaartaja (1968) and Vaartaja & Bumbieris (1964) detected *P. oligandrum* in forest nursery soils quite frequently, but the pH of all these soils was above 5.5, and the soils had recently been disturbed due to planting. Similar evidence was given by Barton (1958) that the pH and the disruption of soils affected the occurrence of *P. oligandrum*. He found that the acidity of natural soils, if less than 5.5, could prevent establishment and survival of *Pythium* spp., and *P. mamillatum* in particular, which was the primary focus of his study.

Soil type and pH would also affect the combinations of mycoparasites occurring in a soil, but assessment of this is only possible by using agar colonised by several host fungi (Table 3.6). *P. oligandrum* usually appeared first on the agar sectors, acting as a mycoparasite or as a secondary coloniser on the low level of residual nutrients. *Papulaspora* and *Trichoderma* appeared next, followed by *Gliocladium*. *Gliocladium* was frequently observed in combination with the other mycoparasites. There was no single host sector that supported growth of all four mycoparasites although almost any combination of two or three mycoparasites could occur. However, *Trichoderma* and *Papulaspora* were never observed on the same host sector, and the occurrence of *P. oligandrum* with *Trichoderma* or *Papulaspora*, with or without *Gliocladium*, was rare. There were also no observations of *P. oligandrum*, *Trichoderma* and *Papulaspora* occurring together. Indeed *Papulaspora* was never detected on *F. culmorum*, the best detector host for *P. oligandrum* (Table 3.4 and Table 3.9).

The combinations of occurrences of these fungi on host-colonised agar suggest population succession (the sequence of fungi colonising the agar sector changing

as the type and supply of nutrients changes), but the lack of some possible combinations of mycoparasites on individual host sectors when they were known to be present in a soil is indicative of competition on host sectors. The lack of *Papulaspora* on plates with *P. oligandrum* was investigated further (section 3.2.2.2) using agar colonised with *B. cinerea* or *F. culmorum,* with or without metalaxyl which inhibits growth of *P. oligandrum*. Then it was found that *P. oligandrum* prevented the detection of *Papulaspora* on host sectors of *B. cinerea* whereas the failure to detect *Papulaspora* on agar colonised by *F. culmorum* was due to the unsuitability of this host for supporting growth of *Papulaspora*.

Preliminary studies *in vitro* have shown that *P. oligandrum* was able to parasitise *Papulaspora* on agar-coated coverslips, and *Trichoderma* also could antagonise *Papulaspora*. *Trichoderma* can also antagonise *P. oligandrum* yet rarely occurred on agar sectors with it, perhaps because *P. oligandrum* rapidly overgrew the host-colonised agar and thus prevented the establishment of *Trichoderma* by competition. By the same token, *P. oligandrum* and *Trichoderma* would be expected rarely to occupy the same microniches in soil, due to the inability of each to colonise a substratum that the other had previously colonised. Substrate possession is discussed later in section 5.3 (and see Bruehl, 1975). *P. oligandrum* and *Trichoderma* were also detected preferentially on different hosts: *P. oligandrum* on *F. culmorum* which did not detect *Trichoderma*, and *Trichoderma* on *R. solani* which did not detect *P. oligandrum*.

It is possible that yet other mycoparasites occurred in the soils used in this study but were not detected because of competition from those that were detected on the hosts used. The increased detection of *Papulaspora* when *P. oligandrum* was prevented from growing by use of metalaxyl is indicative of this.

For example, *P. mycoparasiticum* has only been reported to grow on colonies of *Phialophora* sp. in culture (Laing & Deacon, 1990) and the incidence of detection of *P. mycoparasiticum* was believed to be underestimated due to

competition from *P. oligandrum*. Initially in this study *Phialophora* was used as a host fungus and attempts were made to isolate from host sectors bearing smooth-walled oogonia in the expectation that *P. mycoparasiticum* would be found. However, only *P. oligandrum* was isolated into culture from such host sectors, either because the smooth-walled oogonia represented immature oogonia of *P. oligandrum* or because *P. oligandrum* outgrew *P. mycoparasiticum* on the PDA plates.

In further work on *P. mycoparasiticum*, E. E. Jones (pers. comm.) examined arable (cereal or potato) soils known to contain high or low numbers of *P. oligandrum*. She found that on the tested fungal hosts - *B. cinerea, F. culmorum, Phialophora* sp.- using soils with little detectable *P. oligandrum* - *P. mycoparasiticum* was detected frequently on all the hosts but infrequently in soils with high levels of *P. oligandrum*. Also, it was observed that if the soil with high levels of *P. oligandrum* was serially diluted with sand, then *P. mycoparasiticum* was detected progressively more at the higher dilution levels.

Competition from *P. oligandrum* therefore appears to exclude *P. mycoparasiticum* from precolonised agar plates and thus the latter would only be expected to be detected on host-colonised agar from arable soils with low incidence of *P. oligandrum*, or from soils with high incidence of *P. oligandrum* after they had been diluted with sand. However, *P. mycoparasiticum* was not detected in the one soil (soil 32) with low incidence of *P. oligandrum* and since the soils were not diluted with sand in this study so it was not possible to deduce if *P. mycoparasiticum* was present in the soils with high incidence of *P. oligandrum*.

P. oligandrum was the only mycoparasitic *Pythium* species detected and isolated in this study. Taxonomically this mycoparasite is very similar to both *P. acanthicum* and *P. periplocum*, but reports of the others, and indeed the occurrence of all three in any one soil, are rare. Ribeiro & Butler (1992)

detected *P. oligandrum, P. acanthicum* and *P. periplocum* in 80%, 27% and 13% of soils tested using a sclerotium baiting technique, while Foley & Deacon (1985) recorded, from a possible 164 samples, *P. oligandrum* in 47 soil samples, *P. acanthicum* in 2 samples and *P. periplocum* not at all. Similar studies have reported *P. acanthicum* and *P. periplocum* to be detected infrequently in soil (Plaats-Niterink, 1975). However it is possible that *P. acanthicum*, and *P. periplocum* especially, are uncommon in British soils, or may differ in habitat requirements from *P. oligandrum*. The latter possibility is suggested by the findings of Dick & Ali-Shtayeh (1986), who assessed the distribution and frequency of *Pythium* spp. in parkland and farmland soils. These workers detected *P. oligandrum* more than *P. acanthicum*, while *P. periplocum* was rarely detected. Their results showed, with a few exceptions, that *P. oligandrum* was usually detected in different sites from those that contained *P. acanthicum*.

Another mycoparasite, *Verticillium biguttatum*, was not detected in this study, but had previously been isolated from Dutch soils using agar plates precolonised by *R. solani* (Boogert & Jager, 1983). This mycoparasite is commonly detected in soils where certain forms of *R. solani* are present - notably anastomosis group 3, which is pathogenic to potatoes (Boogert & Saat, 1991). Morris *et al.* (1993) found that *V. biguttatum* is indigenous in British soils cropped with potatoes; he isolated *V. biguttatum* from sclerotia of *R. solani* which had been removed from potato tubers in the soil. *V. biguttatum* is preferentially parasitic on AG3 of *R. solani*, and does not grow (or at least sporulate) on AG5. The reason why it was not detected in this study, even from fields cropped to potatoes, is perhaps because *R. solani* AG5 was used as the precolonising detector host.

The results of this study and those of the others discussed above suggest that the precolonised plate method is applicable for determining the mycoparasitic spectrum of soils. But even more hosts than were used here might have increased the spectra of detected mycoparasites, and the spectra might have been increased yet further by (a) selectively inhibiting some of the common

mycoparasites, such as *P. oligandrum*, and (b) diluting soil samples so as to enable other mycoparasites, such as *P. mycoparasiticum*, to grow.

Baiting and enrichment of soil have both been used to increase the detection of mycoparasites (Boogert & Jager, 1983). In this study the ability of mycoparasites to overgrow host-colonised agar (section 3.2.1) suggests that they might exhibit this ability on other substrata. This was examined by adding cellulose film or wheat leaves to soil, with or without prior colonisation of the substrata by two cellulolytic fungi *Humicola grisea* and *Rhizoctonia oryzae*. Both of these cellulolytic fungi had been isolated frequently from soil using cellulose and cellulose-mineral agar (section 4.2.1). Of the mycoparasites detected, *P. oligandrum* and *Papulaspora* were not cellulolytic (Deacon, 1976; section 5.2.4), so if they occurred on cellulose film or wheat leaves in soil then they presumably did so by using cellulolysis breakdown products generated by other fungi or by parasitism of the fungal mycelia. *Gliocladium roseum* and *Trichoderma* spp. are, in general, cellulolytic (Domsch *et al.*, 1980), so they might occur on cellulosic substrata as a direct result of degrading the substrata.

P. oligandrum, Gliocladium and *Papulaspora* were found abundantly in soil no. 11, and *Trichoderma* had been recorded there, but not abundantly (Tables 3.1 - 3.4). When this soil was baited with cellulose or wheat leaves *Papulaspora* and *Trichoderma* both were detected abundantly on retrieved strips of cellulose film, and *Papulaspora* and *P. oligandrum* were detected abundantly when wheat leaves were the bait. *Gliocladium roseum*, however, was detected in low amounts when baiting was employed, in contrast to its greater abundance in non-baited soil. The type of fungus used to precolonise the buried baits also influenced the subsequent detection of mycoparasites, most notably when *Trichoderma* was selectively favoured by precolonisation of cellulose film with *R. oryzae*, and *Papulaspora* by precolonisation with *H. grisea*, whereas *P. oligandrum* was not favoured by this precolonising treatment as much as on uninoculated cellulose film. With regard to mycoparasitic *Pythium* spp., Ribeiro

& Butler (1992) found that baiting of soil with sclerotia of *Sclerotinia sclerotiorum* was suitable for detection of *P. acanthicum, P. oligandrum* and *P. periplocum* when the sclerotia were subsequently plated onto agar, but these workers did not imply that the sclerotial baits acted by providing fungal host material for parasitism by the pythia; sclerotia were used merely for convenience.

The precolonised plate method was used here to determine the population level of *P. oligandrum* in soil. This was found to increase with repeated addition of organic supplements - especially when wheat leaves were added to soil (Fig. 3.7). In soil with no amendments the population of *P. oligandrum* remained relatively stable. *P. oligandrum* is non-cellulolytic (Tribe, 1961) so probably would not benefit from cellulolytic components of these substrata, but might have used other components in competition with the resident soil microflora or grown at the expense of other fungi that used these materials, as in many dual culture studies on cellulolytic substrata (Al-Hamdani & Cooke, 1983; Laing & Deacon, 1990). The interesting and unexplained feature of this experiment was that the population of *P. oligandrum* increased quite rapidly in response to a single supplement of dried wheat leaves, but not at all in response to green grass leaves, perhaps owing to competition from many microorganisms that would be able rapidly to exploit green leaf material. Yet *P. oligandrum* did respond to a second addition of grass leaves. This cannot be explained by hypothesising that the resident soil population of *P. oligandrum* was initially dormant (constitutively dormant oospores) and required an ageing period before germination could occur (Ayers & Lumsden, 1975) because there was an early response to the wheat leaf supplement. Instead, it might be suggested that the initial grass supplement 'primed' the population of *P. oligandrum* so that it could respond to a further supplement.

The addition of nutrients to soil in an attempt to enhance soil suppressiveness to pathogens has been investigated by many workers. For example, soil

suppressiveness to *P. ultimum* was found to be associated with chemical (chloride) and biological (*P. oligandrum*) factors (Martin & Hancock, 1986). High chloride levels in soil were found to be inhibitory to *P. ultimum* and reduced its colonisation of added leaf debris while simultaneously increasing the saprophytic colonisation of the leaf debris by chloride-tolerant *P. oligandrum*. Colonisation by *P. oligandrum* was more effective when the *P. ultimum* inoculum densities were lower. It was postulated that competition rather than parasitism was occurring on the leaf debris, but in further experiments on the effects of crop debris on the populations of *P. oligandrum* and suppression of *P. ultimum*, F. N. Martin (pers. comm.) could not explain the effects solely in terms of competition for the crop residues and considered that mycoparasitism by *P. oligandrum* could have displaced *P. ultimum* after its initial colonisation of the debris.

Similarily, Paulitz & Baker (1987a,b, 1988) extensively studied the effects of organic amendments and *P. nunn* on suppression of a phytopathogenic *Pythium* sp. They found that *P. nunn* required organic substrates in order to increase its population saprophytically and therefore out-compete *P. ultimum*. *P. nunn*, in this case, did not appear to survive by parasitism but by competition, utilising the nutrients that would otherwise be available to the phytopathogen. No differences were observed between types of organic amendment (Paulitz & Baker, 1987b), but repeated additions of organic amendments were necessary for disease suppression. This correlated with the results of Lifshitz *et al.* (1984b) who added bean leaf meal to soils which contained low initial population levels of *P. nunn.* Repeated addition of the substrate decreased the population of the plant pathogenic *Pythium* while increasing the population of *P. nunn*.

The results from these studies agree with those in section 3.2.5.1 (Fig. 3.7) where a repeated addition of organic supplements progressively increased the populations of *P. oligandrum*. Although the population of a plant pathogen was

not monitored, the results show the potential of manipulating the soil conditions by addition of nutrients to increase the population of *P. oligandrum*.

In a different approach, the addition of oospores of *P. oligandrum* to soil, and their subsequent survival and germination, was investigated (section 3.2.5.2, Fig. 3.8). The oospores were of a fungicide-tolerant mutant of *P. oligandrum*, marked so that the population level could be monitored separately from that of the resident population of *P. oligandrum*. Addition of the oospores increased the total detectable population of *P. oligandrum* to a level higher than that of the resident population alone, and the effect was evident even after 240 days in soil. The results showed the ability of oospores added to soil to survive and maintain a population tolerant to metalaxyl. The persistence of such fungicide-tolerant populations in soil was reported by White *et al.* (1988, 1992), where metalaxyl-tolerant populations of *P. oligandrum* were found to abound in soils that had been treated with metalaxyl in attempts to control *P. violae,* the cause of cavity spot of carrot.

Paulitz & Baker (1987a) found that when oospores of *P. nunn* were added to soil, no increase in the population of *P. nunn* was detected for the first 30 days, but a ten-fold increase occurred after the addition of 0.3% ground bean leaves, and remained high after 50 days. If the raw soil had been treated with aerated steam, then initial populations of *P. nunn* increased to an even greater degree than after the addition of the bean leaves alone. Also, the addition of oospores of *P. nunn* to the soil increased the original level significantly, and the high levels were sustained for at least 60 days.

In the experiment in section 3.2.5.2, it was observed that the fungicide-tolerant mutant of *P. oligandrum* was rarely observed on plates of *F. culmorum* inoculated with soil from sample day zero. Yet detection of *P. oligandrum* was observed on plates colonised by *F. culmorum* when inoculated with soil samples taken after 10 days incubation. Lack of maturation of the oospores could

explain their lack of germination when first added to soil (Ayers & Lumsden, 1975) but the oospores were harvested after 7 weeks in culture so they would have been relatively mature. In addition, results (not shown) demonstrated that they germinated easily on PDA without further maturation.

In order to germinate, oospores may need to be activated from their dormancy by an external factor, such as a particular nutrient (Garraway & Evans, 1984b). PDA provides such nutrients whereas the nutrient levels on agar precolonised by a host fungus may be insufficient. However, once the oospores have been in soil it seems that their dormancy is broken, or their germinability enhanced by other factors, so that they are then detectable when soil is placed on the precolonised plates. Activation in soil could result from the activities of the soil microflora, and their enzymes acting on the oospore wall, possibly making the wall more permeable to exogeneous nutrients and thus inducing activation (Stanghellini & Burr, 1973).

Stanghellini & Russell (1973) demonstrated an increased germination of oospores that had been passed through snail intestines, in comparison to those that were untreated. They suggested that the enzymes present in the snail intestine increased the permeability of the oospore wall. The oospores of *Pythium aphanidermatum* were found to require an external source of calcium and carbohydrate in order to germinate. Stanghellini & Russell (1973) suggested that the calcium was taken up by the oospore which induced absorption of the endospore wall and hence broke the dormancy rendering the oospore active. The production of a germ tube then followed and this was dependent on an external source of carbohydrate.

If it is anticipated that oospores of mycoparasitic *Pythium* spp. could be added to soil to increase the potential degree of soil suppressiveness, or even to introduce fungicide-tolerant strains for use in conjunction with fungicides, then the effects of different methods of production of oospores should be examined.

Hendrix (1970) considered that oospores produced artificially might not act in a biologically similar manner to those produced naturally. Several workers have found that a period of water-storage of oospores of *P. oligandrum*, after removal from the media in which they were produced, can enhance the degree of germination (Walter & Gindrat, 1987a), perhaps paralleling the effect that an initial 10 days incubation in soil had on enhancing germination (detection) of oospores in this work.

Irrespective of the above, the use of a marked strain of *P. oligandrum* has applications in the study of germination of oospores in soil. Foley & Deacon (1985) compared germination of oospores of *P. oligandrum* on host-colonised agar plates to that on PDA, and thereby calculated the efficiency of detection of germinable oospores from soil placed on host-colonised agar plates. They found the efficiency of detection from soil to be 71-88% when correction was made for the detection (germination) efficiency on precolonised agar. If known numbers of 'marked' oospores were added to soil treated with various supplements, then their germination could be followed by taking similar factors into account.

The findings from the experiments in which soil was supplemented with oospores of *P. oligandrum*, or supplemented with organic substrata, show promise for attempts to manipulate the soil population of *P. oligandrum*. It should also be recognised that the success of these treatments is dependent on the inoculum density of a pathogen, and the ability of the antagonist to compete with the pathogen (Paulitz & Baker, 1987a,b). Nevertheless an enhancement of the populations of metalaxyl-tolerant strains of mycoparasites in soil, following introduction of such strains where they do not exist naturally, could be a useful practical adjunct to the use of metalaxyl for control of phytopathogenic *Pythium* spp.

4. INTERACTIONS BETWEEN FUNGI

4.1 Introduction

P. oligandrum is known to parasitise a wide range of fungi and has potential as a biocontrol agent of several fungal plant pathogens (Vesely, 1977; Whipps *et al.*, 1988; Lewis *et al.*, 1989; Laing & Deacon, 1990). But its activities in soil have received little attention, and even its influence on the common soil saprophytes has received less study than its effects on plant pathogens. Thus, we do not know if *P. oligandrum* is a significant regulator of the populations of soil fungi. In the first studies of *P. oligandrum* in soil, Tribe (1961) found *P. oligandrum* growing in association with other fungi on buried cellulose film and suggested that it grew by utilising the small organic substrates released from cellulose by the activities of cellulolytic fungi. This mode of behaviour had earlier been described as secondary sugar saprophytism (Garrett, 1951). Tribe considered it to be a form of parasitism, but not aggressive, invasive parasitism of the hyphae of the cellulolytic fungi. He also noted that *P. oligandrum* grew well when inoculated on cellulose film *in vitro* in the presence of some cellulolytic fungi but not others (Tribe, 1966). Deacon (1976) confirmed this for the same cellulolytic fungi that Tribe had used. Since then there seem to have been no further studies on the interaction of *P. oligandrum* with fungi isolated from cellulosic substrates in soil.

The work in this section examined the interaction of *P. oligandrum* with a range of soil fungi in an attempt to determine whether *P. oligandrum* can significantly affect the activities of cellulolytic soil fungi. These studies involved interactions of whole colonies and also of individual hyphae.

4.2 Results

4.2.1 Isolation of cellulolytic fungi from soil

A range of potentially cellulolytic fungi were isolated from soil no. 11 and tested for cellulolytic activity, as described in section 2.4.2. All the fungi isolated were cellulolytic with the exception of *Mortierella*-like fungi and *P. oligandrum*. These fungi were very fast growing and the *Mortierella* spp. were perhaps isolated from spores that contaminated the cellulose film or, like *P. oligandrum*, might have been growing in association with cellulolytic fungi, utilising some of the cellulose breakdown products. The most common cellulolytic fungi isolated from this method were referable to the genera *Humicola*, *Rhizoctonia*, *Fusarium* and *Chaetomium*; a range of less common fungi, usually with sterile mycelia, could not be identified. For future experiments three of the fungi were selected: a *Fusarium* sp., *Humicola grisea* and *Rhizoctonia oryzae*. These three fungi were the most frequently detected when cellulose film was buried in the soil, and they had characteristic, recognisable growth forms on cellulose film in pure culture, which enabled their naturally occurring colonies to be identified when cellulose film was retrieved from soil.

4.2.2 Interactions with cellulolytic fungi in media containing different cellulose sources

The effects of *P. oligandrum* on the cellulolytic activities of *Fusarium* sp., *Humicola grisea* and *Rhizoctonia oryzae* were tested *in vitro* using different cellulose sources.

4.2.2.1 Interactions on cellulose film

Agar plates bearing films of cellulose were inoculated with fungi, alone or in combination, and the cellulolytic activity of the fungi was assessed as described in section 2.4.8.1. *H. grisea*, *Fusarium* sp. or *R. oryzae* were inoculated alone or in combination with *P. oligandrum*. *P. oligandrum* was also inoculated alone. Four replicate plates were used in all cases and paired-samples *t*-tests were applied to

the results, using arcsine values of percent reduction of cellulolysis caused by each fungus when *P. oligandrum* was present.

As shown in Table 4.1, each cellulolytic fungus alone caused major strength loss of the cellulose film, whereas *P. oligandrum* alone caused virtually no strength loss. In the presence of *P. oligandrum*, the cellulolytic activity of both *H. grisea* and *Fusarium* sp. was significantly reduced, indicating that these fungi were susceptible to attack from the mycoparasite whereas there was no significant effect of *P. oligandrum* on the cellulolytic activity of *R. oryzae*.

4.2.2.2 Interactions on filter paper

The three cellulolytic fungi were inoculated into flasks alone, or in combination with *P. oligandrum* or *P. ultimum*. The *Pythium* spp. were also inoculated alone. The methods were as described in section 2.4.8.2, with three replicate flasks per treatment, and sampling every 2 weeks. However it was soon evident that *R. oryzae* could not utilise the inorganic nitrogen source in the medium, and thus could not degrade the filter paper, so for this fungus the experiment was repeated using L-asparagine (1.51g l^{-1}) as an organic nitrogen source and the flasks were sampled every 4 weeks. One-way analysis of variance was carried out on all results at each sample time.

The cellulolytic activity of *H. grisea* (Fig. 4.1) was sustained for at least 8 weeks, whereas the *Pythium* spp. alone caused negligible weight loss of the filter paper. Analysis of variance showed that, at all sampling times from 2 weeks onwards, the weight loss of the flask contents caused by *H. grisea* was significantly reduced in the presence of *P. oligandrum*. In the presence of *P. ultimum* the cellulolytic activity of *H. grisea* was significantly reduced at 2 weeks but the weight loss of flask contents was enhanced at 4 and 6 weeks compared with that caused by *H. grisea* alone, and then significantly reduced at 8 weeks.

Table 4.1. Percent reduction in strength of cellulose film when inoculated with cellulolytic fungi alone or in combination with *P. oligandrum*; means ± SEM for 4 replicates.

Cellulolytic fungus	With P. oligandrum	Percent cellulose strength loss*	Significance+ (P)
Humicola grisea	No	80.8 ± 1.4	
	Yes	36.5 ± 4.0	0.05
Fusarium sp.	No	84.9 ± 0.4	
	Yes	50.3 ± 7.2	0.05
Rhizoctonia oryzae	No	66.7 ± 5.8	
	Yes	72.6 ± 6.7	nsd
None	Yes	4.0 ± 2.3	

* = Calculated from the weight supported by inoculated cellulose film compared with the weight supported by uninoculated film, using a penetrometer at 7 assessment points on each strip of film.

+ = Significance of difference (P) for each cellulolytic fungus in the presence or absence of *P. oligandrum* is shown; nsd = no significant difference.

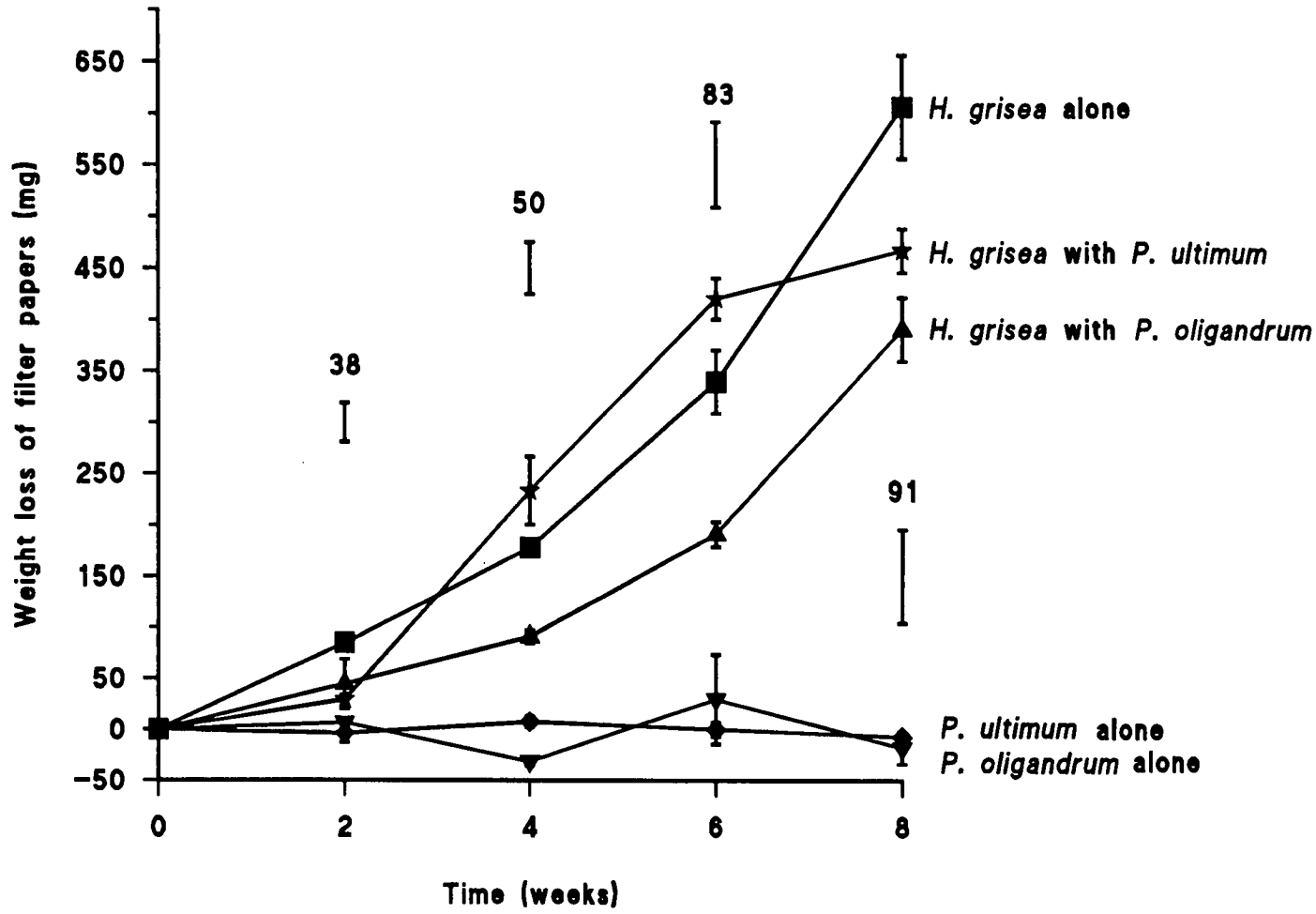


Fig. 4.1. Weight loss of filter papers over 8 weeks when inoculated with *Humicola grisea* in the presence of absence of *P. oligandrum* or *P. ultimum*; means ± SEM for 3 replicates. Numbered bars show 5% LSD at each sampling time based on analysis of variance.

Fusarium sp. alone (Fig. 4.2) continued to degrade the filter paper during 8 weeks. The weight loss of flask contents caused by *Fusarium* sp. was consistently lower in the presence of *P. ultimum*, but most of this reduction of cellulolytic activity occurred in the first 4 weeks, after which the rate of weight loss was similar in the presence and absence of *P. ultimum*. In contrast, *P. oligandrum* caused a major progressive reduction in the weight loss of flask contents and thus in the cellulolytic activity of *Fusarium* sp.

In contrast to the effects on *H. grisea* and *Fusarium* sp., *P. oligandrum* had only a slight effect on *R. oryzae* (Fig. 4.3).

4.2.3 Video analysis of hyphal interactions between P. oligandrum and three cellulolytic fungi

Agar-coated coverslips were prepared as described in section 2.4.9 and inoculated with *P. oligandrum* opposed to each of three cellulolytic fungi: *H. grisea, Fusarium* sp. or *R. oryzae*. The coverslips were incubated on agar plates and transferred to observation chambers as colonies approached one another. Video recordings were usually begun just before individual hyphae made contact with one another, either when a tip of the mycoparasite approached the side of a cellulolytic fungus (tip to host side) or when a tip of a cellulolytic fungus approached a mycoparasite side (side to host tip). Tip to tip interactions were infrequent and were disregarded; most observations involved contact of a mycoparasite tip with a host side.

The main damaging parasitic actions of the mycoparasite on any of the hosts were as follows.

1. Lysis: sudden expulsion of a large amount of cytoplasmic content from the attacked compartment at the point of contact of the host hypha.
2. Leakage lysis: expulsion of a small amount of the host cytoplasm from the attacked compartment at the point of contact with the host hypha.

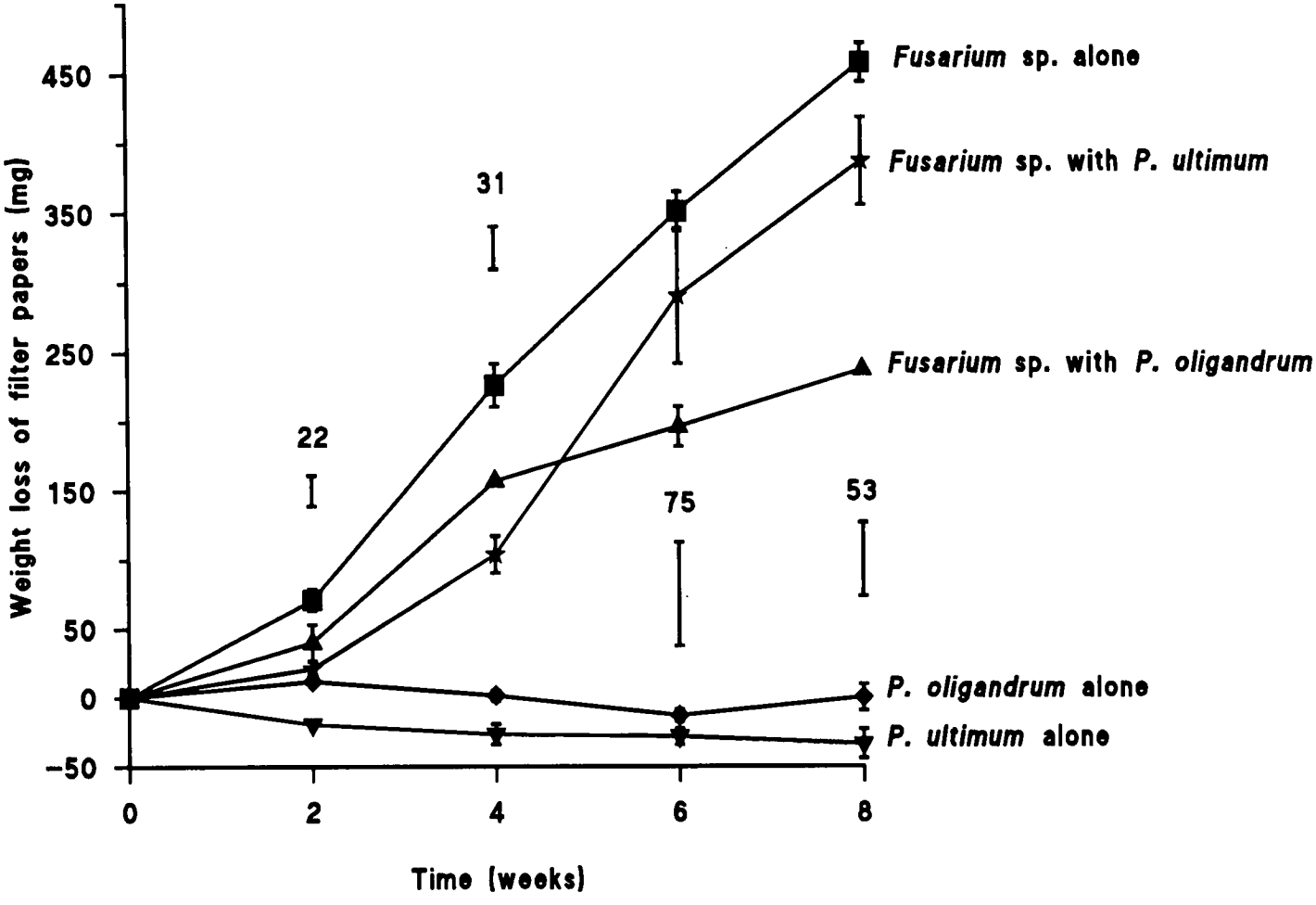


Fig. 4.2. Weight loss of filter papers over 8 weeks when inoculated with *Fusarium* sp. in the presence of absence of *P. oligandrum* or *P. ultimum*; means ± SEM for 3 replicates. Numbered bars show 5% LSD at each sampling time based on analysis of variance.

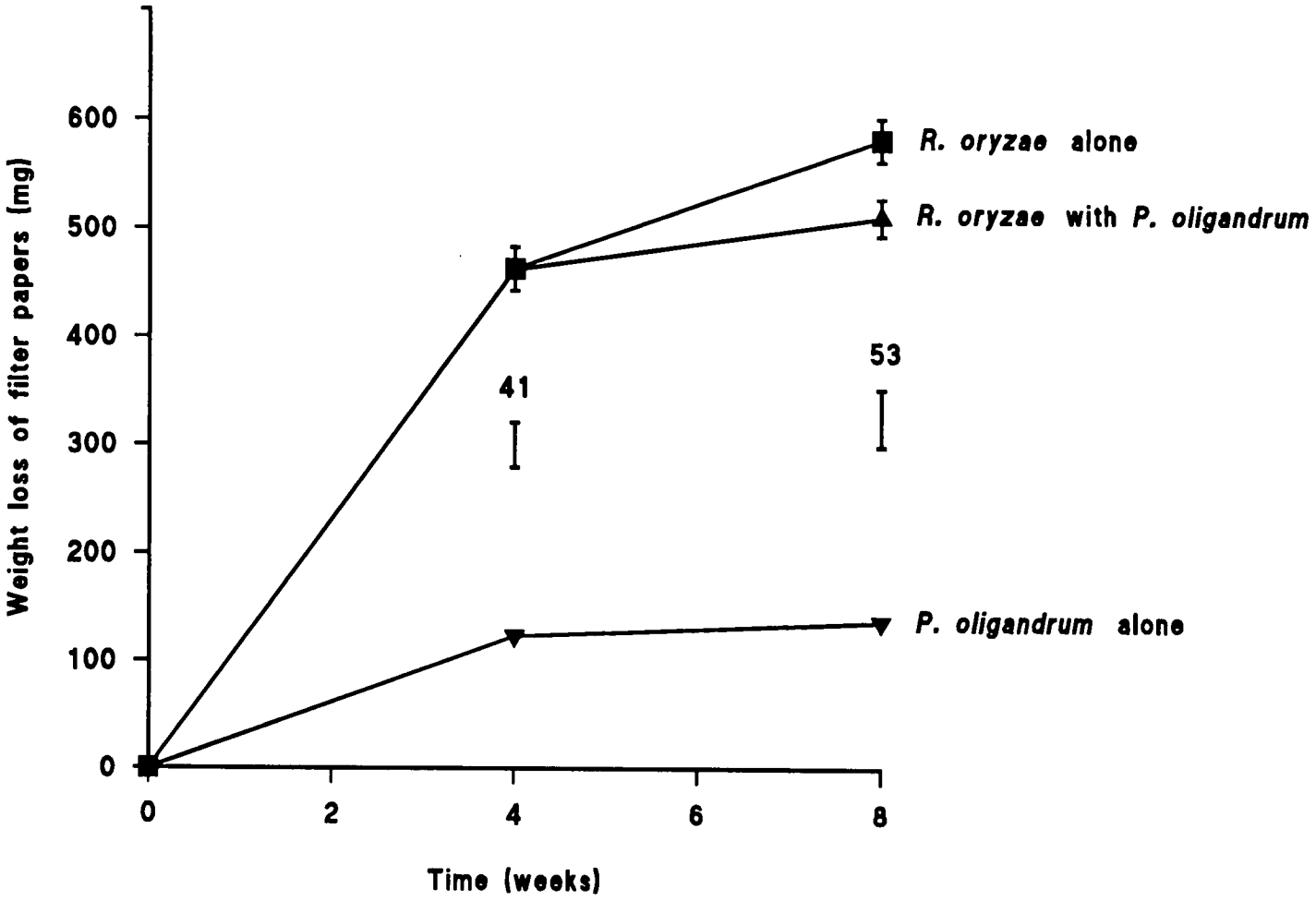


Fig. 4.3. Weight loss of filter papers over 8 weeks when inoculated with *Rhizoctonia oryzae* in the presence of absence of *P. oligandrum*; means ± SEM for 3 replicates. Numbered bars show 5% LSD at each sampling time based on analysis of variance.

3. Coagulation: coagulation or appearance of granulation or vacuolation of the host cytoplasm in the contacted hyphal compartment, occurring some time after host and parasite contact, but appearing rapidly in much or all of the host compartment after it was initiated.

These events were usually independent of each other, although occasionally after a lysis occurred any cytoplasm remaining in the damaged hypha coagulated. The occurrence of any of the above sometimes was associated with penetration of the host hypha by a hypha of the mycoparasite. Although these three major effects - lysis, leakage lysis and coagulation - were found in the different pairings of fungi, their incidence and timing in replicate interactions differed considerably and there were also differences between the host fungi in these respects. The following sections analyse these differences in more detail.

4.2.3.1 Interactions of P. oligandrum *with* Fusarium *sp.*

Fusarium sp. was found to be susceptible to attack from the mycoparasite, with lysis or coagulation as the main conspicious damaging events. As shown in Table 4.2, a total 10 interactions were recorded in detail for incidence and timing of different events. Many similar interactions were observed but not recorded in detail. Nine of the ten recorded contact events led to rapid disruption of the host hypha after contact: lysis or coagulation of the host compartment was observed within 4 - 6 min of contact. Contact between mycoparasite tips and host sides resulted in the tips of the mycoparasite hyphae growing on past the contact point and sometimes resulted in induction of branching of the mycoparasite at the point of contact (in 2 of 4 instances). This branching was only observed in 1 of the 6 contacts involving the side of the mycoparasite with the tip of the host. In mycoparasite tip to host side interactions where the mycoparasite branched, the original (contacting) tip of the mycoparasite usually grew on past the point of contact but was sometimes observed to stop upon parasitism of the host. The growth of the mycoparasite was not affected in mycoparasite side to host tip interactions or when no branching occurred in mycoparasite tip to host side interactions. Host tips did

Table 4.2. Summary of the timed events after hyphal contact in interactions between *Fusarium* sp. and *P. oligandrum*.

Event	Mycoparasite tip to host side		Mycoparasite side to host tip	
	Incidence	**Time (min) post-contact**	**Incidence**	**Time (min) post-contact**
Lysis	3 (1)*	4 - 6	4	4 - 6
Coagulation	1 (1)	nd	1 (1)	4
No effect	0	na	1	na
Total	**4**		**6**	

nd = Not determined.
na = Not applicable.
* = Number of penetrations.

not usually stop growing upon contact with the mycoparasite but did so later upon being parasitised. In mycoparasite tip to host side interactions parasitism only resulted in stoppage of the host if the side of the tip cell was contacted. A representative example of the types of interaction occurring is described below, and shown in Fig. 4.4.

Specific example

A mycoparasite tip contacted the side of the host hypha, *ca* 37μm behind the host tip (Fig. 4.4a). The hypha of the mycoparasite continued to grow along the host hypha for 2 min at a rate of 37μm min^{-1} and then a branch formed, emerging at the initial point of contact between the mycoparasite and host (Fig. 4.4b). During branch emergence and growth of the branch the tip of the mycoparasite hypha grew at a diminished rate (13μm min^{-1}) and arched over the host hypha (Fig. 4.4c). A second branch emerged further along from the point of contact at *ca* 5 min post-contact (Fig. 4.4d). A surge of protoplasm towards the host tip was evident at 5 min 12 sec post-contact, and subsequent minor surges were seen at 7 min 26 sec and at 9 min 17 sec. The original tip of the mycoparasite and that of the host stopped growing upon surging of the host cytoplasm. Coagulation of host cytoplasm was then seen towards the first branch at 9 min 58 sec post-contact (Fig. 4.4e). This coagulation caused loss of cytoplasmic movement in the host hypha from the host tip back to the position of the first mycoparasite branch, but host cytoplasm further back from this point was unaffected. Host lysis then occurred at 10 min 2 sec post-contact (Fig. 4.4f). It occurred at the position of the 2nd branch of *P. oligandrum* and resulted in evacuation of the fluid host protoplasm back from this point. No penetration by *P. oligandrum* was seen, even at 25 min post-contact.

Penetration was not observed in this example, and was only found to occur in 3 of the 9 possible instances of timed interactions. Penetration of the host hypha from a branch resulted in growth of the mycoparasite hypha through the host. Upon meeting each septum, the mycoparasite hypha caused coagulation of the

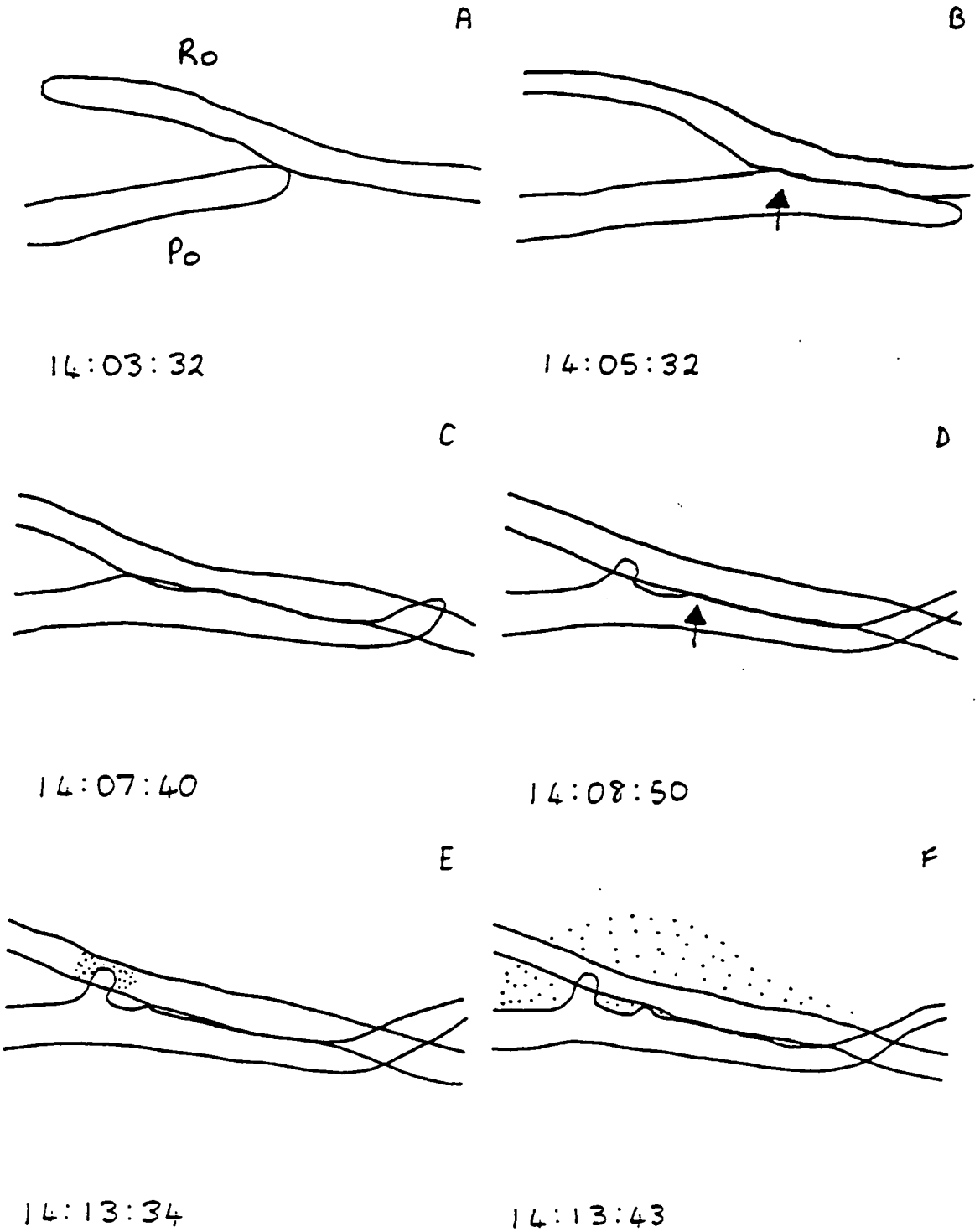


Fig. 4.4 (a) Tip of a hypha of *P. oligandrum* (Po) contacts the side of a hypha of *R. oryzae* (Ro). (b) A branch from the mycoparasite (arrowed) forms at the point of contact between the host and the mycoparasite. (c) Tip of the mycoparasite continues to grow. (d) A second branch emerges from *P. oligandrum* (arrowed) at a subsequent point along the mycoparasite hypha. (e) Coagulation of the host cytoplasm (indicated by dots in the host hypha) occurs at the first point of contact . (f) Lysis of the remaining host cytoplasm (lysate indicated by dots around the host hypha) occurs at the second point of contact.

adjoining compartment before it grew through the septum and on to the next. Internal growth rate of the mycoparasite hypha was measured at 27μm min^{-1} in representative examples; this rate did differ from typical external rates of growth of *P. oligandrum* (32μm min^{-1}) on water agar films.

Thus *Fusarium* was susceptible to attack, with most parasitic events occurring within 4 - 6 mins, and the damage to the host in interactions was not restricted to either type of contact between host and mycoparasite.

4.2.3.2 Interactions of P. oligandrum *with* H. grisea

H. grisea was also found to be susceptible to parasitism by *P. oligandrum*, with lysis and coagulation as the damaging conspicious events. A total 12 interactions were recorded, as shown in Table 4.3, and in only 1 of these did the interaction not have an effect on the host. In 10 of the other 11 interactions host damage occurred in 3 - 14 min of contact, and in the other interaction coagulation took place 28 min post-contact. The interactions were similar to those described for *Fusarium*, with branching of the mycoparasite occurring in 3 of 9 possible instances of contact between mycoparasite tips and host sides, but not at all in contacts between mycoparasite sides and host tips. Penetration of the host hypha resulted twice after lysis and 3 times after coagulation, but it was only observed in mycoparasite tip to host side interactions.

4.2.3.3 Interactions of P. oligandrum *with* R. oryzae

Originally two strains of *R. oryzae* were used - J336 and S1,23 - but no differences were observed in their interactions with *P. oligandrum* so only strain S1,23 was used for detailed study of hyphal interactions with the mycoparasite. Two approaches to recording interactions between the host and the mycoparasite were used: either each interaction was recorded from 5 - 10 min pre-contact until 90 min post-contact, usually by time-lapse recording, or interactions were allowed to take place and then each coverslip was scanned and the different types of interactions were recorded. The main parasitic events

Table 4.3. Summary of the timed events after hyphal contact in interactions between *H. grisea* and *P. oligandrum*.

Event	Mycoparasite tip to host side		Mycoparasite side to host tip	
	Incidence	**Time (min) post-contact**	**Incidence**	**Time (min) post-contact**
Lysis	4 (2)*	3 - 10	2	14
Coagulation	5 (3)	5 - 28	0	na
No effect	0	na	1	na
Total	**9**		**3**	

na = Not applicable.
* = Number of pentrations.

in each interaction could still be observed when scanning was used. Furthermore, when individual interactions were followed in detail, the timing of parasitic events was so variable that it was unrealistic to calculate means for each type of interaction; however, where appropriate, the time range after which the events occurred is mentioned. Thus the frequency rather than timing of events in replicate interactions provided a more realistic appraisal of parasitism of *R. oryzae*, using Chi-squared analysis to test for significance. It should be noted, however, that no control experiment was carried out to investigate the effect of contact on *R. oryzae* when opposed to itself rather than *P. oligandrum*.

In total, 80 incidences of contact were observed between the two fungi. They included 52 instances in which the tip of a mycoparasite hypha contacted the lateral wall of a host hypha (usually in the apical or sub-apical compartment of the host) and 28 instances in which a host tip contacted the side of *P. oligandrum*. The events in these two types of contact are summarised in Figs 4.5 and 4.6. The interactions between *P. oligandrum* and *R. oryzae* were termed 'resistant' if the contact did not have a damaging effect on the hypha of *R. oryzae* (in 19 instances), and 'susceptible' if the compartment of *R. oryzae* in contact with *P. oligandrum* was adversely affected (61 instances). In some instances, the hypha of *P. oligandrum* branched at the point of contact, and sometimes this branch coiled round the hypha of the host. Subsequent parasitic events included fast lysis, leakage lysis or coagulation of the host cytoplasm. Thereafter *P. oligandrum* sometimes penetrated the host hypha, but in other cases the contact led to apparently permanent stoppage of growth of the mycoparasite hyphae. Further details are given below.

Pre-contact events

In mycoparasite tip to host side interactions the extension rates of hyphal tips of *P. oligandrum* were found to be similar before contact and after contact. This was also true for the host if it resisted parasitism, but if it was susceptible then

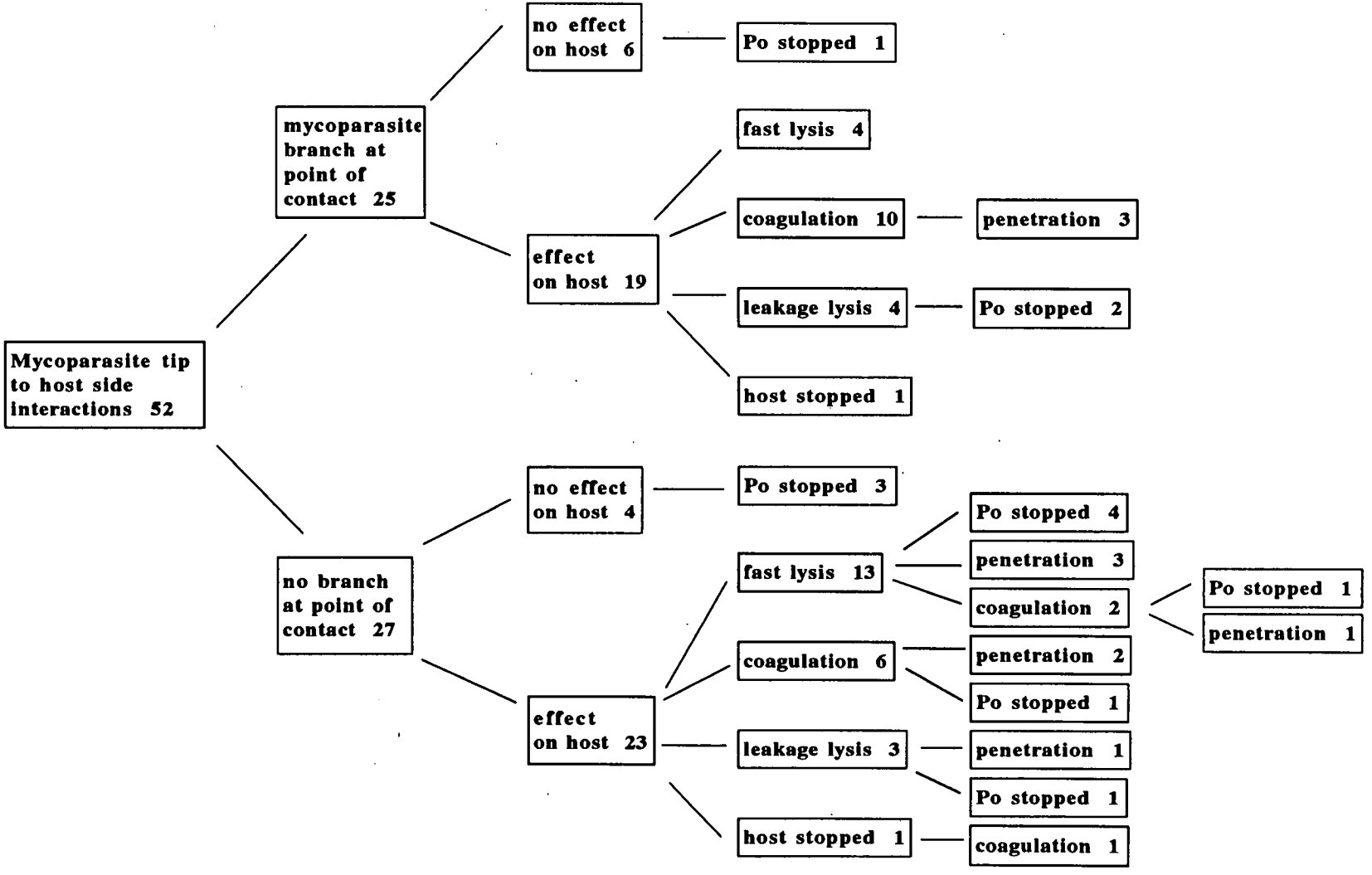


Fig 4.4. Outcome of 52 incidences of contact between the tip of the mycoparasite (*P. oligandrum*, Po) and the side of the host fungus (*R. oryzae*).

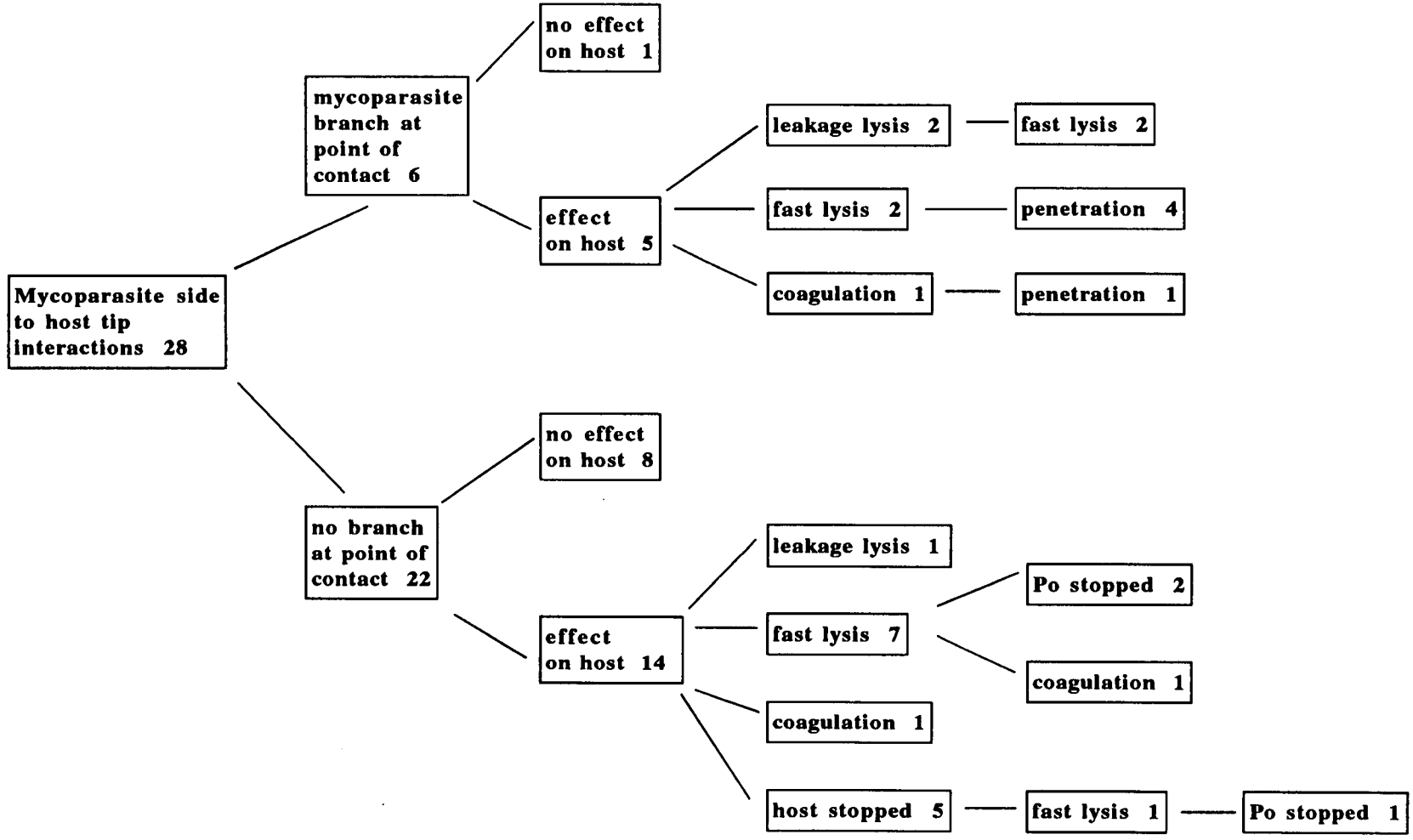


Fig. 4.5. Outcome of 28 incidences of contact between the side of the mycoparasite (*P. oligandrum*, Po) and the tip of the host fungus (*R. oryzae*).

the hypha of *R. oryzae* stopped growing upon or just after contact with *P. oligandrum*. Growth rates were found to vary considerably between interactions but were consistent in each interaction. There was no evidence of pre-contact tropism by either *P. oligandrum* or *R. oryzae*. Instead, the interacting hyphae contacted each other seemingly at random.

Post-contact events

1. *Effect of contact on growth of P. oligandrum*. In mycoparasite side to host tip interactions, growth of the main *P. oligandrum* hyphae was unaffected when contacted by *R. oryzae*. In mycoparasite tip to host side interactions, the behaviour of *P. oligandrum* was of two types - the mycoparasite either branched at the point of contact or it did not branch (see below). When it branched this usually occurred at the contact point but behind the extending mycoparasite tip, which continued to grow on after contact. However, if *P. oligandrum* did not branch at the point of contact then the tip of the mycoparasite usually stopped growing, although visible cytoplasmic movement was still observed within the tip.

2. *Effect of contact on growth of R. oryzae*. In mycoparasite tip to host side interactions, the host hypha was not stopped by contact if a sub-apical compartment was contacted by the mycoparasite; however, if the mycoparasite tip contacted the side of a tip cell of *R. oryzae* (two instances) then extension of the host was stopped. In one of these instances no conspicious damage occurred to the host hypha, but in the other instance coagulation resulted (Fig. 4.5). In mycoparasite side to host tip interactions, the tip of *R. oryzae* behaved in either of two ways. (1) It stopped growing upon or just after contact with the mycoparasite (5 instances); in one such case this was a prelude to fast lysis whereas in the other 4 instances no further effect was observed on the host (Fig. 4.6). (2) The host tip continued to grow until a main damaging event occurred to its hypha (which was usually soon after contact - see later).

3. *Branching of the mycoparasite. P. oligandrum* frequently branched at the point of contact with the host (in a time range of 3 min 6 sec - 13 min for timed interactions). This branching occurred more often in mycoparasite tip to host side interactions (25 of 52 contacts) than in mycoparasite side to host tip interactions (6 of 28 contacts). This difference was significant at $P < 0.05$ by Chi-squared analysis. It indicates that the tips of *P. oligandrum* were more responsive to contacts with *R. oryzae* than were the sides of *P. oligandrum* hyphae when contacted by *R. oryzae*. Induction of branching, where it occurred, was always at the first point of contact between the fungi although sometimes further branches formed at subsequent points along the contact zone between the fungi, i.e. when the contacting tip grew along the hypha of the other fungus.

4. *Effect of P. oligandrum on the host.* Overall, contacts between *P. oligandrum* and *R. oryzae* led to damage to the host in 61 of the recorded contact events - in 19 of the 28 mycoparasite side to host tip interactions, and 42 of the 52 mycoparasite tip to host side interactions (no significant difference by x^2). Thus, the type of contact between the fungi did not influence the success of parasitism. Also, overall, damage to the host occurred in 24 of the 31 instances when the mycoparasite branched at the point of contact, compared with 37 of the 49 instances when the mycoparasite was not seen to branch; again there was no significant difference in this respect. This indicates that branching by the mycoparasite was incidental to damaging effects on the host, even though damage was sometimes initiated by the branch (see later).

5. *Localisation of attack.* If *P. oligandrum* successfully parasitised the host then the effect, whether lysis, coagulation, or leakage lysis, was almost always limited to the contacted host compartment. For example, in cases of lysis or coagulation, the delimiting septa that separated the damaged host compartment from the healthy ones were seen to bulge into the damaged host compartment indicating that the cells on either side of this retained their turgor and the septal pores had become plugged (Fig. 4.7).

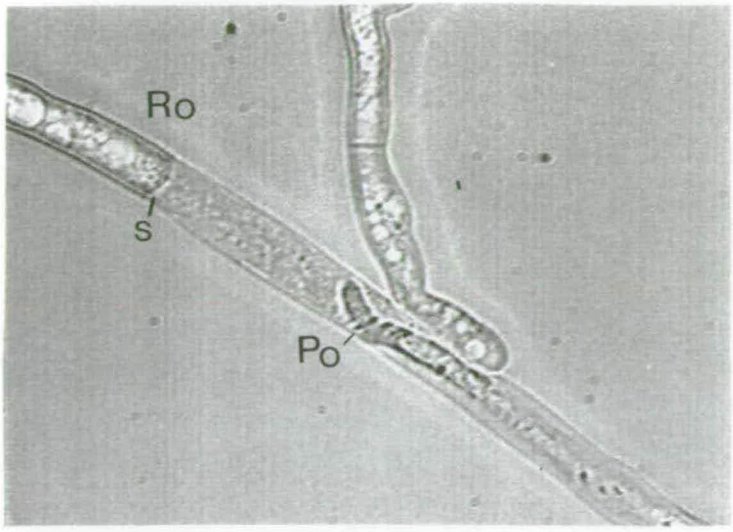


Fig. 4.7. Internal growth of *P. oligandrum* (Po) in a hypha of *R. oryzae* (Ro). Damage was localised to a single compartment of the host, delimited by a bulging septum (s).

6. *Surge of cytoplasm*. A slight or conspicious surge of host protoplasm was usually the first observed event in interactions where the host was damaged by *P. oligandrum*. It was seen at times ranging from 5 sec - 12 min 33 sec post-contact in timed interactions. No difference was observed in the number of incidences of cytoplasmic surging between tip to side and side to tip interactions. Overall, conspicuous surging occurred in about half of the incidences where contact of the two fungi led to an adverse effect on the host.

7. *Fast lysis*. Fast, explosive lysis occurred in 29 of the total 80 interactions, representing 29 of the 61 interactions where there was an effect on the host, within the time range of 5 sec to 12 min 33 sec (in timed interactions). This was observed to be a violent expulsion of cytoplasmic contents, from the narrow area of the point of initial contact between the fungi. Sometimes fast lysis was sporadic, with an initial release of cytoplasm followed by subsequent discharges. This was probably due to some of the contents of the cytoplasm impeding movement through the lesion in the hyphal wall. The cytoplasm was only ever discharged from the attacked compartment. The number of incidences of fast lysis in mycoparasite tip to host side interactions (17 in 42 instances where contact with *P. oligandrum* had an effect on the host), was significantly ($P < 0.05$) less than in mycoparasite side to host tip interactions (12 of 19 instances) where the host was affected. This indicates that the host tips were more susceptible to lysis than were the host lateral walls. When these instances were re-analysed for contact that involved mycoparasite branching (8 of 24 instances leading to lysis) and no branching (21 of 37 instances) there was also a significant difference, indicating that if the mycoparasite branched then the number of incidences of lysis as the main damaging event was less.

8. *Leakage lysis*. Leakage lysis was the main damaging event in only 8 of the 61 incidences of contact which were detrimental to the host, and occurred twice as a prelude to fast lysis (these latter incidences were not included in the analysis of leakage lysis for this reason). It occurred at times from 1 min 13 sec - 12 min

51 sec post-contact. It was observed once in a mycoparasite side to host tip interaction as the main damaging event, and 7 times in mycoparasite tip to host side interactions. Leakage lysis was observed as a single discharge of only a small amount of cytoplasm through a wall lesion at the point of contact. Sometimes this expulsion was violent but usually it was observed as a slow release.

9. *Coagulation.* Conspicious coagulation, granulation or vacuolation of host cell contents occurred as the main damaging event in 19 of the total 61 incidences of contact that had an effect, within a time range of 2 min 2 sec - 15 min 30 sec post-contact (in timed interactions). It was found to occur significantly more often in mycoparasite tip to host side interactions (17 of 42 incidences) than in mycoparasite side to host tip interactions (2 of 19 instances); incidence of branching was not associated with the frequency of coagulation.

10. *Penetration.* Penetration of the host by the mycoparasite occurred in 15 of the 61 interactions that had an effect on the host, and was usually preceded by fast lysis (8 instances) or coagulation (6 instances), and only once by leakage lysis. Penetration was observed as internal growth of the hypha of *P. oligandrum* through the damaged compartment of *R. oryzae* at a rate of *ca* 8μm min^{-1} (Fig. 4.7). However internal growth of the hypha of the mycoparasite rarely reached the delimiting septa separating the infected compartment from the adjacent healthy ones; usually growth of the internal hypha was stopped before then or the mycoparasite re-emerged as a branch through the host hyphal wall. No significant difference was observed in the incidence of penetration between side to tip and tip to side interactions.

11. *Stoppage of P. oligandrum.* In 13 of the total 80 interactions, contact between the mycoparasite with the host was detrimental to *P. oligandrum*, observed 3 min 55 sec - 14 min after contact with the host. Of these 13 events the mycoparasite had no effect on the host in only one case whereas the remaining

12 incidences occurred after lysis (8), coagulation (1) and leakage lysis (3). Analysis of the total incidences of each damaging event showed that stoppage of the mycoparasite occurred significantly more often after incidences of lysis or leakage lysis than it did after coagulation. Stoppage was observed as either (1) a surge of cytoplasm in the hypha of *P. oligandrum*, followed by coagulation of the cytoplasm and no extension of the hypha (observed in timed interactions only), or (2) lack of further growth of the hypha of the mycoparasite and coagulation of its cytoplasm (scanned interactions). Stoppage of *P. oligandrum* only occurred in the region of the interaction because further away from the site of contact the mycoparasite was still evidently healthy.

Evidence of resistance of *R. oryzae* to parasitism by *P. oligandrum*

The focus of the account above was on the damage to hyphae of *R. oryzae* following contacts with *P. oligandrum*. But there was also evidence of resistance of this host to parasitism from *P. oligandrum*, which served to limit the degree of damage to contacted host compartments or even to prevent damage to those host compartments. These aspects are now recorded.

1. *Regrowth of the host hypha.* Sometimes after lysis of a compartment of the host hypha, the host regrew within it by formation of a new hyphal tip from the septum separating a healthy compartment from the damaged compartment (Fig. 4.8). This was observed in at least 7 of 80 instances, but the growth rate of the new, internal host hyphal tip was often slow, about 2μm min^{-1}, compared with normal extension rates of *ca* 9μm min^{-1} for the tips of *R. oryzae*.

2. *Formation of an "optically dense region".* Sometimes when the side of a mycoparasite hypha was contacted by the tip of *R. oryzae* (7 in 80 instances), and occasionally when the mycoparasite tip contacted the side wall of a hypha of *R. oryzae* (2 in 80 instances), an optically dense region was evident at the point of contact. The formation of this structure is best described by citing representative examples.

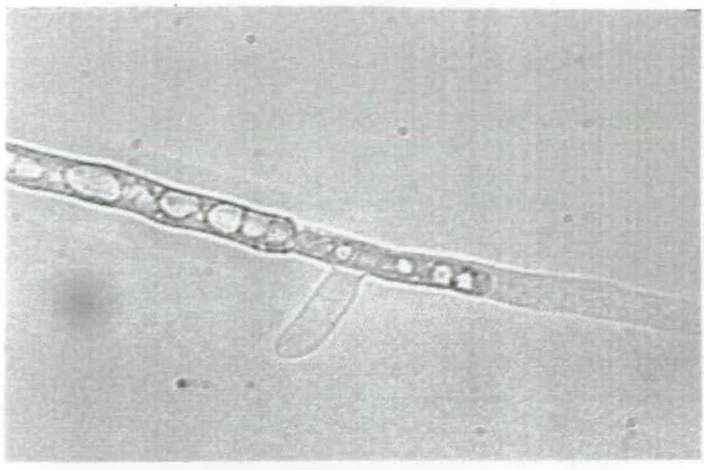


Fig. 4.8. Damage to a compartment of *R. oryzae*, due to lysis, resulting in regrowth of the hypha of the host from the delimiting septum, between an undamaged and damaged compartment, into the damaged compartment.

Example 1. (Fig. 4.9 a-f)

The tip of hypha 1 of *R. oryzae* (see Fig. 4.9a) was recorded as it approached a hypha of *P. oligandrum*; the tip of hypha 2 contacted the side of the mycoparasite before recording began. The growth of hypha 1 was seen to stop soon after contact with the mycoparasite, and this stoppage coincided with surging of the host cytoplasm at 70 sec post-contact. An optically dense region was observed at the contact point at 2 min 40 sec after contact (Fig. 4.9b). No further events were observed until 8 min 23 sec post-contact when a surge of host cytoplasm was evident, followed by a subsequent surge 12 sec later. Leakage lysis then occurred at 9 min post-contact but the remaining host cytoplasm still retained more or less normal movement and structure (Fig. 4.9c). Further surging of the host cytoplasm was observed at both 10 min 57 sec and 11 min 9 sec post-contact. Then, at 11 min 27 sec after contact a branch of *P. oligandrum* became evident in the area of the optically dense region (Fig. 4.9d). At 16 min 34 sec post-contact the branch from the mycoparasite hypha was dislodged sideways from the tip of the host hypha, leaving behind a papilla-like structure at the tip of the host hypha (Fig. 4.9e). At 19 min 35 sec post-contact the cytoplasm of the host surged towards the tip and this was followed by leakage lysis 3 sec later from the tip at the point of contact (Fig. 4.9f). An explosive lysis then occurred 57 sec later (Fig. 4.9g). This did not affect the hypha of the mycoparasite. No penetration of the host hypha occurred and there was no change in the interaction even after 1 h post-contact.

While these events were occurring, hypha 2 of *R. oryzae* had stopped growing after contact with the side of the mycoparasite hypha. An optically dense region was seen on the tip of this host cell (Fig. 4.9a,b) and the tip swelled (Fig. 4.9c,d), followed by leakage lysis at approximately 16 min after the start of observations (Fig. 4.9e). As the host hyphal tip shrank back from its original position a branch of the mycoparasite hypha became evident in the optically dense region (Fig. 4.9e,f). But the optically dense region, resembling a papilla, was still evident in the tip of the host (Fig. 4.9f,g).

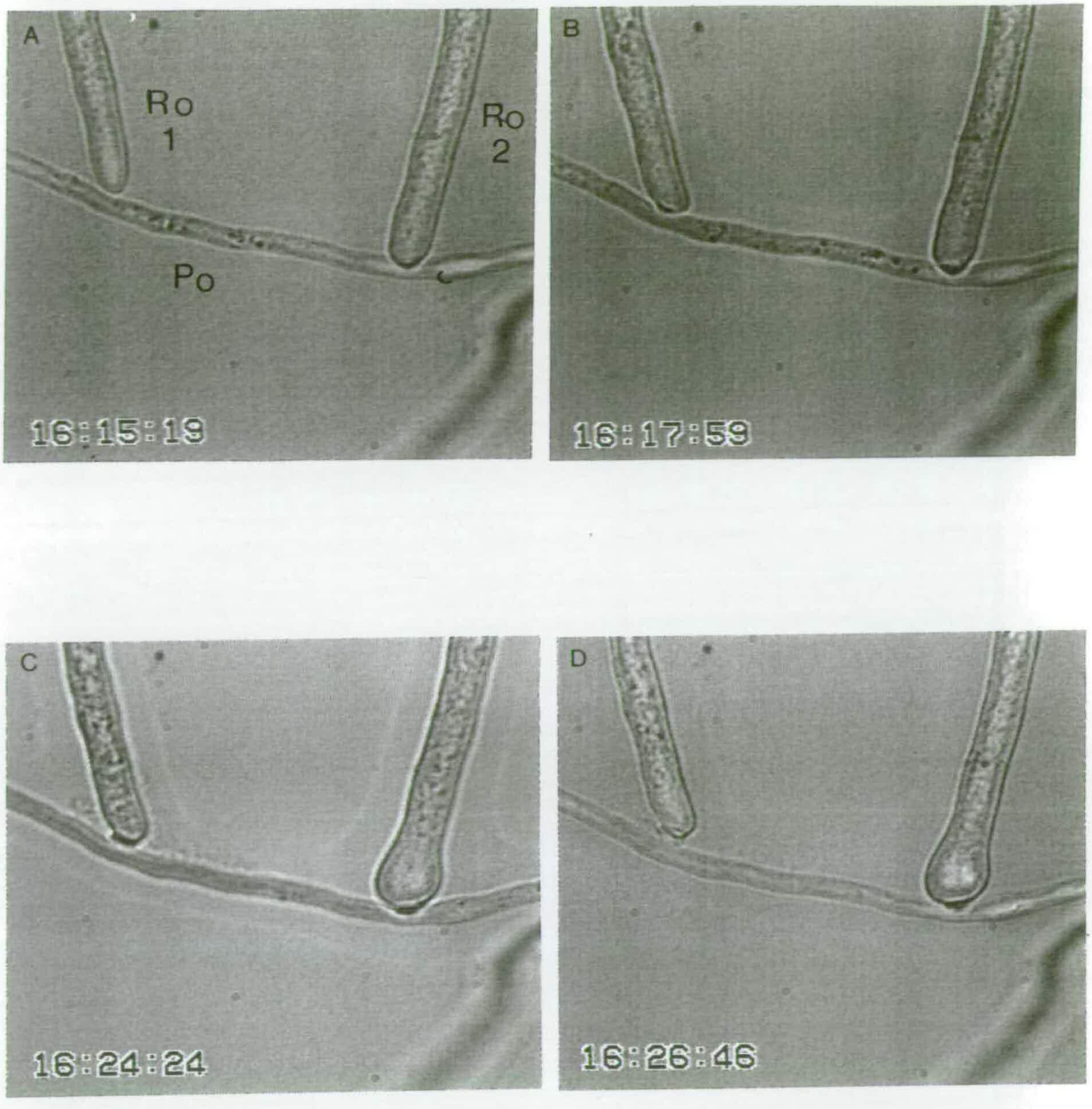


Fig. 4.9 (a) Tip of hypha 1 of *R. oryzae* (Ro) contacts the side of the hypha of *P. oligandrum* (Po). Tip of hypha 2 of the host had already contacted the mycoparasite and an optically dense region was evident on the tip. (b) An optically dense region forms at the contact point between hypha 1 of the host and the mycoparasite. (c) Leakage lysis occurs in host hypha 1 but the host cytoplasm retained almost normal movement and structure. Host hypha 2 swells at its tip. (d) A branch from the hypha of the mycoparasite becomes obvious in the area of the optically dense region.

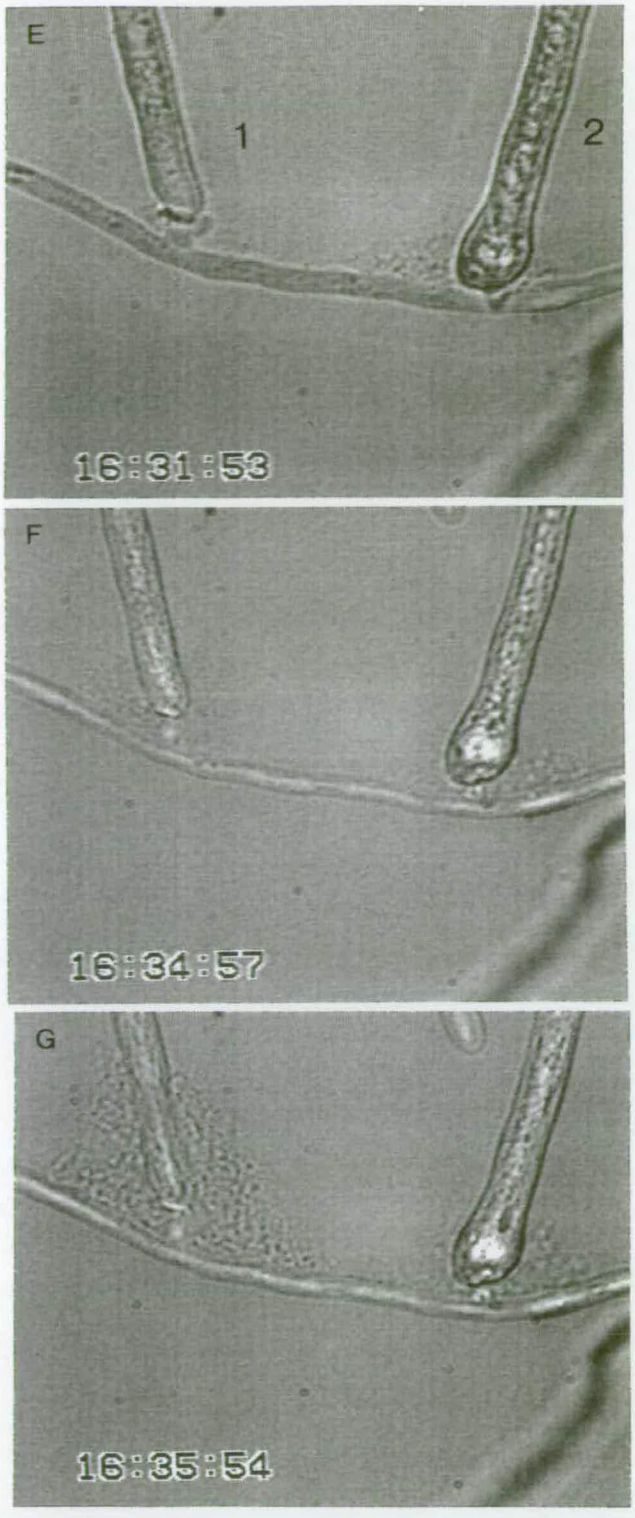


Fig. 4.9 (e) The branch from the mycoparasite breaks from the tip of host hypha 1, leaving behind a papilla-like structure at the tip of host hypha 1. Leakage lysis occurs at the tip of host hypha 2 and a branch from the hypha of the mycoparasite becomes obvious. (f) Leakage lysis occurs at the tip of host hypha 1. (g) Explosive lysis then occurs at the tip of host hypha 1.

Other similar examples of optically dense regions were seen when host tips contacted the sides of mycoparasite hyphae. In all such cases it was difficult to discern the details of these regions, but they appeared to represent a combination of localised branching from the mycoparasite at the point of contact and papilla formation in the tip of the host, coinciding with attempted penetration from the mycoparasite branch.

3. *Evidence of physical force exerted upon contact between the host and the mycoparasite.* Sometimes physical pressure was evident in interactions between the host and the mycoparasite. In 3 instances of a possible 80, the hyphae of *R. oryzae* were displaced following contact by a tip of the mycoparasite, and *P. oligandrum* was similarly displaced in 7 instances from a possible 80 upon contact by a host hyphal tip. Examples of these interactions are shown in Figs. 4.10 & 4.11 and the events are briefly described below. The significance of these in comparison with interactions of *P. oligandrum* with *Fusarium* sp. or *H. grisea* is that they indicate some resistance on the part of *R. oryzae*. For example, they indicate that the tips of *R. oryzae* were not always susceptible to immediate stoppage on contact with the mycoparasite, and similarly the mycoparasite sometimes had evident difficulty in penetrating a hypha of *R. oryzae*.

Example 2. (Fig. 4.10a-g)

The tip of *P. oligandrum* grew at a rate of 9μm min^{-1} until it contacted the tip of a hypha of *R. oryzae* and stopped extending (Fig. 4.10a). *R. oryzae* continued to grow at an unaltered rate (*ca* 9μm min^{-1}) for 57 sec after contact until a surge of cytoplasm was seen in the host tip and it then stopped growing. At 1 min 32 sec post-contact an optically dense region was seen at the tip of the host hypha (Fig. 4.10b). By 6 min 12 sec a bulge had appeared in the apical region of the mycoparasite, behind its tip (Fig. 4.10c) suggesting that the tip of *P. oligandrum* was still growing but its movement forwards was prevented by the host tip. Thereafter [e.g. 17 min 32 sec post-contact (Fig. 4.10d) and 23 min 15 sec post-contact (Fig. 4.10e)] the continued growth of the mycoparasite tip led to the

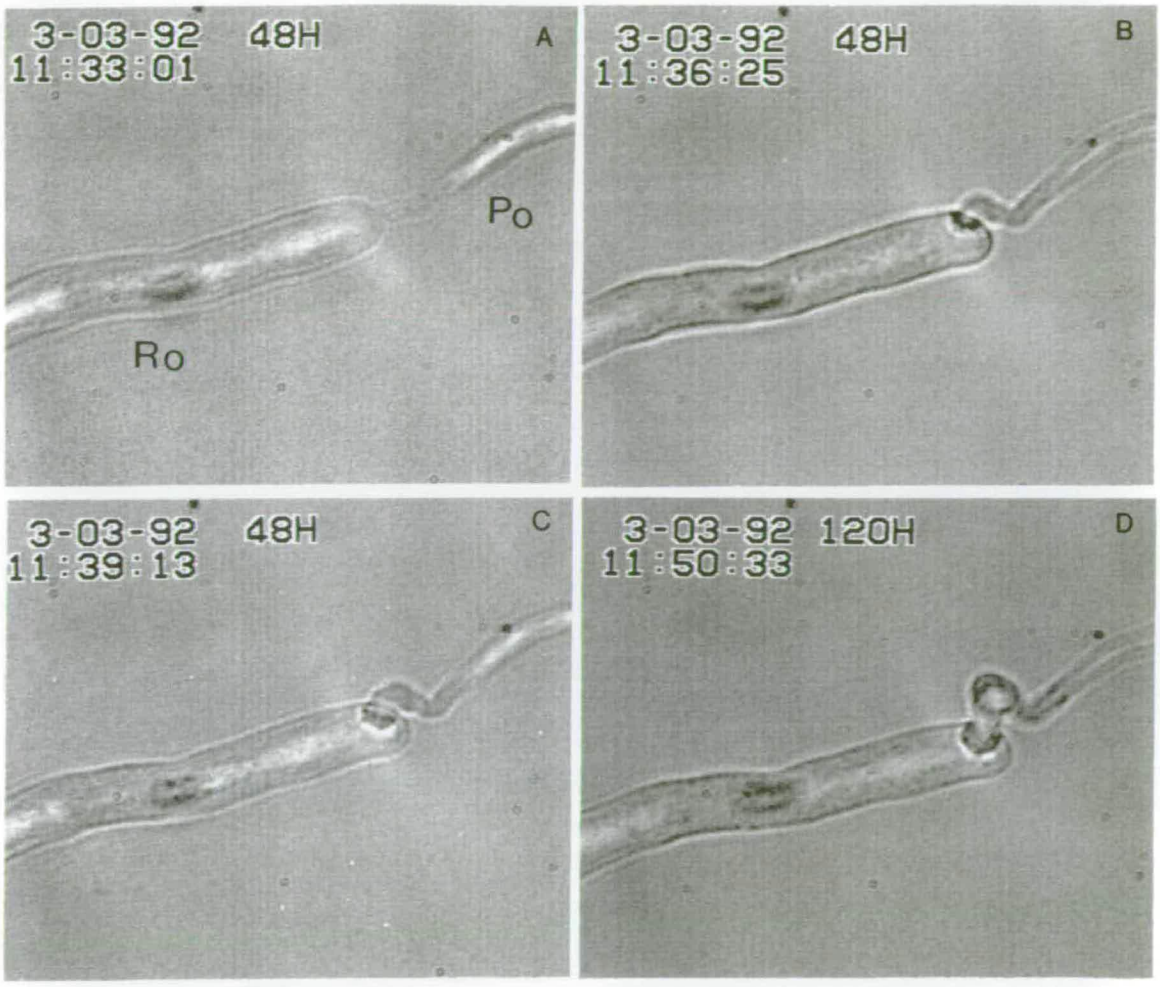


Fig. 4.10 (a) Tip of *P. oligandrum* (Po) contacts the tip of *R. oryzae* (Ro). (b) An optically dense region forms on the tip of the host hypha. (c) A bulge appears in the apical region behind the mycoparasite tip. (d) Hypha behind the tip region of mycoparasite appears to buckle as it swells.

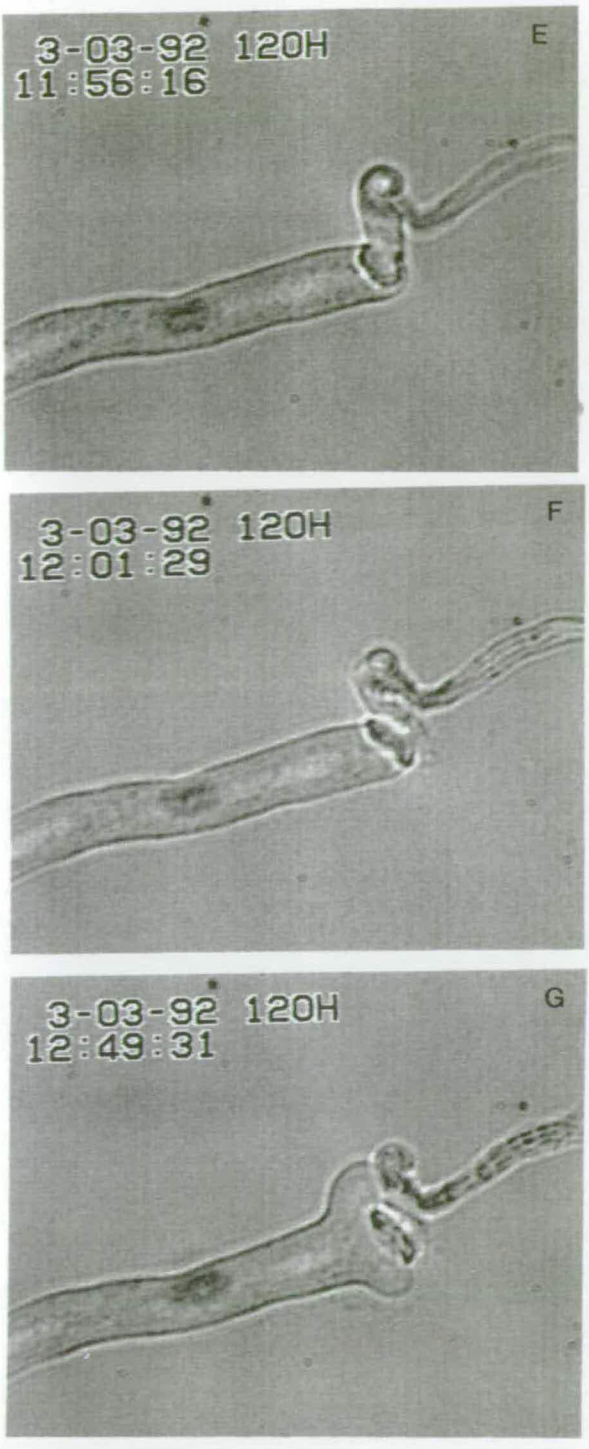


Fig. 4.10 (e) Continued growth causes the mycoparasite hypha to arch into the air. (f) Tip of mycoparasite hypha breaks from host tip causing collapse of the mycoparasite hypha and leakage of some cytoplasm. Papilla-like material evident on the host tip. (g) Host hyphal tip resumes growth and bifurcates around the area of papilla-like formation.

buckling and arching-up of the apical region of the mycoparasite. Finally, by 28 min 28 sec post-contact (Fig. 4.10f) the tip of the mycoparasite suddenly was forced away from the host tip, causing collapse of the mycoparasite hypha and leakage of some of its cytoplasm; papilla-like material was then evident in the tip of the host (Fig. 4.10f). This suggested that the pressure involved at growing at such an angle caused the hypha of the mycoparasite to break. At 40 min post-contact the tip of the host started to regrow and bifurcate around the area of papilla-like formation (Fig. 4.10g).

Example 3. (Fig. 4.11a-e)

The second example of displacement was different from that above and is recorded here mainly because it involved a rarely recorded event - the association between rapid surging of mycoparasite protoplasm and lysis of a host hypha. In this example, the tip of a hypha of *R. oryzae* grew at a rate of 11μm min^{-1} until it contacted the side of a hypha of *P. oligandrum*. After contact (Fig. 4.11a) the tip of the host was displaced for a distance of 8μm (towards the top of Fig. 4.11b) and as it continued to grow it pushed the hypha of the mycoparasite in the direction that the host was extending, for a distance of 14μm (Fig. 4.11b). The tip of the host then grew over the hypha of the mycoparasite at a rate similar to the pre-contact rate (13μm min^{-1}). The tip of a second hypha of *R. oryzae* also grew and contacted the side of the mycoparasite hypha 5 min 13 sec after the first hypha had contacted the mycoparasite (Fig. 4.11c). At 6 min 39 sec post-contact, surging of the cytoplasm in the first host hypha occurred and this hypha stopped extending although cytoplasmic movement was still evident within it. No further changes were observed until 12 min 43 sec after contact (Fig. 4.11d), when a rapid flow of cytoplasm was evident in the hypha of the mycoparasite and this was followed by lysis of the first host hypha 4 sec later. The flow of mycoparasite cytoplasm occurred through the whole of the hyphae visible in Fig. 4.11(d) and was not directed towards the contact point. Lysis caused both host hyphae to be displaced by *ca* 32μm, back to near the original direction in which they had been growing pre-

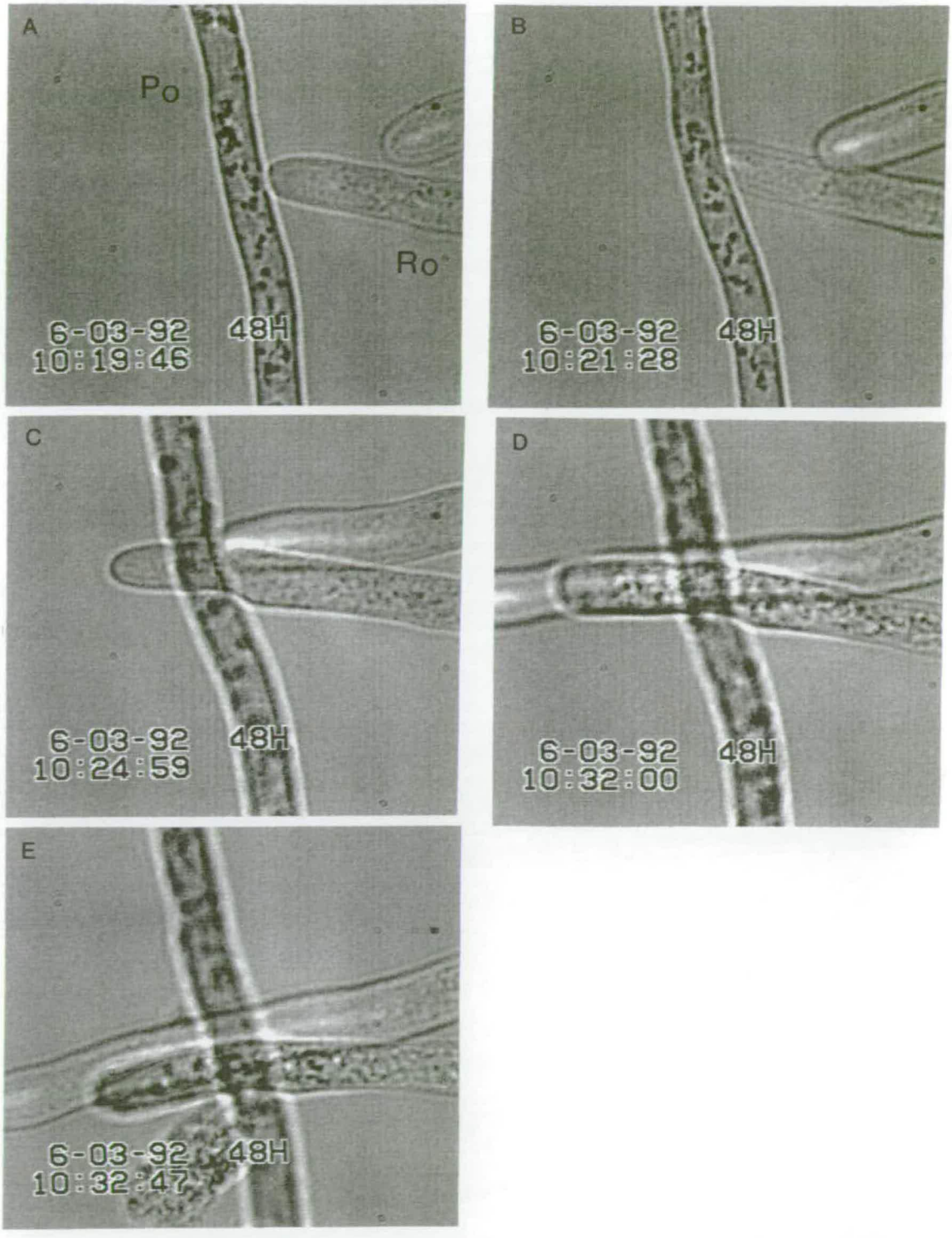


Fig. 4.11 (a) Tip of a hypha of *R. oryzae* (Ro) contacts the side of a hypha of a mycoparasite (Po). (b) This caused the tip of the host to be displaced towards the top of figure (b) (compared with figure (a)), and as it continued to grow the hypha of the mycoparasite is pushed in the direction of host extension. (c) Tip of a second hypha of *R. oryzae* contacts the side of the mycoparasite hypha. (d) Surging of the cytoplasm in host occurs resulting in stoppage of growth of host. (e) Lysis of first host hypha caused both host hyphae to be displaced to near their original direction of growth.

contact (Fig. 4.11e). By 1 min 36 sec later, the cytoplasm in the mycoparasite hypha had stopped flowing rapidly and returned to its normal rate of movement. The tip of the second host hypha grew on regardless of contact. By 22 min post-contact there was no change in the interaction: the second host hypha was still growing, the first had lysed and stopped growing and the cytoplasm in the mycoparasite hypha was still moving slowly.

4.2.4 Determination of the pH of the contents of hyphae of R. oryzae

Agar discs (7mm diam.) of *R. oryzae* were inoculated into 200ml medical flats containing 8ml of PD broth, as described in section 2.4.10. Two strains of *R. oryzae* were used, S1,23 and J336, with 3 replicates per strain. After 7 days at 25°C, the mycelium in each flat was collected under vacuum on Whatman cellulose nitrate membrane filters (pore size 0.45μm). The mycelium was transferred to a test-tube and homogenised with an Ultra-Turrax blender at 25,000 rpm for 90 sec. The pH of the resulting homogenates were found to be 5.31 (strain S1,23) and 5.00 (strain J336), respectively.

4.2.5 Demonstration of a possible inhibitor in hyphae of R. oryzae

To investigate whether the contents of the hyphae of *R. oryzae* contained an inhibitor to growth of *P. oligandrum* the following experiment was done. Various host fungi were inoculated into medical flats containing PDB, with 20 replicate flats per host fungus, and after 10 days incubation the mycelial mats were removed (section 2.4.10). The fungi used were *R. oryzae* (strains S1,23 and J336), *F. oxysporum* and *R. solani* (AG5). The mycelia of each host were pooled, frozen using liquid nitrogen and then ground to a fine powder. Samples were allowed to thaw, giving 25ml of homogenate for each fungus, and these were filtered through a double layer of muslin, then filter paper, and finally through a millipore filter (0.45μm). Aliquots of the homogenates were then dispensed into small (5cm) Petri dishes and inoculated with agar discs of *P. oligandrum*. There was 3 replicates per host fungus.

As shown in Table 4.4 and Fig. 4.12, *P. oligandrum* was able to grow to at least some degree in all culture homogenates. However it grew in only one of the three replicate plates containing culture filtrate from *R. oryzae* (strain S1,23) and only to 8mm diam. in this plate. Analysis of variance showed a significant difference in growth of *P. oligandrum* in the homogenate from *R. oryzae* (strain S1,23) compared with in that from any other fungus, suggesting that the nutrients in the homogenate derived from strain S1,23 of *R. oryzae* were not sufficient enough to support growth of *P. oligandrum*.

4.3 Discussion

Of many fungi isolated from strips of cellulose film in soil (section 4.2.1), three were selected for further study because of their demonstrated cellulolytic activities. *Humicola grisea* is commonly found in soil and is highly cellulolytic (Domsch *et al.*, 1980). *Rhizoctonia oryzae* is less often detected than *R. solani*, but has been reported from sites associated with barley stunt disease (Burton *et al.*, 1988). *Fusarium* spp. are common soil saprophytes or plant parasites and are also widely known for their cellulolytic activities (Domsch *et al.*, 1980). The experiments in this section investigated the relationship between the interactions of these fungi with *P. oligandrum* on cellulosic substrata and in pairings of colonies on water agar films, as studied by videomicroscopy.

On both cellulose film and filter paper, *P. oligandrum* reduced the activities of the three cellulolytic fungi to different degrees. *Fusarium* sp. was most markedly affected, its cellulolytic activity being reduced by as much as 48% when coinoculated with *P. oligandrum* on filter paper (section 4.2.2.2) and 59% when coinoculated with *P. oligandrum* on cellulose film (section 4.2.2.1). *H. grisea* was less markedly affected but still significantly so, and *R. oryzae* was least affected by the mycoparasite. In the particular conditions of these experiments cellulose was provided as the sole organic carbon source and nitrate as the sole nitrogen source. *P. oligandrum* cannot grow on either of these nutrient sources and so

Table 4.4. Colony diameter (mm) of *P. oligandrum* after 2 days at 25°C in mycelial homogenates of four fungi; mean ± SEM for 3 replicates.

Source of culture homogenates	**Colony diameter ± SEM**
R. oryzae (S1,23)	2.7 ± 2.7[a]
R. oryzae (J336)	26.3 ± 0.3[b]
F. oxysporum	36.0 ± 1.0[c]
R. solani	25.3 ± 1.4[b]

Values followed by same letter do not differ significantly from each other, by one-way analysis of variance (5% LSD = 5.2).

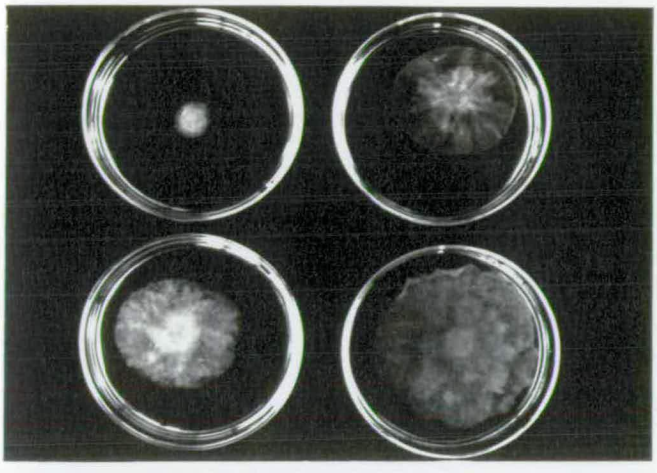


Fig. 4.12. Mycelial growth of *P. oligandrum* in mycelial macerates of, from top left clockwise, *R. oryzae* (S1,23), *R. oryzae* (J336), *F. oxysporum* and *R. solani*.

would be dependent on its cellulolytic and nitrate-utilising partner. It might obtain its necessary nutrients (simple sugars, organic nitrogen) by parasitism or by secondary sugar saprophytism (Garrett, 1951; Tribe, 1966). In the latter case it could obtain sugars released from cellulose by the actions of enzymes released by other fungi. Its ability to obtain organic nitrogen would be more difficult to explain unless organic nitrogen was released from the hyphae of other fungi during autolysis as the hyphae aged. In any case, on this basis, it would not be easy to explain how *P. oligandrum* could markedly reduce the activities of the other fungi. So the evidence from the experiments on cellulosic substrata is most easily interpretated in terms of parasitism by *P. oligandrum*, in which case the other fungi could be assumed to differ in susceptibility to parasitism. Similar arguments were advanced by Deacon (1976) and Tribe (1966).

There was some support for this view in the videotaped sequences of mycoparasitism because the individual hyphae of both *Fusarium* sp. and *H. grisea* were rapidly disrupted after contacts with hyphae of *P. oligandrum*. Typically, the hyphae of *Fusarium* sp. and *H. grisea* showed localised lysis or coagulation of their cytoplasm within 4 - 6 and 3 - 14 min, respectively, after contact with the mycoparasite. *R. oryzae* was also antagonised (in 5 sec - 15 min post-contact) but differences were observed between this host and the others in the inter-hyphal interactions with *P. oligandrum*. In particular, any damage of the hyphae of *R. oryzae* caused by *P. oligandrum* was always localised to the hyphal compartment in which contacts occurred. The adjacent compartments retained normal, healthy, streaming cytoplasm and their septa bulged into the damaged compartment. Any internal growth of the mycoparasite in the hyphae of *R. oryzae* was always confined to the damaged compartment whereas for both *Fusarium* sp. and *H. grisea* it was observed that an internal mycoparasite hypha could grow progressively through the host, disrupting successive septa and damaging successive host compartments.

The restricted type of parasitism shown here with *R. oryzae* had not been reported until recently when Berry *et al.* (1993) found that *P. oligandrum* could parasitise some of the plant pathogenic *Pythium* spp. (*P. ultimum* and *P. vexans*) but was resisted by *P. graminicola* (sometimes) and *P. aphanidermatum* (usually). The interactions involved extensive coiling of the mycoparasite around the host hyphae and damage was localised to the region of coiling by the production of barrier zones of densely coagulated host cytoplasm. Berry *et al.* (1993) suggested that the plant pathogenic pythia might outgrow parasitism if their tips were far enough away from the contact site. This would also be applicable to *R. oryzae*; however it was also observed that this host had the ability to regrow into the damaged compartments from a septum separating the damaged from the healthy compartments (Fig. 4.8), thus alleviating the effect of parasitism.

R. oryzae also showed evidence of resistance to mycoparasitism even at its tips, because these sometimes developed an optically dense zone, suggesting the deposition of a papilla-like structure, and sometimes the hyphae of *R. oryzae* and *P. oligandrum* were forced apart after contact, as if the mycoparasite had difficulty in invading the host.

Papillae in mycoparasite interactions are difficult to visualise by conventional light microscopy or videomicroscopy, but Hoch & Fuller (1977) described their development in detail during invasion of hyphae of *Corticium sensu lato* by *P. acanthicum*. This mycoparasite is similar to *P. oligandrum* in all important behavioural respects (Haskins, 1963; Foley & Deacon, 1986b). The *Corticium* sp. used by Hoch & Fuller (1977) has since been re-identified as *Laetisaria arvalis* Burdsall (H. C. Hoch, pers. comm.) - a basidiomycetous fungus like *R. oryzae*. They observed formation of papillae in the host hyphae upon contact with the mycoparasite. Attack by *P. acanthicum* resulted in penetration of the host cell wall, and of the papilla by an invading hyphal tip of *P. acanthicum*. The mycoparasite then grew through the cytoplasm and the septa separating the

damaged and healthy compartments. Formation of these papillae suggests initial resistance exhibited by the host fungus upon attack from the mycoparasite. However, the precise roles of papillae in host resistance have been questioned. For example, Manocha & Graham (1982) observed extensive formation of a papilla in a hypha of *Phascolomyces articulosus* where attack by the mycoparasite (*Piptocephalis unispora*) failed; in contrast, Jeffries & Young (1978) observed degenerate haustoria in the host hypha without formation of a papilla.

Formation of papillae in interactions between plant hosts and parasitic fungi has also been investigated. For example, Skou (1981) discussed the formation of the papillae (termed lignitubers) in cereals in response to attack from the take-all fungus *Gaeumannomyces graminis*. These structures were taken as evidence of resistance displayed by the host plant, as the development of papillae apparently delayed advancement of the internal hyphae of the pathogen through the host cell wall; however, with time the pathogen hypha usually penetrated the apex of a papilla leading to host cell death. Ride & Pearce (1979) found that the papillae formed in cereal leaves in response to infection by *Botrytis cinerea* were highly resistant to enzymatic degradation and thus might limit or slow the penetration of pathogens if this depends on enzyme degradation. Also, the papillae might incorporate additional chemical moieties, such as phenolic polymers in cereals, which are fungitoxic (Speakman & Lewis, 1978; Ride & Pearce, 1979) and could block access of pathogen-derived enzymes to the normal host wall constituents.

In all these respects papillae serve to delay the invasion of parasites and so their role in resistance might be combined with other factors such as mobilisation of host chemical defences or general unsuitability of the host's internal chemical milieu. In this respect there was preliminary evidence that the hyphal contents of at least one strain of *R. oryzae* were inhibitory to growth of *P. oligandrum*, or did not provide the nutrients that could support growth of the mycoparasite

(Table 4.4). This internal inhibitory environment, as evidenced by the *in vitro* study, could help to explain the poor internal growth of *P. oligandrum* when it invaded damaged hyphal compartments of *R. oryzae*. The finding that growth of *P. oligandrum* hyphae was sometimes halted by *R. oryzae*, especially following lysis, would also be compatible with release of an inhibitory factor in the lysate, although it might sometimes also have been caused by physical disruption resulting from the violent discharge of host cytoplasm. Hyphal tips are known to be highly sensitive to disturbance, including changes of external osmotic potential (Robertson, 1965). In any case, the internal pH of the hyphae of *R. oryzae* was probably not a factor in the halting of the growth of *P. oligandrum* because the internal pH was determined to be *ca* 5.0 - 5.5 which is within the range in which *P. oligandrum* can grow (Foley & Deacon, 1985).

It may be noted that hyphal tips of *P. oligandrum* were not inhibited as they approach hyphae of *R. oryzae*, so this seems to preclude the release of an inhibitor by *R. oryzae* - at least on water agar films. The poor growth of *P. oligandrum* inside the hyphae of this host would thus have to be explained either in terms of an internal antibiotic-like substance or in terms of the release or production of an inhibitor as a result of cytoplasmic damage. Other studies report effects relevant to these points. Burton & Coley-Smith (1985), Walther & Gindrat (1987b) and Burton & Coley-Smith (1993) all have reported that *R. oryzae* produces low levels of antibiotics active against *P. oligandrum*. Walther & Gindrat (1987b) even showed that *Rhizoctonia cerealis*, *R. fragariae* and *R. solani* antagonised *P. oligandrum* by attenuation of its cell wall and vacuolation of its cytoplasm, suggesting possible antibiosis, while Tribe (1966) suggested that there might be some specific antimicrobial agent responsible for lack of growth of *P. oligandrum* in association with *R. solani* when the fungi were opposed on agar plates.

Burton & Coley-Smith (1985, 1993) studied antibiotic production by *R. cerealis*, *R. oryzae-sativae* and *R. tuliparum*, to determine if the antibiotics were present

throughout the growth phases of these fungi. They found that the fungi retained antibiotic activity in the mycelium and if it was released it was rapidly degraded; there was little leakage and they suggested that lysis of sections of *R. oryzae* hyphae followed by leakage of antibiotic material could have a localised inhibitory effect on other soil fungi. Burton & Coley-Smith (1993) also observed that less antibiotic was present in the tips than in older regions of hyphae and that most was concentrated in the sclerotia.

In a different context, Fravel & Roberts (1991) reported that glucose oxidase is involved in biocontrol of verticillium wilt (*V. dahliae*) by *Talaromyces flavus*. They suggested that *T. flavus* produced glucose oxidase in the root zone and that the action of this enzyme on glucose released hydrogen peroxide which is toxic to *V. dahliae*. Hydrogen peroxide and oxygen free radicals can be expected to result from cell damage, when separately compartmentalised enzymes and substrates are brought together. So perhaps this occurs in the damaged compartments of *R. oryzae*, leading to inhibition of *P. oligandrum*. It would not, however, explain why *P. oligandrum* can grow well in damaged hyphae of other host fungi.

To investigate the relationship between *P. oligandrum* and *R. oryzae* further, *P. oligandrum* was inoculated into culture homogenates of *R. oryzae* and other fungi, as controls (section 4.2.4). Little or no growth of *P. oligandrum* was observed in the homogenate from strain S1,23 of *R. oryzae*. This strain was used in the video studies with *P. oligandrum* and thus the results imply that *R. oryzae* has a cytoplasm-contained inhibitor active against *P. oligandrum*. This is in agreement with the work by Burton & Coley-Smith (1985, 1993).

To some degree, the observations by videomicroscopy supported the contention that the three host fungi - *Fusarium* sp., *H. grisea* and *R. oryzae* - differed in resistance to parasitism by *P. oligandrum*. But such differences cannot entirely explain the different degrees of reduction of cellulolysis by the host fungi in the

presence of *P. oligandrum*. *R. oryzae* had to be supplied with organic nitrogen in the cellulolysis studies and this might have altered its susceptibility to parasitism by *P. oligandrum*. Studies on other fungal host-mycoparasite systems have shown that the degree of host susceptibility is influenced by nutritional status (Whipps, 1987a). This is probably of more importance in biotrophic mycoparasitism where the mycoparasite loses the ability to synthesise certain nutrients and therefore depends on the fungal host for survival. Identification of the nutrient that the mycoparasite can no longer utilise is necessary before the mycoparasite can be grown axenically (Jeffries, 1985).

An equally important general factor affecting interactions between whole colonies on cellulosic substrata might be the differences in hyphal extension rates of the host fungi. Hyphae of *Fusarium* sp. extended very slowly ($1\mu m\ min^{-1}$) across agar and cellulosic substrata, so that its hyphae would have little chance of escaping parasitic challenge by *P. oligandrum*, which has a colony extension rate of 8 - 36μm min^{-1} on agar. *H. grisea* grew faster ($3\mu m\ min^{-1}$) than *Fusarium* sp., giving more opportunity for escape of individual hyphae from parasitism. *R. oryzae* also extended faster ($9\mu m\ min^{-1}$). Deacon (1976) likened these effects to disease escape in plant-pathogenic interactions, as discussed by Garrett (1970). Both the host growth rates and environmental conditions contribute to disease escape: essentially, a host is most seriously affected by a pathogen when environmental conditions are changed to favour the pathogen more than they favour the host. It is thus dangerous to extrapolate from studies on mycoparasitism in artificial conditions, such as those used here, to conditions on cellulosic substrata in soil.

The work on videomicroscopy described here revealed several features that were previously described in detail by Laing & Deacon (1991). Notable was the fact that mycoparasitic attack was almost always initiated at the point of first contact between *P. oligandrum* and its host, even when the hypha of *P. oligandrum* grew on past this point before any evidence of mycoparasitism was

seen. Also, there was no obvious relationship between branch initiation by the mycoparasite and the occurrence of a damaging effect on the host. It is often difficult to observe the early stages of branching by mycoparasites, especially at points of contact with a host, because of optical resolution. But some of the faster lytic events observed here, especially involving host hyphal tips of all three hosts, were unlikely to have been caused by attempted invasion from mycoparasite branches. Laing & Deacon (1991) suggested that in at least some cases the host hyphal lysis observed at points of contact could involve wall lytic enzymes of the hosts themselves, rather than, as often suggested in mycoparasitism, production of wall lytic enzymes by mycoparasites (Adams & Ayers, 1983; Ko & Lockwood, 1970). The findings in this section would be compatible with the suggestion of Laing & Deacon (1991).

The work on *R. oryzae* described here has contributed new information on the mycoparasite-host interactions involving *P. oligandrum* because it gives several lines of evidence relating to a resistant host. For example, it provides the first evidence of physical resistance to parasitic attack by *P. oligandrum* at the hyphal tips of *R. oryzae*. This resistance was so great in some cases that it caused hyphae of *P. oligandrum* to buckle as they were displaced backwards while the tip continued to extend and was held stationary by a hypha of the host. It would now be valuable to study such interactions by electron microscopy, as in the work of Hoch & Fuller (1977), to confirm the possible involvement of papillae in host resistance to *P. oligandrum* and to examine in detail any changes in host and mycoparasite hyphal walls in the contact zones, as evidence relating to the roles of host or mycoparasite-derived wall lytic enzymes.

5. NUTRITION AND PHYSIOLOGY OF PAPULASPORA

5.1 Introduction

The precolonised plate method, described in section 2.4.1, revealed the presence of a fungus, referable to *Papulaspora* Preuss, with surprising frequency in a range of soils. It was detected most often on agar colonised by *B. cinerea*, but also on *T. aureoviride* and *R. solani* plates, and was identified by its characteristic production of bulbils.

Only limited research has been done on fungi of this type, most of the information on *Papulaspora* being collected in the first half of this century. Also, most of that information concerns the classification of *Papulaspora* and related fungi (Hotson, 1917; Weresub & LeClair, 1971; Watanabe, 1991), and their distributions, but little had been done on the ecological relationships with other fungi.

The genus *Papulaspora* belongs to the Deuteromycetes. Hotson (1912) assigned a range of fungi to this genus on the basis of sterile mycelia and production of 'bulbils'. His definition of the term 'bulbils' was "*reproductive bodies of more or less definite form, composed of a compact mass of homogeneous or heterogeneous cells which may be few or many in number, but which are usually developed from primordia of more than one cell*". Hotson (1917) revised the genus because previously the distinctions between the genera *Papulaspora* and *Helicosporangium* were not well defined and he felt that it was necessary to separate them. He redescribed the genus *Papulaspora* to include all imperfect fungi that produce bulbils. More recently, Weresub & LeClair (1971) distinguished between papulaspores and bulbils, revising the taxonomy of the fungi on this basis. The different structures were distinguished as follows.

Papulaspore: "*initiated when prospective central cells are encircled by branches which arise primarily from the hyphae on which the central cells are borne. And thus we have a ball of cells differentiated soon after inception into a core of one-to-several enlarged, presumably germinable, central cells, with wall more or less thickened and pigmented, and a sheath of cells usually abruptly smaller, thinner-walled, purportedly sterile*".

Bulbil: "*pseudoparenchymatous propagules without structural differentiation . . . discretely homogeneous*".

Weresub & LeClair, using these definitions, removed from Hotson's classification of *Papulaspora* any fungi that did not produce 'papulaspores', thus producing a more homogeneous taxon. "*These papulaspores are recognized by their early development whether or not central and sheathing cells are distinguishable at maturity*" (Weresub & LeClair, 1971). Thus the two bulbilliferous basidiomycete genera *Burgoa* and *Minimedusa* were removed from the genus since they produce bulbils, not papulaspores.

All isolates of *Papulaspora* obtained by the precolonised plate method in this thesis had identical morphology. The fungus produced abundant, flocculent, white mycelium, extending across plates of PDA at a rate of *ca* 15mm per day. Production of bulbils followed the leading hyphae *ca* 36 hours later. Bulbils were produced abundantly from usually an intercalary primordium, or occasionally a terminal primordium, on the hyphae; their formation was initiated by the development of swollen cells which grew and divided to form the cells of the bulbil (Fig. 5.1, and see Fig. 3.4). The bulbil is heterogenous with large central cells (usually 1 - 4) developing among smaller cells; the whole propagule is *ca* 110 - 130μm diameter and initially colourless, it then becomes pigmented with maturity. Mature bulbils are orange-brown.

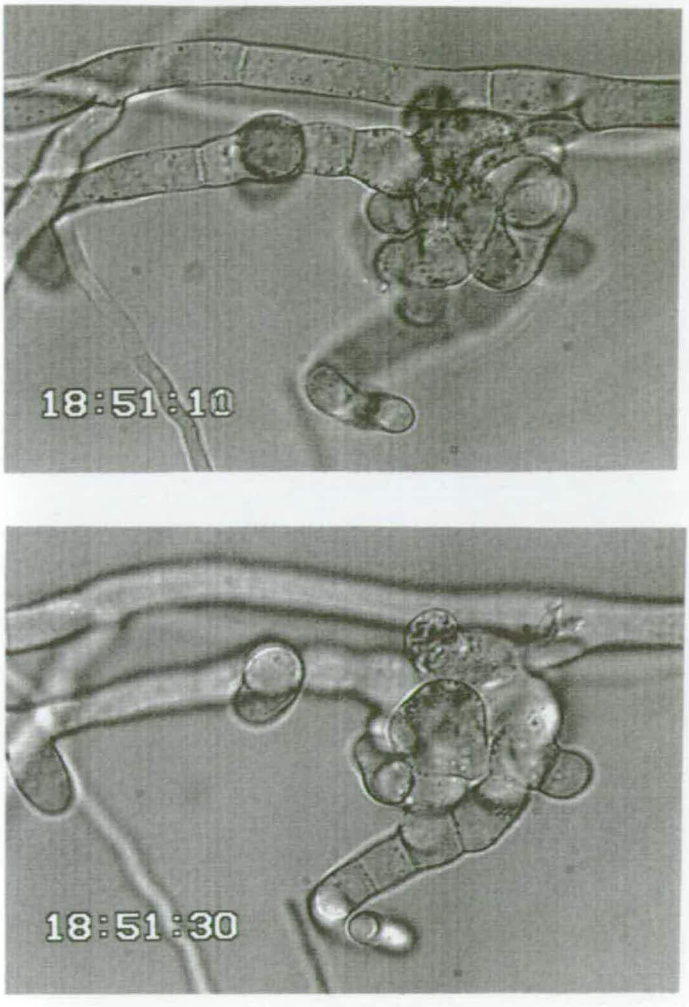


Figure 5.1. Development of a bulbil of *Papulaspora* sp. - two different planes of focus shown for the same bulbil structure.

This fungus did not belong to the basidiomycetes: hyphae did not have clamp connections, nor dolipore septa. Indeed rapid cytoplasmic streaming was observed through simple septa more characteristic of the ascomycetes. Thus identification of this fungus was made, on the basis of the heterogenous bulbils and the lack of basidiomycetous characteristics, and the fungus was termed *Papulaspora* Preuss (Weresub & LeClair, 1971). This would mean that the propagules produced by the fungus might, strictly, be termed 'papulaspores' and not 'bulbils'. However the term bulbil will be retained in this thesis because the distinction between the two terms is somewhat confused in the descriptions of Weresub & LeClair (1971), with various intermediate forms that are not easily referable to one or other type. Furthermore, the variation that exists in these vegatative hyphal bodies is much less than that found in sclerotia (for which a simple term is used) and they are probably not spores (papulaspores) in any case. In short, for purposes of this thesis, the isolates have been assigned to *Papulaspora* (sensu Hotson, 1912) and their heterogenous propagules have been termed 'bulbils'. A representative strain has been deposited in the culture collection of the International Mycological Institute, and has been assigned the culture reference number IMI 357504.

Papulaspora (sensu Hotson, 1912) seems to exist as a saprophyte and has been isolated from dung, decaying plant material and from soil (Domsch *et al.*, 1980). Some species have been reported to be mycoparasitic or plant-parasitic. For example, Hotson (1917) suggested that *P. parasitica* and *P. dahliae* were parasitic towards 'beets' and roots of dahlias, respectively. Hotson (1942) implied that *P. gladioli* (= *P. dodgei*) might be parasitic on *Gladiolus* bulbs but plant pathogenicity tests were negative. This fungus, along with two other *Papulaspora* spp., was found on decayed bulb material that had been infected with *Botrytis*, so the *Papulaspora* spp. were possibly secondary colonizers of the diseased material.

There have been two reports of the mycoparasitic nature of *Papulaspora* spp. Hotson (1917) reported that *P. magnifica* was either a parasite of the ascomycete *Ascobolus magnificus* or was an asexual form of this fungus, while *P. stoveri* was reported to be parasitic towards *R. solani* which infected sugar beets (Warren, 1948). In the latter example the mode of action of *Papulaspora* was to coil around the hyphae of the host fungus and cause disintegration of host cytoplasm, leaving the hyphal walls (Warren, 1948). In the same study *Papulaspora* was found to be effective in reducing black root (*R. solani*) of sugar beet, but not all strains of *R. solani* were found to be susceptible.

In addition to these reports, the bulbilliferous basidiomycete, *Minimedusa polyspora*, was implicated recently as a biological control agent of *Fusarium oxysporum* f. sp. *narcissi*, which causes basal rot of *Narcissus* (Beale & Pitt, 1990). Preliminary work by Beale & Pitt (1992) suggested also that it is effective against other soilborne plant pathogens, namely *F. oxysporum* f. sp. *dianthi* and *Pythium violae*. Beale & Pitt (1992) reported that the mode of action of *M. polyspora* against other fungi appeared to be antibiosis and mycoparasitism but that more studies were required to assess the full potential of the fungus.

Against this background, the experiments reported here were designed to investigate the behaviour of the *Papulaspora* sp. isolated by the precolonised plate method, with special regard to its nutrition, relationship with other fungi and the feasibility of it being used as a biocontrol agent due to possible mycoparasitic activity.

5.2 Results

5.2.1 Detection of growth of Papulaspora across potential host fungi

Sectors of fungus-colonised agar, prepared as described in section 2.4.1, were inoculated centrally with water agar discs (5mm) or 50µl of a bulbil suspension of *Papulaspora*. The bulbil suspension was prepared (section 2.4.11) to a

concentration of 242 or 484 bulbils ml^{-1} of sterile distilled water so each sector of colonised agar received a mean 12 or 24 bulbils. The potential host fungi used for both types of *Papulaspora* inoculum were *Botrytis cinerea, Fusarium culmorum, F. oxysporum, Phytophthora cinnamomi, Pythium aphanidermatum, Rhizoctonia solani* and *Trichoderma aureoviride.* In addition, the following fungi were inoculated with only agar discs of *Papulaspora*: *Chaetomium* sp., *Fusarium* sp. (from soil no. 11), *Gaeumannomyces graminis, Humicola grisea, Mucor hiemalis, Phialophora* sp., *Rhizoctonia oryzae, Sclerotinia sclerotiorum* and *Zygorhynchus moelleri.* Replication of 'host' sectors was 10-fold for agar disc inocula of *Papulaspora*, and 6-fold for bulbil inocula.

It was impossible to quantify the extent of growth of *Papulaspora* across 'host' sectors, so sectors were deemed to have been colonised if bulbils of *Papulaspora* appeared outwith one microscope field of view (*ca* 2mm diameter) from the edge of the agar block (or bulbils suspension) when the plates were examined at x100 magnification. As shown in Table 5.1, *Papulaspora* colonised only *B. cinerea, F. culmorum, F. oxysporum, P. cinnamomi, P. aphanidermatum, T. aureoviride* and *Z. moelleri* from agar inocula and only *B. cinerea, F. oxysporum* and *T. aureoviride* from bulbil inocula. Notably, in all cases where *Papulaspora* grew from agar inocula it colonised all or nearly all of the replicate 'host' sectors, but from bulbil inocula it only colonised all the sectors of *B. cinerea*, and fewer of the sectors of *F. oxysporum* and *T. aureoviride.* The remaining fungi did not support growth of *Papulaspora.*

5.2.2 Quantification of growth of Papulaspora across fungus-colonised plates

The growth of *Papulaspora* across fungus-colonised agar plates was measured by removing replicate agar strips from these plates which had been inoculated with *Papulaspora* at one side, then cutting the strips into 5mm segments and placing these on discs of agar colonised by *B. cinerea.* These discs were examined microscopically for the presence of bulbils of *Papulaspora* (section 2.4.12) after 7, 14 and 21 days incubation. Recording of presence or absence of bulbils on *B.*

Table 5.1. Detection of growth of *Papulaspora* across fungus-colonised agar from agar discs (maximum 10) or bulbil suspensions (maximum 6).

Test fungus	Number of agar sectors on which Papulaspora grew		
	From agar (max. 10)	From bulbil suspension ($242\ ml^{-1}$) (max. 6)	From bulbil suspension ($484\ ml^{-1}$) (max. 6)
B. cinerea	10	6	6
F. culmorum	10	0	0
P. cinnamomi	10	0	0
P. aphanidermatum	10	0	0
Z. moelleri	10	nt	nt
T. aureoviride	9	3	2
F. oxysporum	8	0	1
R. solani	0	0	0
Chaetomium sp.	0	nt	nt
Fusarium sp.	0	nt	nt
G. graminis	0	nt	nt
Humicola sp.	0	nt	nt
M. hiemalis	0	nt	nt
Phialophora sp.	0	nt	nt
R. oryzae	0	nt	nt
S. cepivorum	0	nt	nt

nt = Not tested.

cinerea, where *Papulaspora* had grown from each 5mm segment, allowed the distance that *Papulaspora* had grown as mycelia on the original agar plates to be calculated. The potential host fungi, *B. cinerea, F. culmorum, F. oxysporum, P. cinnamomi, P. aphanidermatum, R. solani* and *T. aureoviride*, were used with 5 replicate plates per host. *Papulaspora*, on previous evidence (Table 5.1), does not grow across *R. solani* so this fungus was included as a control.

As shown in Table 5.2, the 'host' fungi varied in their ability to support growth of *Papulaspora*. *B. cinerea* was the only fungus that was rapidly and completely colonised by *Papulaspora*. On plates of *F. culmorum*, *Papulaspora* grew slower but managed to extend across approximately half of each plate (*ca* 40mm), while on *F. oxysporum, P. cinnamomi* and *T. aureoviride*, *Papulaspora* was able to grow to only some degree (*ca* 15mm). No growth of *Papulaspora* was evident on agar colonised by *P. aphanidermatum* or *R. solani*.

Also, as shown in Table 5.2, *F. oxysporum* was recorded as making some growth after 7 days, but not after 14 or 21 days. It is possible that this discrepancy was due to poor initial growth, as mycelia on the 'host' colonies, but that the *Papulaspora* hyphae had died by 14 days, being unable to obtain further nutrients or to form bubils on *F. oxysporum*. A similar though less marked inability of *Papulaspora* to sustain growth was also seen on agar precolonised by *P. cinnamomi* and *T.aureoviride* (Table 5.2).

5.2.3 Nutritional requirements

The nutritional requirements of *Papulaspora* were investigated using liquid culture as described in section 2.4.13, with four replicate medical flats per treatment. For all the experiments described a one-way analysis of variance was applied to the results, using a completely randomised design.

Table 5.2. Mean extent of growth (mm ± SEM) of *Papulaspora* across host-colonised agar (max. 75mm) after 7, 14 and 21 days incubation, with 5 replicates per host.

	Mean extent of growth ± SEM		
'Host' fungus	**7 days***	**14 days**	**21 days**
B. cinerea	75 ± 0	75 ± 0	75 ± 0
F. culmorum	12 ± 5.6	45 ± 5.0	36 ± 5.6
F. oxysporum	16 ± 5.6	0	0
P.cinnamomi	35 ± 10.0	18 ± 4.9	14 ± 2.4
P.aphanidermatum	0	0	0
R. solani	0	0	0
T. aureoviride	15 ± 4.7	14 ± 3.3	14 ± 4.3

* = Days after inoculation of *Papulaspora* at which the sample strip was removed from the agar plate.

5.2.3.1 Nitrogen requirements

Papulaspora was inoculated and incubated for 14 days in liquid medium supplemented with glucose (20g l^{-1}) and containing (1) no nitrogen or vitamin supplement; (2) no nitrogen but with Difco yeast nitrogen base as a vitamin source; (3) sodium nitrate but no vitamin supplement; (4) sodium nitrate and yeast nitrogen base; (5) L-asparagine but no vitamin supplement; and (6) L-asparagine and yeast nitrogen base.

Very little growth was observed in the absence of a nitrogen source or yeast nitrogen base (vitamins and growth factors) and very little also when the medium contained nitrate-nitrogen with or without yeast nitrogen base (Table 5.3). Substantial growth was found on the medium supplemented with L-asparagine, but most growth occurred in the presence of both asparagine and yeast nitrogen base. It was therefore concluded that *Papulaspora* requires for good growth, both a source of growth factors and vitamins and a source of nitrogen, other than nitrate-nitrogen.

5.2.3.2 Carbon requirements

A similar type of experiment was done to that described above, but this time the effect of the different carbon sources on growth of *Papulaspora* was investigated. The carbon sources were glucose, mannitol and trehalose. L-asparagine (1.51g l^{-1}) was added to the media as the nitrogen source, and the carbon source was varied in each treatment, with or without the addition of yeast nitrogen base.

Papulaspora grew very poorly in media with no carbon or yeast base, or when trehalose or mannitol were used as the carbon sources (Table 5.4). Growth increased significantly when glucose was present as the carbon source, and also when trehalose was used as the carbon source with supplementary yeast nitrogen base. But optimum growth was observed when glucose was added to the medium with yeast nitrogen base. It seems therefore that *Papulaspora* can

Table 5.3. Mean mycelial dry weight (mg ± SEM) of *Papulaspora* after 14 days in glucose-salts medium supplemented with different nitrogen sources, with or without vitamins and growth factors from Difco yeast nitrogen base, with 4 replicates per treatment.

Treatment	**Mycelial Dry Weight (mg)**
Control (no nitrogen or yeast base)	4.2 ± 1.2[a]
Yeast base (no nitrogen)	3.8 ± 0.6[a]
Sodium nitrate	4.8 ± 1.1[ab]
Nitrate + yeast base	5.5 ± 0.9[ab]
L-asparagine	13.8 ± 2.3[b]
L-asparagine + yeast base	37.2 ± 6.9[c]

Values followed by the same letter do not differ significantly from one another at $P < 0.05$, as assessed by one-way analysis of variance (5% LSD = 9.2).

Table 5.4. Mean mycelial dry weight (mg ± SEM) of *Papulaspora* after 14 days in asparagine-salts medium supplemented with different carbon sources, with or without vitamins and growth factors from Difco yeast nitrogen base, with 4 replicates per treatment.

Treatment	**Mycelial Dry Weight (mg)**
Control (no carbon or yeast base)	2.5 ± 0.3[a]
Yeast base (no carbon)	2.2 ± 1.0[a]
Mannitol	3.2 ± 0.5[a]
Mannitol + yeast base	5.0 ± 1.2[a]
Trehalose	3.8 ± 0.8[a]
Trehalose + yeast base	12.8 ± 2.3[b]
Glucose	10.2 ± 1.9[b]
Glucose + yeast base	26.5 ± 2.2[c]

Values followed by the same letter do not differ significantly from one another at $P < 0.05$, as assessed by one-way analysis of variance (5% LSD = 4.3).

use trehalose as a sole available carbon source, but not as well as it uses glucose, and that utilisation of trehalose is dependant on a supply of vitamins or growth factors in yeast base. In contrast, it can grow on glucose in the absence of vitamins and growth factors, although its growth is enhanced by these nutrients.

5.2.3.3 Vitamin requirements

Growth of *Papulaspora* was assessed in media containing glucose and asparagine, with different combinations of vitamins. Treatments were the addition of biotin, thiamine and yeast nitrogen base, singly or in combination.

The results presented in Table 5.5 show that *Papulaspora* had the ability to grow in media containing glucose and asparagine with no supplementary vitamins, but grew best in the presence of yeast nitrogen base. The addition of biotin or thiamine, alone or in combination, sometimes enhanced growth but not as well as did the yeast base.

5.2.4 Cellulolytic activity

The ability of *Papulaspora* to utilise cellulose as a carbon source was examined using a modification of the method described in section 2.4.8.1. MNA plates were supplemented with L-asparagine (1.51g l^{-1}), instead of sodium nitrate, and each strip of cellulose film was inoculated with a PDA disc (5mm) of *Papulaspora* or left uninoculated (control). Each plate contained 1 strip of cellulose, with 4 replicate plates per treatment. The mean weight required to puncture the strips of cellulose film at 7 points was expressed as a percentage of the weight required to puncture 7 points in an uninoculated strip.

After 8 days incubation, the strips inoculated with *Papulaspora* still supported 95.60% ± 1.66 (SEM) of the weight supported by control strips, indicating no significant cellulolytic activity.

Table 5.5. Mean mycelial dry weight (mg ± SEM) of *Papulaspora* after 14 days in asparagine-glucose-salts medium supplemented with different vitamins, or with vitamins and growth factors from Difco yeast nitrogen base, with 4 replicates per treatment.

Treatment	**Mycelial Dry Weight (mg)**
Control (no vitamins)	17.0 ± 2.6[a]
Biotin + thiamine	17.0 ± 2.7[a]
Biotin	25.2 ± 0.5[ab]
Thiamine	25.8 ± 4.2[b]
Biotin + thiamine + yeast base	31.8 ± 2.8[b]
Yeast base	32.2 ± 3.0[b]

Values followed by the same letter do not differ significantly from one another at $P < 0.05$, as assessed by one-way analysis of variance (5% LSD = 8.4).

5.2.5 Determination of the pH range for growth of Papulaspora

Medical flats were prepared as described in section 2.4.13 and inoculated with *Papulaspora* with 4 replicates per treatment. The mycelial dry weights were determined across the pH range 3.0 - 8.0. In order to achieve this range two buffers were used: citrate-phosphate (pH 2.6 - 7.0) and phosphate-phosphate (pH 5.8 - 8.0). Overlap between these two buffers was desirable so that effects of pH could be separated, at least partly, from effects of the buffer components *per se*.

The buffers were added to media before autoclaving, at nominal pH intervals of 0.5 units, but assessment of pH after autoclaving showed that these intervals changed, as shown in Table 5.6. This narrowed the pH range tested to 3.12 - 7.25. As the results presented in Fig. 5.2 show, *Papulaspora* did not grow at pH 3.12, and made little or no growth above pH 7. But it grew between pH 4.0 and 6.9, with a broad optimum of pH 4.0 - 5.5. The decline in growth between pH 5.5 and *ca* 6.5 was evident from the results for both buffer systems.

5.2.6 Videotaped interactions

Hyphal interactions were studied on water-agar-coated coverslips, as described in section 2.4.9, between *Papulaspora* and the test fungi *B. cinerea, F. oxysporum* and *R. solani,* with 3, 6 and 5 interactions recorded for these respective fungi. The recorded observations covered a combination of contacts such as *Papulaspora* tip to 'host' side and vice versa.

These was no observed effect of *Papulaspora* on the other fungi in any interaction: no evidence of hyphal parasitism was observed nor was there any sign of other types of antagonism. The growth rate of *Papulaspora* hyphal tips was similar before contact and after contact with any of the fungi. Indeed, the colonies in all interactions grew over and past each other with no detrimental effect.

Table 5.6. The pH of buffered media, before and after autoclaving.

pH before autoclaving	**pH after autoclaving**	
	citrate-phosphate	*phosphate-phosphate*
3.0	3.12	na
4.0	nt	na
4.5	4.53	na
5.0	4.97	na
5.5	5.51	na
6.0	5.96	5.91
6.5	6.37	6.50
7.0	na	6.90
7.5	na	7.14
8.0	na	7.25

na = Not applicable.

nt = Not tested.

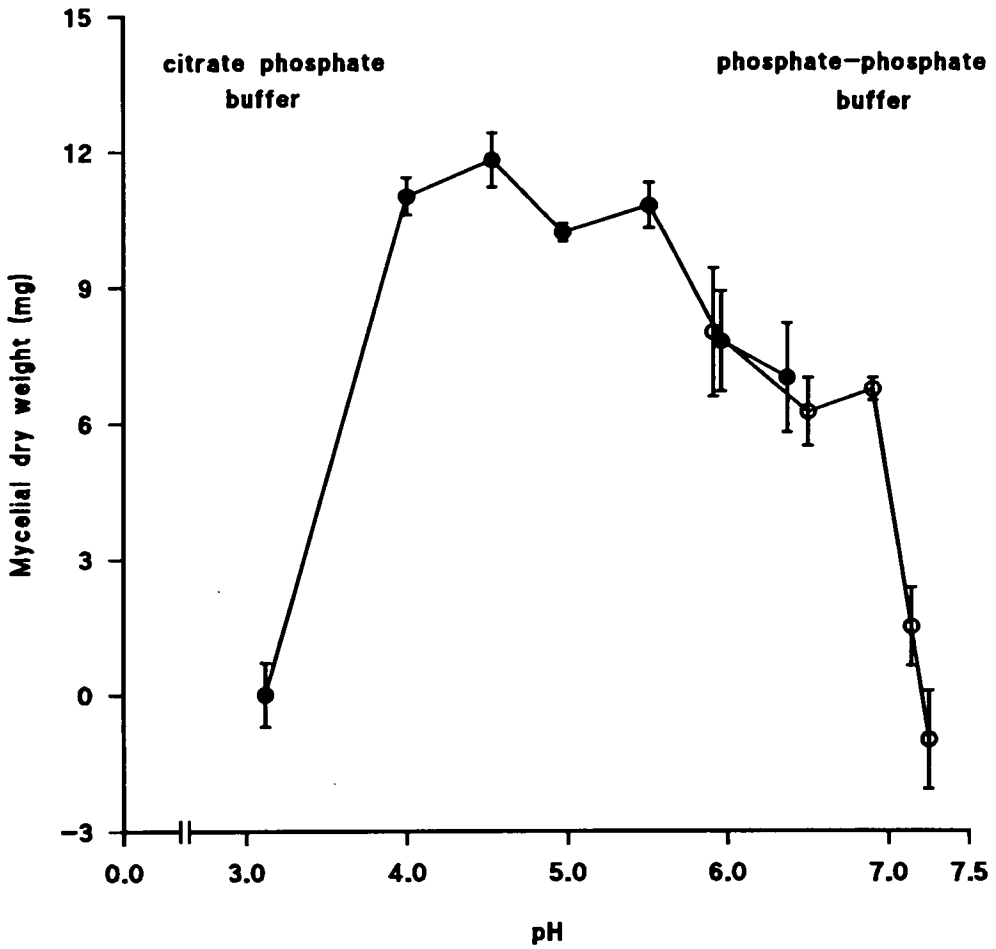


Figure 5.2. Effect of measured pH on growth of *Papulaspora* in buffered potato-dextrose broth; data points are means ± SEM for 4 replicate culture bottles.

5.2.7 Production of non-volatile metabolites

PDA plates completely overlaid with cellulose film were inoculated with *Papulaspora* or a 'host' fungus and incubated for different times. Then the cellulose and its overlying colony was removed before the agar was inoculated with another fungus and the linear growth extension was recorded (section 2.4.14). The host fungi used were *B. cinerea, R. solani* and *T. aureoviride*, with 4 replicates used per treatment. Student's *t*-tests were performed on the results to assess significant differences in growth caused by any residual non-volatile metabolites of the initial colonisers.

As shown in Table 5.7, pre-inoculation of PDA plates with *Papulaspora* resulted in significant increases in linear extension of all the test fungi across the agar in comparison to extension across plates pre-inoculated with the test fungus itself. If non-volatile inhibitory compounds had been produced by *Papulaspora* then growth of the hosts should have been reduced in their presence. Instead the presence of *Papulaspora* on the agar plates prior to inoculation with a test fungus had a stimulatory effect on growth of that fungus.

5.2.8 Production of volatile compounds

PDA plates were inoculated with *Papulaspora* for 7 - 10 days or with a test fungus for 2 - 5 days. Then PDA plates inoculated with the test fungi 1 day beforehand were inverted over the older plates (with lids removed) and taped together (section 2.4.15). The test fungi were those used in section 5.2.7, with 4 replicates per treatment. The linear growth of the colonies was measured and comparisons made between the fungi opposed to themselves and opposed to *Papulaspora*, using Student's *t*-tests.

The results shown in Table 5.8 show that some of the treatments resulted in significant increases in linear extension of the test fungi, in comparison to the controls (the test fungi inoculated against itself). In the presence of 7-day old

Table 5.7. Mean linear extension (mm ± SEM) of various fungi on agar plates which had previously been inoculated with *Papulaspora* or the same test fungus (self) on cellulose film overlays, with 4 replicates per treatment.

Test fungus	Pre-inoculated fungus		Significant difference*
	Self	*Papulaspora*	
B.cinerea	53.6 ± 1.1	70.2 ± 0.8	0.05
R.solani	62.3 ± 0.6	64.8 ± 0.6	0.05
T.aureoviride	57.5 ± 1.6	71.5 ± 2.1	0.05

* = Significant increase in linear extension of the test fungus on agar preinoculated with *Papulaspora*, compared with on agar precolonised by itself, as assessed by Student's *t*-tests ($P < 0.05$).

Table 5.8. Mean linear extension (mm ± SEM) of test fungi on PDA plates inverted over PDA plates inoculated with the same test fungus 2 - 5 days earlier (self), or with *Papulaspora* 7 or 10 days earlier, with 4 replicates per treatment.

Test fungus	Age of *Papulaspora* colony	Mean linear extension of test fungus when opposed to Self	Mean linear extension of test fungus when opposed to *Papulaspora*	Significant difference*
B.cinerea	7	29.2 ± 1.0	33.8 ± 3.4	nsd
	10	30.8 ± 0.7	35.5 ± 0.6	0.05
R.solani	7	51.6 ± 1.5	51.6 ± 1.6	nsd
	10	48.8 ± 0.8	49.6 ± 1.6	nsd
T.aureoviride	7	40.5 ± 0.8	49.5 ± 1.0	0.05
	10	45.9 ± 0.5	54.2 ± 0.4	0.05

* = Significant difference in linear extension of the host when opposed to itself or *Papulaspora*, as assessed by Student's *t*-tests ($P<0.05$); nsd = no significant difference.

Papulaspora colonies the extension of *T. aureoviride* across PDA increased significantly, and both *T. aureoviride* and *B. cinerea* showed significant increases in extension when 10-day old colonies of *Papulaspora* were present. In contrast the extension rate of *R. solani* was unaffected by colonies of *Papulaspora*. There was thus no evidence that *Papulaspora* produced volatile inhibitory compounds that affected any of the test fungi.

5.2.9 Growth of Papulaspora in liquid culture with other fungi

The ability of *Papulaspora* to grow in liquid media previously colonised or currently occupied by other fungi was investigated. *Papulaspora* was inoculated into medical flats containing liquid mineral salts medium, supplemented with L-asparagine (1.51g l^{-1}) or sodium nitrate (2g l^{-1}), and which either (1) contained colonies of other fungi inoculated 12 days earlier or (2) had been colonised by other fungi but the mycelia had been removed after 12 days (section 2.4.16). Three replicate flats were prepared for each 'host' fungus, for each source of nitrogen and for each subsequent treatment. The fungi used were *B. cinerea, F. culmorum, P. aphanidermatum, R. solani* and *T. aureoviride*.

The extent of growth of *Papulaspora* across the existing 'host' colonies was measured in each flat, from the edge of the *Papulaspora* inoculum block to the furthest visible bulbil, as an indication of the linear extent of growth of *Papulaspora*. Other flats were used to obtain dry weights of the test fungi in the different media, and also for *Papulaspora* inoculated into media that had previously supported other fungi or that had not been previously colonised. A one-way analysis of variance was carried out on the results to compare the growth of *Papulaspora* in different treatments.

Growth of *Papulaspora* across the host fungi was found to be very selective. *Papulaspora* grew, and colonised completely, all replicate flats that still contained colonies of *B. cinerea*, on either of the nitrogen sources, but it only partially colonised one replicate flat that still contained *T. aureoviride*, which

had been grown on asparagine-amended media. No production of bulbils was observed in the flats containing the other fungal colonies for any nitrogen source.

As shown in Table 5.9, all 'host' fungi with the exception of *R. solani* grew well in the liquid media supplemented with either nitrogen source. *R. solani* grew poorly, producing sparse mycelium. The medium previously inoculated with this fungus might thus be expected to contain higher levels of residual nutrients than the media colonised by other fungi.

Mycelial dry weights of *Papulaspora* revealed that this fungus grew best in the original liquid medium supplemented with asparagine and significantly less well when sodium nitrate was the nitrogen source ($P < 0.05$, as assessed by Student's *t*-test). Also, variable growth of *Papulaspora* was evident in media that had previously supported the different fungi. In asparagine-amended media *Papulaspora* grew poorly when the media had previously supported growth of *P. aphanidermatum* or *T. aureoviride,* but well when *B. cinerea*, *F. culmorum* or *R. solani* had precolonised the medium. Growth of *Papulaspora* in these cases was comparable to that in the original asparagine-amended medium.

When sodium nitrate was the nitrogen source then *Papulaspora* grew best in the medium that had previously supported growth of *R. solani*; there was no significant difference between this and growth of *Papulaspora* in the original medium. *Papulaspora* also grew well on medium that had supported *B. cinerea*, but poorly in equivalent media from *F. culmorum, P. aphanidermatum* or *T. aureoviride.* These results, except in the case of *F. culmorum*, were similar to those obtained for the asparagine-amended media.

Within each type of medium (nitrate or asparagine), Table 5.9 shows that growth of *Papulaspora* was not obviously related to the amount of previous

Table 5.9. Mycelial dry weights (mg ± SEM) of various fungi in glucose-salts media containing sodium nitrate or asparagine, and of *Papulaspora* inoculated into media in which the other fungi had grown, with 3 replicate medical flats per treatment.

Test fungus	Mycelial dry weight (mg) of 'host'		Mycelial dry weight (mg) of Papulaspora	
	Asparagine	Nitrate	Asparagine	Nitrate
B. cinerea	50.3 ± 2.1	63.0 ± 5.5	9.6 ± 0.7^{a}	4.6 ± 0.3^{bc}
F. culmorum	55.0 ± 2.7	68.3 ± 2.3	8.3 ± 2.0^{a}	1.6 ± 0.9^{d}
P. aphanidermatum	40.6 ± 9.7	52.6 ± 2.7	3.0 ± 0.9^{b}	2.0 ± 1.2^{cd}
R. solani	15.3 ± 2.0	17.0 ± 0.9	8.0 ± 0.3^{a}	6.6 ± 1.7^{ab}
T. aureoviride	73.6 ± 4.3	66.3 ± 3.0	1.3 ± 1.7^{b}	2.0 ± 0.3^{cd}
None	na	na	11.3 ± 0.6^{a}	7.6 ± 0.3^{a}

In asparagine-amended media, values for the mycelial dry weight of *Papulaspora* followed by the same letter do not differ significantly from one another at $P<0.05$, as assessed by one-way analysis of variance (5% LSD = 3.7).

In nitrate-amended media, values for the mycelial dry weight of *Papulaspora* followed by the same letter do not differ significantly from one another at $P<0.05$, as assessed by one-way analysis of variance (5% LSD = 2.9).

na = Not applicable.

growth by the 'host' fungus with the possible exception of *R. solani* because this fungus grew very poorly in the liquid media.

5.3 Discussion

Papulaspora Preuss has been found to be ubiquitious in soil, being detected in 27 soils of the 34 that had been added to precolonised agar plate (section 3.2.1.3). The soils in which it was found were from a variety of disturbed and undisturbed sites, with a pH range of 3.65 - 7.72. The fungus was detected most often on agar colonised by *B. cinerea*, but also on *T. aureoviride* and sometimes *R. solani*. Previous to this study, *Papulaspora* had been recorded infrequently from soil (Domsch *et al.*, 1980; Foley & Deacon, 1985). The experiments in this section were designed to investigate the biology of the *Papulaspora* isolates from soil, and to address questions such as (1) what role might *Papulaspora* have in interfungal interactions in soil and (2) why is it selectively isolated from soil only on specific 'host' fungi?

With regard to the second question, a range of fungi were tested *in vitro* to investigate the ability of *Papulaspora* to overgrow them on agar and to see if the efficiency of detection from soil could be improved still further by use of agar precolonised by fungi that were not used in the isolation studies (section 3.2.1.2).

The ability of *Papulaspora* to overgrow colonies of 16 fungi from a range of taxonomic groups varied with the source of inoculum: if agar blocks were used then *Papulaspora* was able to grow across more fungal colonies than if bulbil suspensions were used (Table 5.1). In addition, growth across the test fungus from a bulbil suspension only occurred on fungi that supported growth of *Papulaspora* from agar blocks, although fewer replicates were colonised from bulbils. Growth of *Papulaspora* from agar blocks probably occurred more often than from bulbils because the former contained active mycelium that would readily extend out from the inoculum to the new food source. In a bulbil

suspension the bulbils would have to germinate before growth commenced across the host sectors. Although little is known about the germination of bulbils of any *Papulaspora*-like fungus, it seems reasonable to assume that nutrients are a trigger for this. In addition, it is possible that inoculum density or inoculum potential (in the sense of Garrett, 1970, as the energy available for growth) is involved; a minimum inoculum potential might be required for *Papulaspora* to be able to establish itself in a competitive environment. *Papulaspora* probably exists in soil as bulbils rather than active mycelia, so the findings for agar plates inoculated with bulbil suspensions might be more representative of the ability of *Papulaspora* to be able to establish on precolonised agar plates from soil placed on these. Growth across the plates from bulbil suspensions in the dual culture studies (section 5.2.1) did in fact correspond closely to the results from the isolations from soil onto precolonised plates (section 3.2.1.2).

Quantitative growth of *Papulaspora* across fungus-colonised plates was also measured. *Papulaspora* overgrew the fungal colonies selectively and to different extents. *B. cinerea* was the only fungus to support extensive growth of *Papulaspora*; partial colonisation or none was observed on the other fungi. A potentially important observation in this respect was that *Papulaspora* initially colonised the colonies of some fungi from agar disc inocula but then ceased further growth (eg. on *T. aureoviride,* in Table 5.2) or was recorded as having colonised less far after 14 or 21 days than after 7 days (eg. on *P. cinnamomi* or *F. oxysporum*, in Table 5.2). Foley & Deacon (1985) made similar observations for growth of *Pythium oligandrum* across precolonised agar plates. The likely explanation is that the initial growth occurred at the expense of nutrients in the inoculum block (*Papulaspora* or *P. oligandrum*), but that the hyphae could not sustain growth nor persist if they could not produce survival structures such as bulbils (or oogonia of *P. oligandrum*).

Different degrees of growth across fungal colonies have also been recorded for other presumptive mycoparasites. For example, Laing & Deacon (1990) found

that *P. oligandrum* grew across colonies of more host fungi, and more extensively, than did *Pythium* SWO (now called *P. mycoparasiticum*) or *P. nunn*. These different extents of growth across host fungi broadly correlated with sensitivity of the hosts to parasitism in videotaped inter-hyphal interactions (Laing & Deacon, 1991). Also, Whipps (1987a) investigated the effect of nutrition on the interactions between a range of soil-borne glasshouse pathogens and some antagonistic fungi, and found that the ability of the antagonistic fungi to overgrow the colonies of the pathogens was dependent on the nutrients used in the underlying agar. Overgrowth of the pathogens occurred more frequently on nutrient-rich media.

The ability of *Papulaspora* to overgrow fungal colonies implies that *Papulaspora* was obtaining nutrients from either the fungal hyphae or the residual components of the agar media. Pure culture studies showed that the essential nutrients that *Papulaspora* required for growth were provided by mineral salts, an organic nitrogen source (L-asparagine), glucose and a mixture of vitamins and growth factors as supplied by Difco yeast nitrogen base (section 5.2.3). Little or no growth was observed in media containing sodium nitrate as sole nitrogen source, indicating that *Papulaspora* might require organic and not inorganic nitrogen (Table 5.3). However some fungi that cannot utilise nitrate can use ammonium as a sole nitrogen source, because they lack an enzyme (nitrate reductase or nitrite reductase) involved in the reduction of nitrate to ammonium (Smith & Berry, 1976). This was not tested for *Papulaspora*.

In further pure culture studies, *Papulaspora* grew best in media containing glucose with yeast nitrogen base, but also grew to some degree in media with supplementary glucose only (Table 5.4). It thus does not seem to have an obligatory requirement for exogenous vitamins and growth factors, although its growth was enhanced by these (Table 5.5). With regard to carbon sources, *Papulaspora* seemed to have a rather restricted range of abilities. It was found to be non-cellulolytic, even when a relatively easily degradable cellulose source

such as reconstituted cellulose film was used. It grew poorly or not at all on mannitol, with or without yeast nitrogen base and when supplied with organic nitrogen, but it grew on trehalose if supplemented with yeast nitrogen base, but not in the absence of vitamins or growth factors.

This finding is of interest because it demonstrates that *Papulaspora* needs to be supplied with additional medium components in order to utilise the more unusual sugars. Such a requirement for multiple components is not unusual among fungi when they are presented with a sub-optimal carbon and energy source (Garraway & Evans, 1970a). It was also reported for the mycoparasite *Pythium acanthicum*, which was found to utilise mannitol better in the presence than in the absence of sterols (Child *et al.*, 1969). Some biotrophic mycoparasites, such as *Tieghemiomyces parasiticus*, require glycerol for growth in axenic culture, and can apparently utilise common sugars in the presence of glycerol (Jeffries, 1985). In such cases the supplements (sterols, glycerol) might serve a role in facilitating uptake of sugars across the membrane. Yeast nitrogen base might also contain components with this property, although this was not investigated. In any case, trehalose and mannitol were tested as potential substrates for *Papulaspora* because they are common 'fungal carbohydrates', found within the mycelia, spores and resting bodies of many fungi, but not necessarily utilisable as exogenously supplied carbon sources (Jennings, 1974). Foley & Deacon (1986b) had shown that the mycoparasite *Pythium oligandrum* could utilise these carbohydrates whereas plant-pathogenic *Pythium* spp. could not do so, consistent with the types of carbohydrates that these fungi might be expected to encounter from their respective fungal or plant hosts. The failure of *Papulaspora* to utilise mannitol rises doubts about its mycoparasitic activity.

Video microscopical studies of inter-hyphal interactions on water agar films showed no evidence of coiling of *Papulaspora* around hyphae of other fungi, nor any evidence of penetration or even disruption of the growth of other fungal hyphae before or after contact. Thus there was no evidence that *Papulaspora*

was mycoparasitic, or even antagonistic to other fungi, including *B. cinerea* which supports its growth on precolonised agar plates. This does not correlate with the few reports of the mycoparasitic nature of *Papulaspora*. Warren (1948) found *P. stoveri* to be parasitic towards some strains of *R. solani* but the degree of parasitism was dependent on the strains of the host used. *P. magnifica* has also been reported to be mycoparasitic (Hotson, 1917) and the *Papulaspora*-like fungus *Minimedusa polyspora* is said, but without supporting evidence, to be a mycoparasite of *Fusarium oxysporum* f. sp. *narcissi* (Beale & Pitt, 1992). The findings here suggest that not all *Papulaspora*-like fungi are mycoparasites, and even the reported mycoparasitism by other strains, mentioned above, seems to merit re-evaluation.

The precolonised plate method for isolation of *Papulaspora* from soil also commonly selects for the mycoparasites *Pythium oligandrum, Gliocladium roseum* and *Trichoderma* spp. (section 3.2.1.1). These other fungi, when tested against host fungi on water agar films, show various antagonistic activities including delayed post-contact disruption of the contacted compartments of 'host' hyphae, direct penetrative parasitism or pre-contact inhibition of other fungi by release of diffusible metabolites. Deacon & Berry (1992) categorised these modes of behaviour as different types of mycoparasitism, using this term in a loose sense. The fact that *Papulaspora* showed none of these forms of behaviour raised the possibility that it antagonises only when sufficient nutrients are available. Also, tests for antibiotic activity revealed that *Papulaspora* did not release any inhibitory volatile or non-volatile compounds that could prevent or slow the growth of other fungi (Table 5.7 & 5.8). Thus all the evidence suggests that *Papulaspora* is not a true mycoparasite or even a direct antagonist of other fungi.

It was observed when testing *Papulaspora* for production of inhibitory compounds that sometimes growth of the test fungi seemed to be enhanced by the presence of *Papulaspora*. However, these results must be interpreted

cautiously. A possible explanation for the increase in growth by test fungi on media that previously supported *Papulaspora* compared with media that previously supported the test fungi themselves is that the test fungi had depleted more of the nutrients that they require than did pre-inoculated *Papulaspora*. Likewise the results for tests on volatile inhibitory compounds (Table 5.8), where *Papulaspora* seemed to enhance growth of other fungi, might have been due to the lesser growth of *Papulaspora* so that fewer generally inhibitory volatile metabolites accumulated. In any case, there was no evidence from these tests that *Papulaspora* produced especially inhibitory metabolites that would aid the antagonism of other fungi.

The ability of *Papulaspora* to overgrow colonies of other fungi, despite its apparent lack of antagonistic properties, suggests that it is able to exploit the relatively low levels of nutrients in previously colonised agar or, perhaps, nutrients released from hyphae of other fungi as these hyphae autolyse. This was investigated by examining the ability of *Papulaspora* to grow in liquid media in the presence of other fungi, and in media that had previously been exploited by them (section 5.2.9).

An interesting feature of this experiment was that *Papulaspora* could grow in the presence of mycelia of *B. cinerea* in liquid media but not in the presence of mycelia of other fungi (*F. culmorum, P. aphanidermatum, R. solani* and *T. aureoviride*, except for one replicate). This range of 'cohabitant' fungi was narrower than on agar plates, where *Papulaspora* had been found to grow over colonies of *F. culmorum* and *T. aureoviride*. Similar findings were obtained by Foley & Deacon (1986a) in that *P. oligandrum* grew only in the presence of *Phialophora* sp. in liquid medium and not in the presence of *F. culmorum*, which supported or enabled its growth on agar. Evidently the conditions in liquid culture are more demanding for these mycoparasites or presumptive mycoparasites than on agar media. But the most interesting feature of this experiment (Table 5.9) was that *Papulaspora* was able to make at least some

growth in media that had previously supported the growth of other fungi, such as *R. solani* (and *F. culmorum* when organic nitrogen was present), even though *Papulaspora* was not observed to grow in the same media when these fungi were present. This again was found by Foley & Deacon (1986a) for growth of *P. oligandrum*. It is clear that the necessary nutrients for growth of *Papulaspora* were present in the media previously exploited by some other fungi, but the continuing presence of these other fungi - except *B. cinerea* - prevented *Papulaspora* from using these media components.

These findings seem to relate to the principle of fungal possession of substrata, reviewed by Bruehl (1975), who distinguished between passive possession (food stored in resting structures), active possession (the unused components of the substratum are controlled by the hyphae of existing fungi, thereby excluding competitors) and combination possession (a mixture of the two). With the type of fungi and the design of the experiment in section 5.2.9, active possession seems the most likely possibility. It is interesting because it does not necessarily correlate with the production of recognisable and extractable antibiotics. In fact, *Papulaspora* hyphae were unaffected by hyphae of the other fungi on agar films used for video microscopy.

Other examples of this phenomenon include the reported ability of the eyespot fungus, *Pseudocercosporella herpotrichoides*, to slow ('control') the rate of breakdown of cereal straws in which it is already established (Macer, 1961), the ability of *Cephelosporium gramineum* (= *Hymenula cerealis*) to exclude other fungi from precolonised substrata (Bruehl, 1975) and the ability of *Armillaria mellea* to persist in woody substrata, preventing the ingress of *Trichoderma* spp. (Ohr & Munnecke, 1974). In such cases it has sometimes been proposed that the primary coloniser produces highly labile antibiotics that help in substrate possession. It has also been found that even a temporary reduction of activity of the previously established fungus, such as by treatment with a sub-lethal dosage of fumigant (Ohr & Munnecke, 1974; Henis & Papavizas, 1983) or sub-lethal

heat-treatment (Lifshitz *et al.*, 1983; Katan, 1987), was sufficient to weaken the initial occupant and promote its replacement by antagonists. Katan *et al.*, (1992) recently reviewed this 'weakening effect' of sublethal treatments and discussed the difficulties of investigating this phenomenon. Perhaps the type of experiment done here and by Foley & Deacon (1986a) could provide an approach to investigating it in reasonably defined conditions.

In conclusion of this section, it seems that *Papulaspora* can utilise low levels of nutrients in the presence of some, but not all, fungi and that its ability to colonise substrata occupied by other fungi (as in the precolonised plate method) could have implications in plant disease control. If the inoculum density of a pathogen increases through utilisation of available nutrients in the soil, such as inorganic debris, then *Papulaspora* could be one of the fungi that limits this activity. It could lead to control by creating nutrient stress of the pathogen and thereby induce autolysis (Ko & Lockwood, 1970); *Papulaspora* might also then be able to utilise components of the dead mycelia as nutrient sources.

Other mycoparasites or presumptive mycoparasites also have this ability. For example, Paulitz & Baker (1987b) showed that *Pythium nunn* prevented *P. ultimum* from utilising host residues, and thus maintained a low inoculum level of the pathogen; Whipps (1987b) showed that *P. oligandrum* and some other antagonists can colonise crop residues previously colonised by plant-pathogenic fungi; and Martin & Hancock (1986) also showed that *P. oligandrum* can compete for crop residues with phytopathogenic pythia, thereby reducing the buildup of inoculum of plant pathogens in their saprophytic phase on crop residues.

More generally, Stahl & Christensen (1992) considered the mycelial interactions between soil fungi *in vitro*. They concluded that on nutrient-poor substrata "*neutral intermingling*" between opposing fungi was the most common type of hyphal interaction, but if nutrient-rich substrata were present then the mycelia

of the different fungi were usually inhibited from growing into each other's colonies, described as "*deadlock*". A third type of interaction, termed "*replacement*", also occurred; this involved the invasion of a fungal colony by another fungus to the detriment of the invaded fungus. Whipps (1987a) found similar types of interactions between fungal colonies with the interactions between the fungi depending on the isolates of fungi used and the components of the media. Thus the tendency of a few fungi (those that grow on precolonised agar plates) to invade the territory of other fungi can be categorised. This supports previous evidence that overgrowth is a fungus-specific and nutritional-specific effect because the different presumptive mycoparasites are able differentially to grow on agar, or liquid, precolonised by various 'host' fungi.

6. CONCLUDING DISCUSSION

The work in this thesis was carried out to determine the mycoparasite spectra of soils and to investigate the effects of mycoparasites on the activities of other common soil fungi. This work has been discussed in detail in the relevant sections - 3.3, 4.3, 5.3 - so the aim of this section is to draw the separate findings together and to consider future work and its relevance to biocontrol.

In section 3 it was shown that agar colonised by a range of different host fungi could be used to differentially detect the mycoparasites of a soil. Four presumptive mycoparasites were detected - *Gliocladium roseum* (and related species), *Papulaspora* sp., *Pythium oligandrum* and *Trichoderma* spp. (principally *T. harzianum*). The detection of these presumptive mycoparasites on precolonised agar was related to the host fungus (section 3.2.1.2), the presence of other mycoparasites (sections 3.2.1.2 & 3.2.3) and the soil type (section 3.2.1.3). But many other reportedly common mycoparasites were not detected and there are three possible reasons for this.

(a) *Competition.* This was shown to exist between *Papulaspora* and *P. oligandrum*, influencing the detection of *Papulaspora* on host-colonised agar that *P. oligandrum* was also detected on (Table 3.9). Other combinations of mycoparasites never occurred or rarely occurred on single host sectors - for example, *P. oligandrum* with *Trichoderma* (Table 3.6). Thus detection of the mycoparasites was influenced by the presence of other mycoparasites, which is perhaps why other known mycoparasites, such as *V. biguttatum* (Boogert & Jager, 1983), *P. nunn* (Lifshitz *et al.*, 1984c) and *P. mycoparasiticum* (Deacon *et al.*, 1991), were not detected in this study. The use of metalaxyl to inhibit *P. oligandrum* proved valuable in detection of *Papulaspora* sp., revealing that this fungus is much more common in soil than was previously recognised. It might be desirable to investigate the use of other selectively toxic components in precolonised agar plates in attempts to detect yet further mycoparasites.

(b) *Host range.* The value of using a range of hosts for detection of mycoparasites has been demonstrated here, so it is possible that other mycoparasites would have been found by using further types of host fungi. As one example, the mycoparasite *V. biguttatum* was not detected in this study, even though soil from potato crops was placed on plates precolonised by the host *R. solani.* Boogert & Jager (1983) detected this mycoparasite frequently from soil using agar colonised with *R. solani,* but they used strain AG3 of the pathogen whereas in this study the *R. solani* strain was AG5, which is not a preferred host of *V. biguttatum* (Boogert *et al.*, 1989). A possible further example concerns *Talaromyces flavus.* Although this seemingly is not a highly host-restricted mycoparasite, it has been reported to colonise the microsclerotia of *Verticillium dahliae* on roots (Fahima & Henis, 1990) and also antagonise this fungus in soil (Marios *et al.*, 1982). Perhaps *V. dahliae* should be tested on precolonised agar plates for detection of this mycoparasite in soil.

(c) *Soil type or other site factors.* Mycoparasites were perhaps not detected in certain soils because the soils or site factors were ecologically unsuitable for their growth. For example, *P. acanthicum* and *P. periplocum* are taxonomically similar to *P. oligandrum* and would therefore be expected to overgrow the same fungal hosts. The dual-culture studies of Deacon & Henry (1978) suggested this. However *P. acanthicum* and *P. periplocum* were not detected at all in this study. Dick & Ali-Shtayeh (1986) suggested that these mycoparasitic pythia differed in their habitat requirements, as evidenced by a lack of detection of them together in the same sites. To date, *P. nunn* has only been isolated from a site in Colorado (Lifshitz *et al,* 1984c), which might indicate that it is site-restricted. But this more probably reflects the fact that a systematic search has not been made for *P. nunn* elsewhere, using the methods with which it was found at Nunn, Colorado. This raises the important point, that it will only be possible to gain a clear picture of the distribution of different mycoparasites, and of the soil or other site factors that influence them when standardised detection techniques are used in many different geographical areas.

Further methods have been developed in order to detect other potential mycoparasites in soil. One method of improving detection is with the use of baiting (section 1.4.1). In this study, baiting with cellulose film or pieces of wheat leaves (with or without prior colonisation by cellulolytic fungi), was found to increase the detection of *Trichoderma* and *Papulaspora* from soils known to contain them (section 3.2.3). Fungal structures such as sclerotia have also been used as baits (Adams & Ayers, 1981). It would seem that some fungi require more specific detection methods than others; for example *Coniothyrium minitans* was isolated much more frequently from soil by using sclerotia baiting than by use of precolonised agar plates (Sandys-Winsch *et al.,* 1993) whereas the use of precolonised plates above seems satisfactory for detection of *P. oligandrum* from soil (Foley & Deacon, 1985).

A major potential advantage that will come from the further development of detection methods is that the distributions of mycoparasites in different countries will be known. This should aid the registration of biocontrol inocula based on mycoparasites, because indigenous biocontrol agents are more likely to be more readily acceptable than exotic agents. Moreover, for some of the most common and widely distributed mycoparasites a knowledge of their presence or absence in a site should provide an indication of the suitability of site conditions for them. This, in turn, could indicate the likely success of biocontrol inocula applied to those sites because some of the most successful examples of applied biocontrol involve the addition of a resident biocontrol agent to a site where it already exists, but applied at an overwhelming (inundative) level to specific microsites such as seeds or wounded plant surfaces (Deacon, 1991).

The use of the precolonised plate method detected three known types of mycoparasites - *Gliocladium roseum*, *P. oligandrum*, *Trichoderma* spp. - and one presumptive mycoparasite, *Papulaspora* sp., about which little was previously known. Further study of *Papulaspora* (section 5) showed that it did not act as a

mycoparasite, not did it antagonise other fungi by contact-mediated inhibition. It was, however, able to grow at low level of nutrients in the presence of some fungi (e.g. *B. cinerea*) and to tolerate their metabolic by-products, whereas it did not grow in the presence of other fungi, such as *R. solani*.

This ability of mycoparasites and presumptive mycoparasites to overgrow fungus-colonised substrata should parallel the ability to act as a secondary invader of substrate, including host tissues that have been colonised and destroyed by plant pathogens. Indeed, *P. oligandrum* was initially thought to be a plant pathogen because Dreschler (1930) found that it was almost the only fungus that grew from diseased pea roots when he was attempting to isolate the causal agent. Subsequent work led Dreschler (1943) to suggest that the role of *P. oligandrum* was actually as a secondary invader of the plant tissues that had been killed by other *Pythium* spp. *P. oligandrum* was found to coil round the hyphae of other *Pythium* spp. on agar plates, so he suggested that it acted partly as a mycoparasite of the plant pathogens. Since then the role of *P. oligandrum* and other mycoparasitic pythia in soil has been re-investigated. Tribe (1966) found that *P. oligandrum* grew in association with cellulolytic fungi on cellulose in dual culture, although it itself could not use cellulose. He observed no evidence of parasitism and suggested that the fungus grew by secondary sugar saprophytism. Deacon (1976) later showed clearly that *P. oligandrum* can parasitise other fungi, including cellulolytic fungi that Tribe had investigated. Nevertheless, *P. oligandrum* could be an opportunist, sometimes parasitising other fungi and sometimes merely competing with them for nutrients in the underlying substratum.

Paulitz & Baker (1987a,b) investigated disease suppression to *P. ultimum* and found that *P. nunn* competed effectively with the pathogen for organic amendments added to soil. *P. nunn* is, in fact, a weak mycoparasite compared to *P. oligandrum*, and *P. ultimum* is not a highly susceptible host of these mycoparasites in any case (Laing & Deacon, 1990, 1991; Berry *et al.*, 1993).

Moreover, *P. nunn* grows much more slowly than does *P. ultimum*, which can germinate very rapidly in soil from sporangia and colonise fresh organic substrata more rapidly than *P. nunn* (Paulitz & Baker, 1988). So the ability of *P. nunn* to act as a secondary invader of substrata already colonised by *P. ultimum* could be crucial for its role in biocontrol and disease-suppression (Paulitz & Baker, 1987a,b, 1988). Martin & Hancock (1986) similarily found that *P. ultimum* colonised fresh cotton leaf residues ahead of *P. oligandrum* when the residues were added to soils but *P. oligandrum* then displaced *P. ultimum* or at least limited its population increase in the residues. In this case the level of colonisation by *P. oligandrum* was favoured in soils with high chloride content because *P. oligandrum* was more tolerant of high chloride levels than was *P. ultimum*. But the ability of *P. oligandrum* to colonise substrata already occupied by another fungus was a major contributory factor in this example of biocontrol (Martin & Hancock, 1986).

In relation to the displacement of plant pathogens from crop residues, and also presumably the displacement of other fungi from substrata that they have colonised, it is often necessary for the "resident" fungus to be "weakened" in some way. This was discussed by Katan (1992). For example, soil solarisation or soil partial fumigation kills or weakens the resident pathogens (and other organisms) in soil or in buried substrata, and allows proliferation of any antagonists that survive the treatment or that are re-introduced as added inocula. This gives the antagonist (mycoparasite) an advantage in possession of substrata in the soil but the ability of the antagonist to maintain possession depends on the ability of a fungus to defend its captured resource (Bruehl, 1975).

Interaction studies (section 4) showed that *P. oligandrum* is an aggressive mycoparasite against some cellulolytic soil fungi but mycoparasitism as such has not been demonstrated for *P. oligandrum* in soil. It would be useful to investigate further the cellulolytic fungi that predominate in soil, and their

resistance or susceptibility to mycoparasites, including *P. oligandrum*. Many (cellulolytic) plant pathogens are not active resident saprophytes in soil, and only become active when the appropriate fresh plant residues are added to soil or when appropriate plant hosts are present. This could influence the effects of mycoparasites. For example, Velvis *et al.* (1989) found that *V. biguttatum* parasitised a proportion of sclerotia of *R. solani* that survived in soil after harvest of potato crops, but that some sclerotia escaped attack. Those sclerotia which escaped infection became dormant in the absence of the potato crop and were not susceptible to parasitism by the resident population of *V. biguttatum* until they became active again in the presence of the plant. A tri-phasic system of plant, pathogen and mycoparasite was the ideal one for biological control of the soil-borne inoculum, because this was activated by the crop and then became susceptible to attack by the mycoparasite. Boogert & Velvis (1992) also studied population densities of *V. biguttatum* and *R. solani* in soil and found that there was a density-dependent mortality in the mycoparasitic relationship, stemming from the fact that *V. biguttatum* is a biotrophic mycoparasite, dependent on the population of its fungal host.

Strict density-dependent relationships might not be expected for the necrotrophic mycoparasites in the present study. Nevertheless, assuming that mycoparasitism contributes to their activities in soil, a cycle of mycoparasitic activity might exist. If *P. oligandrum* were to grow on cellulosic substrata in association with a susceptible cellulolytic fungus such as *H. grisea* then it might markedly reduce the activity of its host, then become nutrient-limited itself, because it cannot degrade cellulose, and revert to dormant oospores. This could then enable the host fungus to regrow repeating the cycle. A type of density-dependent parasitism could thus be envisaged, and examples of this have been considered in more detail by Jaffee (1993).

Whether mycoparasites exist in soil by mycoparasitism, competition or some other form of antagonism, it is known that mycoparasites can act as population

regulators of other fungi, especially of plant pathogens. Thus for optimum biocontrol it is suggested that the activities of mycoparasites in soil might be promoted purposefully by addition of organic supplements, with or without the simultaneous addition of mycoparasite inoculum, where necessary. Also, integrated control could be achieved by the addition of fungicide-tolerant strains of mycoparasites to soil, for use in conjunction with fungicides aimed at the control of plant pathogens. The current systemic fungicides have single-site modes of action, such that resistance to them can develop quite rapidly in populations of the target pathogens. Potentially, the rate of development of resistance could be reduced if fungicide-tolerant mycoparasites antagonised the pathogen, acting in concert with the fungicide. This approach merits further study - especially since it was shown here that a metalaxyl-tolerant strain of *P. oligandrum* could be recovered from soil at least 8 months after it had been introduced.

Appendix I. Summary of the data from section 3.2.5.1; values are the logarithms of the mean fertile level (x) of *P. oligandrum* determined for each treated soil at each sample time, with 3 replicate soils per treatment.

Time (days)	One addition of			Significance (P)	Two additions of			Significance (P)
	Control	Grass	Wheat		Control	Grass	Wheat	
0	0.76	0.74	0.78	nsd	0.76	0.74	0.78	nsd
10	0.74	0.76	0.80	nsd	0.74	0.76	0.80	nsd
20	0.70	0.65	0.74	nsd	0.70	0.65	0.74	nsd
40	0.70	0.65	0.81	nsd	0.70	0.65	0.81	nsd
80	0.68	0.72	0.83	nsd	0.68	0.72	0.83	nsd
120	0.68	0.76	0.86	nsd	0.68	0.76	0.86	nsd
150	0.71	0.70	0.89	nsd	0.71	0.70	0.89	nsd
160	0.72	0.68	0.90	nsd	0.74	0.86	0.88	nsd
200	0.68	0.72	0.85	nsd	0.60*	0.88	0.90	0.05
255	0.74	0.76	0.92*	0.05	0.65*	0.90	0.93	0.05
280	0.74	0.74	0.86	nsd	0.70	0.86	0.90	nsd

* = Data which differ significantly from the others at that sample day (5% LSD = 0.26).

Appendix II. Summary of the data from section 3.2.5.2; values are the logarithms of the mean fertile level (x) of *P. oligandrum* determined for each treated soil at each sample time, with 3 replicate soils per treatment.

Time (days)	Mean fertile level of Total population	Resistant population	Resident population	Significance (P)
0	0.63	-0.60*	0.65	0.05
10	0.78	0.68	0.57	nsd
20	0.78	0.74	0.60	nsd
40	0.74	0.63	0.57	nsd
80	0.70	0.63	0.60	nsd
120	0.70	0.57	0.65	nsd
160	0.74	0.72	0.57	nsd
200	0.76	0.68	0.63	nsd
240	0.74	0.70	0.60	nsd

* = Data which differ significantly from the others at that sample day (5% LSD = 0.26).

BIBLIOGRAPHY

ADAMS, P. B. (1967). A buried membrane filter method for studying behaviour of soil fungi. *Phytopathology* **57**, 602-603.

ADAMS, P. B. (1979). A rapid method for quantitative isolation of sclerotia of *Sclerotinia minor* and *Sclerotium cepivorum* from soil. *Plant Disease Reporter* **63**, 349-351.

ADAMS, P. B. (1987). Effects of soil temperature, moisture and depth on survival and activity of *Sclerotinia minor, Sclerotium cepivorum,* and *Sporidesmium sclerotivorum*. *Plant Disease* **71**, 170-174.

ADAMS, P. B. (1990). The potential of mycoparasites for biological control of plant disease. *Annual Review* of *Phytopathology* **28**, 59-72.

ADAMS, P. B. & AYERS, W. A. (1981). *Sporidesmium sclerotivorum*: distribution and function in natural biological control of Sclerotial fungi. *Phytopathology* **71**, 90-93.

ADAMS, P. B. & AYERS, W. A. (1983). Histological and physiological aspects of infection of sclerotia of *Sclerotinia* species by two mycoparasites. *Phytopathology* **73**, 1072-1076.

ADAMS, P. B., MAROIS, J. J. & AYERS, W. A. (1984). Population dynamics of the mycoparasite *Sporidesmium sclerotivorum*, and its host, *Sclerotinia minor*, in soil. *Soil Biology and Biochemistry* **16**, 627-633.

AHMAD, J. S. & BAKER, R. (1987a). Rhizosphere competence of *Trichoderma harzianum. Phytopathology* **77**, 182-189.

AHMAD, J. S. & BAKER, R. (1987b). Competitive saprophytic ability and cellulolytic activity of rhizosphere-competent mutants of *Trichoderma harzianum*. *Phytopathology* **77**, 358-362.

AHMAD, J. S. & BAKER, R. (1988a). Implications of rhizosphere competence of *Trichoderma harzianum*. *Canadian Journal* of *Microbiology* **34**, 229-234.

AHMAD, J. S. & BAKER, R. (1988b). Rhizosphere competence of benomyl-tolerant mutants of *Trichoderma* spp. *Canadian Journal of Microbiology* **34**, 694-696.

AL-HAMDANI, A. M. & COOKE, R. C. (1983). Effects of the mycoparasite *Pythium oligandrum* on cellulolysis and sclerotium production by *Rhizoctonia solani*. *Transactions of the British Mycological Society* **81**, 619-670.

AL-HAMDANI, A. M., LUTCHMEAH, R. S. & COOKE, R. C. (1983). Biological control of *Pythium ultimum*-induced damping-off by treating cress seed with the mycoparasite *Pythium oligandrum*. *Plant Pathology* **32**, 449-454.

ALI-SHTAYEH, M. S., LEN, L-H. C. & DICK, M. W. (1986). An improved method and medium for quantitative estimates of *Pythium* species in soil. *Transactions of the British Mycological Society* **86**, 39-47.

ALLEN, M. F., BOOSALIS, M. G., KERR, E. D., MULDOON, A. E. & LARSEN, H. J. (1985). Population dynamics of sugar beets, *Rhizoctonia solani*, and *Laetisaria arvalis*: Responses of a host, plant pathogen and hyperparasite to perturbation in the field. *Applied and Environmental Microbiology* **11**, 1123-1127.

ANDERSEN, A. L. & HUBER, D. M. (1965). The plate-profile technique for isolating soil fungi and studying their activity in the vicinity of roots. *Phytopathology* **55**, 592-594.

AYERS, W. A. & LUMSDEN, R. D. (1975). Factors affecting production and germination of oospores of three *Pythium* species. *Phytopathology* **65**, 1094-1100.

AYERS, W. A. & LUMSDEN, R. D. (1977). Mycoparasitism of oospores of *Pythium* and *Aphanomyces* species by *Hyphochytrium cateniodes*. *Canadian Journal of Microbiology* **23**, 38-44.

BAKER, R. (1987). Mycoparasitism: ecology and physiology. *Canadian Journal of Plant Pathology* **9**, 370-379.

BAKER, R. & SCHER, F. M. (1987). Enhancing the activity of biological control agents. In *Innovative Approaches to Plant Disease Control,* pp. 1-17. Edited by I. Chet. New York: Wiley Interscience.

BARAK, R. & CHET, I. (1990). Lectins of *Sclerotium rolfsii*: its purification and possible function in fungal interactions. *Journal of Applied Bacteriology* **69**, 101-112.

BARNETT, H. L. & BINDER, F. L. (1973). The fungal host-parasite relationship. *Annual Review* of *Phytopathology* **11**, 273-292.

BARTLE, H. (1991). Quiet sufferers of the silent spring. In *New Scientist*, 18th May, pp. 30-35.

BARTON, R. (1958). Occurrence and establishment of *Pythium* in soils. *Transactions of the British Mycological Society* **41**, 207-222.

BEAGLE-RISTAINO, J. E. & PAPAVIZAS, G. C. (1985). Survival and proliferation of propagules of *Trichoderma* spp. and *Gliocladium virens* in soil and in plant rhizospheres. *Phytopathology* **75**, 729-732.

BEALE, R. E. & PITT, D. (1990). Biological and integrated control of *Fusarium* basal rot of *Narcissus* using *Minimedusa polyspora* and other microorganisms. *Plant Pathology* **39**, 477-488.

BEALE, R. E. & PITT, D. (1992). Studies on *Minimedusa polyspora*, a biological control agent of soilborne plant pathogens. In *Biological control of plant diseases,* pp. 249-253. Edited by E. S. Tjamos *et al.* New York: Plenum Press.

BERRY, L. A., JONES, E. E. & DEACON, J. W. (1993). Interaction of the mycoparasite *Pythium oligandrum* with other *Pythium* species. *Biocontrol Science and Technology* **3**, 247-260.

BOOGERT, P. H. J. F. VAN DEN & JAGER, G. (1983). Accumulation of hyperparasites of *Rhizoctonia solani* by addition of live mycelium of *R. solani* to soil. *Netherlands Journal of Plant Pathology* **89**, 223-228.

BOOGERT, P. H. J. F. VAN DEN, REINARTZ, H., SJOLLEMA, K. A. & VEENHUIS, M. (1989). Microscopic observations of the mycoparasite *Verticillium biguttatum* with *Rhizoctonia solani* and other soil-borne fungi. *Antonie van Leeuwenhoek* **56**, 161-174.

BOOGERT, P. H. J. F. VAN DEN & SAAT, T. A. W. M. (1991). Growth of the mycoparasitic fungus *Verticillium biguttatum* from different geographical origins at near-minimum temperatures. *Netherland Journal of Plant Pathology* **97**, 115-124.

BOOGERT, P. H. J. F. VAN DEN & VELVIS, H. (1992). Population dynamics of the mycoparasite *Verticillium biguttatum* and its host, *Rhizoctonia solani. Soil Biology and Biochemistry* **24**, 157-164.

BOOSALIS, M. G. (1956). Effect of soil temperature and green-manure amendment of unsterilized soil on parasitism of *Rhizoctonia solani* by *Penicillium vermiculatum* and *Trichoderma* sp. *Phytopathology* **46**, 473-478.

BRADSHAW-SMITH, R. P., WHALLEY, W. M. & CRAIG, G. D. (1991). Interactions between *Pythium oligandrum* and the fungal footrot pathogens of peas. *Mycological Research* **95**, 861-865.

BRUEHL, G. W. (1975). Systems and mechanisms of residue possession by pioneer fungal colonists. In *Biology and Control of Soil-Borne Plant Pathogens*, pp. 77-83. Edited by G. W. Bruehl. Minnesota: The American Phytopathological Society.

BURGE, M. N. (1988). The scope of fungi in biological control. In *Fungi in Biological Control Systems*, pp. 1-18. Edited by M. N. Burge. Manchester: Manchester Universtiy Press.

BURR, T. J. & STANGHELLINI, M. E. (1973). Propagule nature and density of *Pythium aphanidermatum* in field soil. *Phytopathology* **63**, 1499-1501.

BURTON, R. J. & COLEY-SMITH, J. R. (1985). Antibiotics in sclerotia and mycelium of *Rhizoctonia* species. *Transactions of the British Mycological Society* **85**, 447-453.

BURTON, R. J., COLEY-SMITH, J. R., WAREING, P. W. & GLADDERS, P. (1988). *Rhizoctonia oryzae* and *R. solani* associated with barley stunt disease in the United Kingdom. *Transactions of the British Mycological Society* **91**, 409-417.

BURTON, R. J. & COLEY-SMITH, J. R. (1993). Production and leakage of antibiotics by *Rhizoctonia cerealis, R. oryzae-sativae* and *R. tuliparum*. *Mycological Research* **97**, 86-90.

BUTT, T. M., HOCH, H. C., STAPLES, R. C. & ST. LEGER, R. J. (1989). Use of fluorochromes in the study of fungal cytology and differentiation. *Experimental Mycology* **13**, 303-320.

CHEN, W., HOITINK, H. A. J. & MADDEN, L. V. (1988). Microbial activity and biomass in container media for predicting suppressiveness to damping-off caused by *Pythium ultimum*. *Phytopathology* **78**, 1447-1450.

CHEN, W., HOITINK, H. A. J. & SCHMITTHENNER, A. F. (1987). Factors affecting suppression of *Pythium* damping-off in container media amended with composts. *Phytopathology* **77**, 755-760.

CHESTERS, C. G. C. (1948). A contribution to the study of fungi in the soil. *Transactions of the British Mycological Society* **30**, 100-117.

CHET, I. (1987). *Trichoderma* - application, mode of action, and potential as a biocontrol agent of soilborne plant pathogenic fungi. In *Innovative Approaches to Plant Disease Control*, pp. 137-160. Edited by I. Chet. New York: Wiley Interscience.

CHILD, J. J., DEFAGO, G. & HASKINS, R. H. (1969). The influence of carbon and nitrogen nutrition on growth and sterol-induced sexuality of *Pythium* sp. PRL 2142. *Mycologia* **61**, 1096-1105.

COCHRAN, W. G. (1950). Estimation of bacterial densities by means of the "Most Probable Number". *Biometrics* **6**, 105-116.

COHEN, S. D. (1984). Detection of mycelium and oospores of *Phytophthora megasperma* forma specialis *glycinea* by vital stains in soil. *Mycologia* **76**, 34-39.

COHEN, S. D. & LOCKWOOD, J. L. (1981). Use of vital and fluorescent-antibody stains to detect propagules of *Phytophthora megasperma* f. sp. *glycinea* race 1 in soil. *Phytopathology* **71**, 867.

COOK, R. J. (1988). Management of the environment for the control of pathogens. *Philosophical Transactions of the Royal Society of London B* **318**, 171-182.

DEACON, J. W. (1976). Studies on *Pythium oligandrum*, an aggressive parasite of other fungi. *Transactions of the British Mycological Society* **66**, 383-391.

DEACON, J. W. (1991). Significance of ecology in the development of biocontrol agents against soil-borne plant pathogens. *Biocontrol Science and Technology* **1**, 5-20.

DEACON, J. W. & BERRY, L. A. (1992). Modes of action of mycoparasites in relation to biocontrol of soilborne plant pathogens. In *Biological Control of Plant Diseases: progress and challenges for the future*, pp. 157-167. Edited by E. S. Tjamos, G. Papavizas and R. J. Cooke. New York: Plenum Press.

DEACON, J. W. & HENRY, C. M. (1978). Mycoparasitism by *Pythium oligandrum* and *P. acanthicum*. *Soil Biology and Biochemistry* **10**, 409-415.

DEACON, J. W., LAING, S. A. K. & BERRY, L. A. (1991). *Pythium mycoparasiticum* sp. nov., an aggressive mycoparasite from British soils. *Mycotaxon* **42**, 1-8.

DENNIS, C. & WEBSTER, J. (1971a). Antagonistic properties of species-groups of *Trichoderma*, I. Production of non-volatile antibiotics. *Transactions of the British Mycological Society* **57**, 25-39.

DENNIS, C. & WEBSTER, J. (1971b). Antagonistic properties of species-groups of *Trichoderma*. II. Production of volatile antibiotics. *Transactions of the British Mycological Society* **57**, 41-49.

DENNIS, C. & WEBSTER, J. (1971c). Antagonistic properties of species-groups of *Trichoderma*. III. Hyphal interactions. *Transactions of the British Mycological Society* **57**, 363-369.

DICK, M. W. (1966). The Saprolegniaceae of the environs of Blelham Tarn: sampling techniques and the estimation of propagule numbers. *Journal of General Microbiology* **42**, 257-282.

DICK, M. W. & ALI-SHTAYEH, M. S. (1986). Distribution and frequency of *Pythium* Species in parkland and farmland Soils. *Transactions of the British Mycological Society* **86**, 49-62.

DIFCO MANUAL (1984). Dehydrated culture media and reagents for microbiology, pp. 1135-1141. Detroit: Difco Laboratories Incorporated.

DOMSCH, K. H., GAMS, W. & ANDERSON, T-H. (1980). *Compendium of Soil Fungi*. Edited by K. H. Domsch, W. Gams & T-H. Anderson. London: Academic Press Limited.

DRESCHLER, C. (1930). Some new species of *Pythium*. *Journal of the Washington Academy of Sciences* **20**, 398-418.

DRECHSLER, C. (1943). Antagonism and parasitism among some oomycetes associated with root rot. *Journal of the Washington Academy of Sciences* **33**, 21-28.

EASTBURN, D. M. & BUTLER, E. E. (1988a). Microhabitat characterization of *Trichoderma harzianum* in natural soil: evaluation of factors affecting population density. *Soil Biology and Biochemistry* **20**, 541-545.

EASTBURN, D. M. & BUTLER, E. E. (1988b). Microhabitat characterization of *Trichoderma harzianum* in natural soil: evaluation of factors affecting distribution. *Soil Biology and Biochemistry* **20**, 547-553.

ELAD, Y., LIFSHITZ, R. & BAKER, R. (1985). Enzymatic activity of the mycoparasite *Pythium nunn* during interaction with host and non-host fungi. *Physiological Plant Pathology* **27**, 131-148.

FAHIMA, T. & HENIS, Y. (1990). Interactions between pathogen, host and biocontrol agent: multiplication of *Trichoderma hamatum* and *Talaromyces flavus* on roots of diseased and healthy hosts. In *Biological Control of Soil-Borne Plant Pathogens*, pp. 165-180. Edited by D. Hornby. Wallingford: C. A. B. International.

FAULL, J. L. (1988). Competitive antagonism of soil-borne plant pathogens. In *Fungi in Biological Control Systems,* pp. 125-140. Edited by M. N. Burge. Manchester: Manchester University Press.

FISHER, R. A. & YATES, F. (1963). *Statistical Tables for Biological, Agricultural and Medical Research*. Edited by R. A. Fisher & F. Yates. Edinburgh: Oliver & Boyd.

FLOWERS, R. A. & HENDRIX J. W. (1969). Gallic acid in a procedure for isolation of *Phytophthora parasitica* var. *nicotianae* and *Pythium* spp. from soil. *Phytopathology* **59**, 725-731.

FOLEY, M. F. & DEACON, J. W. (1985). Isolation of *Pythium oligandrum* and other necrotrophic mycoparasites from soil. *Transactions of the British Mycological Society* **85**, 631-639.

FOLEY, M. F. & DEACON, J. W. (1986a). Susceptibility of *Pythium* spp. and other fungi to antagonism by the mycoparasite *Pythium oligandrum*. *Soil Biology and Biochemistry* **18**, 91-95.

FOLEY, M. F. & DEACON, J. W. (1986b). Physiological differences between mycoparasitic and plant-pathogenic *Pythium* spp. *Transactions of the British Mycological Society* **86**, 225-231.

FRAVEL, D. R. (1988). Role of antibiosis in the biocontrol of plant diseases. *Annual Review of Phytopathology* **26**, 75-91.

FRAVEL, D. R., ADAMS, P. B. & POTTS, W. E. (1992). Use of disease progress curves to study the effects of the biocontrol agent *Sporidesmium sclerotivorum* on lettuce drop. *Biocontrol Science and Technology* **2**, 341-348.

FRAVEL, D. R. & ROBERTS, D. P. (1991). *in situ* Evidence for the role of glucose oxidase in the biocontrol of Verticillium wilt by *Taloromyces flavus*. *Biocontrol Science and Technology* **1**, 91-99.

GARRAWAY, M. O. & EVANS R. C. (1984a). Carbon nutrition. In *Fungal Nutrition and Physiology*, pp. 71-95. Edited by M. O. Garraway & R. C. Evans. New York: Wiley Interscience.

GARRAWAY, M. O. & EVANS R. C. (1984b). Spore dormancy, activation and germination. In *Fungal Nutrition and Physiology*, pp. 212-230. Edited by M. O. Garraway & R. C. Evans. New York: Wiley Interscience.

GARRETT, S. D. (1951). Ecological groupings of soil fungi: a survey of substrate relationships. *New Phytologist* **50**, 149-166.

GARRETT, S. D. (1970). Introduction. In *Pathogenic Root-Infecting Fungi*, pp. 1-30. Edited by S. D. Garrett. Cambridge: Cambridge University Press.

GRAEME-COOK, K. A. & FAULL, J. L. (1991). Effect of ultraviolet-induced mutants of *Trichoderma harzianum* with altered antibiotic production on selected pathogens *in vitro*. *Canadian Journal of Microbiology* **37**, 659-664.

HADAR, Y. & MANDELBAUM, R. (1992). Suppressive compost for biocontrol of soilborne plant pathogens. *Phytoparasitica* **20** (supplement), 113-116.

HASKINS, R. H. (1963). Morphology, nutrition, and host range of a species of *Pythium*. *Canadian Journal of Microbiology* **9**, 451-457.

HENDRIX, J. W. (1970). Sterols in growth and reproduction of fungi. *Annual Review of Phytopathology* **8**, 111-130.

HENDRIX, F. F. & CAMPBELL, W. A. (1970). Distribution of *Phytophthora* and *Pythium* species in soils in the Continental United States. *Canadian Journal of Botany* **48**, 377-384.

HENIS, Y. & PAPAVIZAS, G. C. (1983). Factors affecting germinability and susceptibility to attack of sclerotia of *Sclerotium rolfsii* by *Trichoderma harzianum* in field soil. *Phytopathology* **73**, 1469-1474.

HOCH, H. C. & FULLER, M. S. (1977). Mycoparasitic relationships, I. Morphological features of interactions between *Pythium acanthicum* and several fungal hosts. *Archives of Microbiology* **111**, 207-224.

HOTSON, J. W. (1912). Culture studies of fungi producing bulbils and similar propagative bodies. *Proceedings of the American Academy* **48**, 225-306

HOTSON, J. W. (1917). Notes on bulbiferous fungi with a key to described species. *The Botanical Gazette*, **64**, 265-289.

HOTSON, H. H. (1942). Some species of *Papulaspora* associated with rots of gladiolus bulbs. *Mycologia*, **34**, 391-399.

HUANG, H. C. (1978). *Gliocladium catenulatum*: hyperparasite of *Sclerotinia sclerotiorum* and *Fusarium* species. *Canadian Journal of Botany* **56**, 2243-2246.

HUBER, D. M., ANDERSEN, A. L. & FINLEY, A. M. (1966). Mechanisms of biological control in a bean root rot soil. *Phytopathology* **56**, 953-956.

HUNTER, W. E., DUNIWAY, J. M. & BUTLER, E. E. (1977). Influence of nutrition, temperature, moisture & gas composition on parasitism of *Rhizopus oryzae* by *Syncephalis californica*. *Phytopathology* **67**, 664-669.

INBAR, J. & CHET, I (1992). Biomimics of fungal cell-cell recognition by use of lectin-coated nylon fibers. *Journal of Bacteriology* **174**, 1055-1059.

JAFFEE, B. A. (1993). Density-dependent parasitism in biological control of soil-borne insects, nematodes, fungi and bacteria. *Biocontrol Science and Technology* **3**, 235-246.

JAGER, G. & VELVIS, H. (1989). Dynamics of damage from *Rhizoctonia solani* in potato fields. In *Effects of Crop Rotation on Potato Production in the Temperate Zones*, pp. 237-246. Edited by J. Vos, C. D. van Loon & G. J. Bollen. Kluwer: Dordrecht.

JEFFRIES, P. (1985). Mycoparasitism within the Zygomycetes. *Botanical Journal of the Linnean Society* **91**, 135-150.

JEFFRIES, P. & YOUNG, T. W. K. (1975). Scanning electron and light microscopy of appressorium development in *Piptocephalis unispora* (Mucorales). *Archives of Microbiology* **103**, 293-296.

JEFFRIES, P. & YOUNG, T. W. K. (1976). Ultrastructure of infection of *Cokeromyces recurvatus* by *Piptocephalis unispora* (Mucorales). *Archives of Microbiology* **109**, 277-288.

JEFFRIES, P. & YOUNG, T. W. K. (1978). Mycoparasitism by *Piptocephalis unispora* (Mucorales): host range and reaction with *Phascolomyces articulosus*. *Canadian Journal of Botany* **56**, 2449-2459.

JENNINGS, D. H. (1974). Sugar transport into fungi: an essay. *Transactions of the British Mycological Society* **62**, 1-24.

JOHNSON, L. F. & ARROYO, T. (1983). Germination of oospores of *Pythium ultimum* in the cotton rhizosphere. *Phytopathology* **73**, 1620-1624.

KAO, C. W. & KO, W. H. (1983). Nature of suppression of *Pythium splendens* in a pasture soil in South Kohala, Hawaii. *Phytopathology* **73**, 1284-1289.

KAO, C. W. & KO, W. H. (1986a). Suppression of *Pythium splendens* in a Hawaiian soil by calcium and microorganisms. *Phytopathology* **76**, 215-220.

KAO, C. W. & KO, W. H. (1986b). The role of calcium and microorganisms in suppression of cucumber damping-off caused by *Pythium splendens* in a Hawaiian soil. *Phytopathology* **76**, 221-225.

KATAN, J. (1987). Soil solarisation. In *Innovative Approaches to Plant Disease Control*, pp. 75-105. Edited by I. Chet. New York: Wiley Interscience.

KATAN, J., GINZBURG, C. & FREEMAN, S. (1992). The weakening effect as a trigger for biological control and criteria for its evaluation. In *Biological Control of Plant Diseases,* pp. 55-61. Edited by E. S. Tjamos. New York: Plenum Press.

KEINATH, A. P., FRAVEL, D. R. & PAPAVIZAS, G. C. (1991). Potential of *Gliocladium roseum* for biocontrol of *Verticillium dahliae*. *Phytopathology* **81**, 644-648.

KO, W. & LOCKWOOD, J. L. (1970). Mechanism of lysis of fungal mycelia in soil. *Phytopathology* **60**, 148-154.

LAING, S. A. K. & DEACON, J. W. (1990). Aggressiveness and fungal host ranges of mycoparasitic *Pythium* species. *Soil Biology and Biochemistry* **22**, 905-911.

LAING, S. A. K. & DEACON, J. W. (1991). Video microscopical comparison of mycoparasitism by *Pythium oligandrum, P. nunn* and an unnamed *Pythium* species. *Mycological Research* **95**, 469-479.

LEN, L-H. C. & DICK, M. W. (1986). Comparative estimates of minimum propagule densities of *Aplanopsis* and *Pythium* in soil. *Transactions of the British Mycological Society* **87**, 309-340.

LEWIS, K., WHIPPS, J. M. & COOKE, R. C. (1989). Mechanisms of biological disease control with special reference to the case study of *Pythium oligandrum* as an antagonist. In *Biotechnology of fungi for improving plant growth*, pp. 191-217. Edited by J. M. Whipps & R. D. Lumsden. Cambridge: Cambridge University Press.

LEWIS, J. A. & PAPAVIZAS, G. C. (1991). Biocontrol of cotton damping-off caused by *Rhizoctonia solani* in the field with formulations of *Trichoderma* spp. and *Gliocladium virens*. *Crop Protection* **10**, 396-402.

LIFSHITZ, R., DUPLER, M., ELAD, Y. & BAKER, R. (1984a). Hyphal interactions between a mycoparasite, *Pythium nunn*, and several soil fungi. *Canadian Journal of Microbiology* **30**, 1482-1487.

LIFSHITZ, R., SNEH, B. & BAKER, R. (1984b). Soil suppressiveness to a plant pathogenic *Pythium* species. *Phytopathology* **74**, 1054-1061.

LIFSHITZ, R., STANGHELLINI, M. & BAKER, R. (1984c). A new species of *Pythium* isolated from soil in Colorado. *Mycotaxon* **20**, 373-379.

LIFSHITZ, R., TABACHNIK, M., KATAN, J. & CHET, I. (1983). The effect of sublethal heating on sclerotia of *Sclerotium rolfsii*. *Canadian Journal of Microbiology* **29**, 1607-1610.

LIFSHITZ, R., WINDHAM, M. T. & BAKER, R. (1986). Mechanisms of biological control of preemergence damping-off of pea by seed treatment with *Trichoderma* spp. *Phytopathology* **76**, 720-725.

LIU, S. & BAKER, R. (1980). Mechanisms of biological control in soil suppressive to *Rhizoctonia solani*. *Phytopathology* **70**, 404-412.

LLOYD, A. B. & LOCKWOOD, J. L. (1966). Lysis of fungal hyphae in soil and its possible relation to autolysis. *Phytopathology* **56**, 595-602.

LOCKWOOD, J. L. (1960). Lysis of mycelium of plant-pathogenic fungi by natural soil. *Phytopathology* **50**, 787-789.

LODHA, B. C. & WEBSTER, J. (1990). *Pythium acanthophoron*, a mycoparasite, rediscovered in India and Britain. *Mycological Research* **94**, 1006-1008.

LUMSDEN, R. D. (1980). Ecology of mycoparasitism. In *The Fungal Community, its Organization and Role in the Ecosytem*, pp. 295-318. Edited by D. T. Wicklow & G. C. Carroll. New York: Marcel Dekker, Incorporated.

LUMSDEN, R. D. (1981). A nylon fabric technique for studying the ecology of *Pythium aphanidermatum* and other fungi in soil. *Phytopathology* **71**, 282-285.

LUMSDEN, R. D., GARCIA-E, R., LEWIS, J. A. & FRIAS-T, G. A. (1987). Suppression of damping-off caused by *Pythium* spp. in soil from the indigenous Mexican chinampa agricultural system. *Soil, Biology and Biochemistry* **19,** 501-508.

LUTCHMEAH, R. S. & COOKE, R. C. (1984). Aspects of antagonism by the mycoparasite *Pythium oligandrum*. *Transactions of the British Mycological Society* **83,** 696-700.

LUTCHMEAH, R. S. & COOKE, R. C. (1985). Pelleting of seed with the antagonist *Pythium oligandrum* for biological control of damping-off. *Plant Pathology* **34,** 528-531.

MACER, R. C. F. (1961). The survival of *Cercosporella herpotrichoides* Fron in wheat straw. *Annals of Applied Biology* **49,** 165-172.

MALAJCZUK, N., BOWEN, G. D. & GREENHALGH, F. C. (1978). A combined fluorescent antibody and soil sieving technique to count chlamydospores of *Phytophthora cinnamomi* in soil. *Soil Biology and Biochemistry* **10,** 437-438.

MALOY, O. C. & ALEXANDER, M. (1958). The "Most Probable Number" method for estimating populations of plant pathogenic organisms in the soil. *Phytopathology* **48,** 126-128.

MANOCHA, M. S. & GRAHAM, L. (1982). Host cell wall synthesis and its role in resistance to a mycoparasite. *Physiological Plant Pathology* **20,** 157-164.

MARIOS, J. J., JOHNSTON, S. A., DUNN, M. T. & PAPAVIZAS, G. C. (1982). Biological control of *Verticillium* wilt of eggplant in the field. *Plant Disease* **66,** 1166-1168.

MARTIN, F. N. & HANCOCK, J. G. (1986). Association of chemical and biological factors in soils suppressive to *Pythium ultimum*. *Phytopathology* **76,** 1221-1231.

MARTIN, F. N. & HANCOCK, J. G. (1987). The use of *Pythium oligandrum* for biological control of preemergence damping-off caused by *Pythium ultimum*. *Phytopathology* **77**, 1013-1020.

McQUILKEN, M. P., WHIPPS, J. M. & COOKE, R. C. (1990). Control of damping-off in cress and sugar-beet by commercial seed-coating with *Pythium oligandrum*. *Plant Pathology* **39**, 452-462.

McQUILKEN, M. P., WHIPPS, J. M. & COOKE, R. C. (1992a). Use of oospore formulations of *Pythium oligandrum* for biological control of *Pythium* damping-off in cress. *Journal of Phytopathology* **135**, 125-134.

McQUILKEN, M. P., WHIPPS, J. M. & COOKE, R. C. (1992b). Nutritional and environmental factors affecting biomass and oospore production of the biocontrol agent *Pythium oligandrum*. *Enzyme Microbial Technology* **14**, 106-111

MEHROTRA, R. S. (1972). Behavior of zoospores of *Phytophthora megasperma* var. *sojae* and *P. drechsleri* in soil. *Canadian Journal of Botany* **50**, 2125-2130.

MERRIMAN, P. R. (1976). Survival of sclerotia of *Sclerotinia sclerotiorum* in soil. *Soil Biology and Biochemistry* **8**, 385-389.

MIRCETICH, S. M. (1971). The role of *Pythium* in feeder roots of diseased and symptomless peach trees and in orchard soils in peach tree decline. *Phytopathology* **61**, 357-360.

MORQUER, R., VIALA, G., ROUCH, J., FAYRET, J. & BERGE, G. (1963). Contribution a l'etude morphogenique du genre *Gliocladium*. *Bulletin de la Societe Mycologique de France* **79**, 137-241.

MORRIS, R. A. C., COLEY-SMITH, J. R. & WHIPPS, J. M. (1992). Isolation of mycoparasite *Verticillium biguttatum* from sclerotia of *Rhizoctonia solani* in the United Kingdom. *Plant Pathology* **41**, 513-516.

OHR, H. D. & MUNNECKE, D. E. (1974). Effects of methyl bromide on antibiotic production by *Armillaria mellea*. *Transactions of the British Mycological Society* 62, 65-72.

OLSEN, C. M. & BAKER, K. F. (1968). Selective heat treatment of soil, and its effects on the inhibition of *Rhizoctonia solani* by *Bacillus subtilis*. *Phytopathology* **58**, 79-87.

PAPAVIZAS, G. C. (1981). Survival of *Trichoderma harzianum* in soil and in pea and bean rhizospheres. *Phytopathology* **71**, 121-125.

PAPAVIZAS, G. C. (1985). *Trichoderma* and *Gliocladium*: biology, ecology, and potential for biocontrol. *Annual Review of Phytopathology* **23**, 23-54.

PAPAVIZAS, G. C., LEWIS, J. A. & ABD-EL MOITY, T. H. (1982). Evaluation of new biotypes of *Trichoderma harzianum* for tolerance to benomyl and enhanced biocontrol capabilities. *Phytopathology* **72**, 126-132.

PAPAVIZAS, G. C. & LUMSDEN, R. D. (1980). Biological control of soilborne fungal propagules. *Annual Review of Phytopathology* **18**, 389-413.

PAPAVIZAS, G. C. & LUMSDEN, R. D. (1982). Improved medium for isolation of *Trichoderma* spp. from soil. *Plant disease* **66**, 1019-1020.

PARK, Y., STACK, J. P. & KENERLEY, C. M. (1992). Selective isolation and enumeration of *Gliocladium virens* and *G. roseum* from soil. *Plant Disease* **76**, 230-235.

PAULITZ, T. C. & BAKER, R. (1987a). Biological control of Pythium damping-off of cucumbers with *Pythium nunn*: population dynamics and disease suppression. *Phytopathology* **77**, 335-340.

PAULITZ, T. C. & BAKER, R. (1987b). Biological control of Pythium damping-off of cucumbers with *Pythium nunn*: influence of soil environment and organic amendments. *Phytopathology* **77**, 341-346.

PAULITZ, T. C. & BAKER, R. (1988). The formation of secondary sporangia by *Pythium ultimum*: the influence of organic amendments and *Pythium nunn*. *Soil Biology and Biochemistry* **20**, 151-156.

PEARSON, J., PIKE, D. J. & DICK, M. W. (1991). A model for short-term fluctuations of *Pythium* propagules in soil. *Mycological Research* **95**, 324-328.

PERSSON, Y., NORDBRING-HERTZ, B. & CHET, I. (1990). Effect of polyoxin D on morphogenesis of the nematode-trapping fungus *Arthrobotrys oligospora*. *Mycological Research* **94**, 196-200.

PLAATS-NITERINK, A. J. VAN DER' (1975). Species of *Pythium* in the Netherlands. *Netherland Journal of Plant Pathology* **81**, 22-37.

PLAATS-NITERINK, A. J. VAN DER (1981). Monograph of the Genus *Pythium*. *Studies in Mycology* **21**. Edited by W. Gams & R. P. W. Jacobs. Centraalbureau Voor Schimmelcultures Baarn, Netherlands: Institute of the Royal Netherlands Academy of Science and Letters.

RIBEIRO, W. R. C. & BUTLER, E. E. (1992). Isolation of mycoparasitic species of *Pythium* with spiny oogonia from soil in California. *Mycological Research* **96**, 857-862.

RIDE, J. P. & PEARCE, R. B. (1979). Lignification and papilla formation at sites of attempted penetration of wheat leaves by non-pathogenic fungi. *Physiological Plant Pathology* **15**, 79-92.

RIDINGS, W. H., GALLEGLY, M. E. & LILLY, V. G. (1969). Thiamine requirements helpful in distinguishing isolates of *Pythium* from those of *Phytophthora*. *Phytopathology* **59**, 737-742.

RIFAI, M. (1969). A Revision of the Genus *Trichoderma*. *Mycological Papers* **116**.

RISTAINO, J. B., PERRY, K. B. & LUMSDEN, R. D. (1991). Effect of solarization and *Gliocladium virens* on sclerotia of *Sclerotium rolfsii*, soil microbiota, and the incidence of Southern blight of tomato. *Phytopathology* **81**, 1117-1124.

ROBERTSON, N. F. (1965). The mechanism of cellular extension and branching. In *The Fungi, Volume I, The Fungal Cell*, pp. 613-623. Edited by G. C. Ainsworth & A. S. Sussman. London: Academic Press.

ROUSE, D. I. & BAKER, R. (1978). Modeling and quantitative analysis of biological control mechanisms. *Phytopathology* **68**, 1297-1302.

SANDYS-WINSCH, C., WHIPPS, J. M., GERLAGH, M. & KRUSE, M. (1993). World distribution of the sclerotial mycoparasite *Coniothyrium minitans*. *Mycological Research* **97**, 1175-1178.

SCHER, F. M. & BAKER, R. (1983). Fluorescent microscopic technique for viewing fungi in soil and its application to studies of a *Fusarium*-suppressive soil. *Soil Biology and Biochemistry* **15**, 715-718.

SCHIPPERS, B. (1992). Prospects for management of natural suppressiveness to control soilborne pathogens. In *Biological Control of Plant Diseases*, pp. 21-34. Edited by E. S. Tjamos, G. Papavizas and R. J. Cooke. New York: Plenum Press.

SCHMITTHENNER, A. F. (1962). Isolation of *Pythium* from soil particles. *Phytopathology* **52**, 1133-1138.

SKOU, J. P. (1981). Morphology and cytology of the infection process. In *Biology and Control of Take-All*, pp. 175-197. Edited by M. C. Asher & P. J. Shipton. London: Academic Press.

SMITH, J. E. & BERRY, D. R. (1976). Nitrogen metabolism. In *The Filamentous Fungi, II, biosynthesis and Metabolism*, pp. 159-237. Edited by J. E. Smith & D. R. Berry. Bristol: Edward Arnold (Publishers) Limited.

SMITH, V. L., WILCOX, W. F. & HARMAN, G. E. (1990). Potential for biological control of Phytophthora root and crown rots of apple by *Trichoderma* and *Gliocladium* spp. *Phytopathology* **80**, 880-885.

SNEH, B., HUMBLE, S. J. & LOCKWOOD, J. L. (1977). Parasitism of oospores of *Phytophthora megasperma var. sojae, P. cactorum, Pythium* sp., and *Aphanomyces euteiches* in soil by oomycetes, chytridiomycetes, hyphomycetes, actinomyces and bacteria. *Phytopathology* **67**, 622-628.

SODERSTROM, B. E. (1977). Vital staining of fungi in pure cultures and in soil with fluorescein diacetate. *Soil Biology and Biochemistry* **9**, 59-63.

SPEAKMAN, J. B. & LEWIS, B. G. (1978). Limitation of *Gaeumannomyces graminis* by wheat root responses to *Phialophora radicicola. New Phytologist* **80**, 373-380.

STACK, J. P. & MILLAR, R. L. (1985). Isolation and characterization of a metalaxyl-insensitive isolate of *Phytophthora megasperma* f. sp. *medicaginis. Phytopathology* **75**, 1387-1392.

STAHL, P. D. & CHRISTENSEN, M. (1992). *In vitro* mycelial interactions among members of a soil microfungal community. *Soil Biology and Biochemistry* **24**, 309-316.

STANGHELLINI, M. E. & BURR, T. J. (1973). Germination in vivo of *Pythium aphanidermatum* oospores and sporangia. *Phytopathology* **63**, 1493-1496.

STANGHELLINI, M. E. & RUSSEL, J. D. (1973). Germination *in vitro* of *Pythium aphanidermatum* oospores. *Phytopathology* **63**, 133-137.

STEWART, A. (1993). The use of vital fluorochromes as tracers in fungal growth studies. In press.

STOLP, H. & STARR, M. P. (1963). *Bdellovibrio bacteriovorus* gen. et sp. n. a predatory ectoparasitic and bacteriolytic microorganism. *Antonie van Leeuwenhoek* **29**, 217-248.

TRIBE, H. T. (1961). Microbiology of cellulose decomposition in soil. *Soil Science* **92**: 61-77.

TRIBE, H. T. (1966). Interactions of soil fungi on cellulose film. *Transactions of the British Mycological Society* **49**, 457-466.

TRUTMANN, P. & KEANE, P. J. (1990). *Trichoderma koningii* as a biological control agent for *Sclerotinia sclerotiorum* in Southern Australia. *Soil Biology and Biochemistry* **22**, 43-50.

TSAO, P. H. (1970). Applications of the vital fluorescent labeling technique with brighteners to studies of saprophytic behavior of *Phytophthora* in soil. *Soil Biology and Biochemistry* **2**, 247-256.

TZEAN, S. S. & ESTEY, R. H. (1992). *Geotrichopsis mycoparasitica* as a destructive mycoparasite. *Mycological Research* **96**, 263-269.

VAARTAJA, O. (1968). *Pythium* and *Mortierella* in soils of Ontario forest nurseries. *Canadian Journal of Microbiology* **14**, 265-269.

VAARTAJA, O. & BUMBIERIS, M. (1964). Abundance of *Pythium* species in nursery soils in South Australia. *Australian Journal of Biological Sciences* **17**, 436-445.

VELVIS, H., BOOGERT, P. H. J. F. VAN DEN & JAGER, G. (1989). Role of antagonism in the decline of *Rhizoctonia solani* inoculum in soil. *Soil Biology and Biochemistry* **21**, 125-129.

VESELY, D. (1977). Potential biological control of damping-off pathogens in emerging sugar beet by *Pythium oligandrum* Drechsler. *Phytopathologische Zeitschrift* **90**, 113-115.

VESELY, D. (1978). Parasitic relationships between *Pythium oligandrum* Drechsler and some other species of the Oomycetes class. *Zentralblatt fur Bakteriologie parasitenkunde, Infektionskrankheiten und Hygiene. Zweite Abteilung Bd.* **133**, S. 341-349

VESELY, D. & HEJDANEK, S. (1984). Microbial relations of *Pythium oligandrum* and problems in the use of this organism for the biological control of damping-off in sugar-beet. *Zentralblatt fur Mikrobiologie* **139**, 257-265.

WALTHER, D. & GINDRAT, D. (1987a). Biological control of *Phoma* and *Pythium* damping-off of sugar-beet with *Pythium oligandrum*. *Journal of Phytopathology* **119**, 167-174.

WALTHER, D. & GINDRAT, D. (1987b). Antagonism of *Rhizoctonia* spp. to *Pythium oligandrum* and damping-off fungi. *Journal of Phytopathology* **119**, 248-254.

WARREN, J. R. (1948). An undescribed species of *Papulospora* parasitic on *Rhizoctonia solani*. *Mycologia* **40**, 391-401.

WATANABE, T. (1991). New species of *Oedocephalum* and *Papulaspora* from Japanese soils. *Mycologia* **83**, 524-529.

WERESUB, L. K. & LECLAIR, P. M. (1971). On *Papulaspora* and bulbilliferous basidiomycetes *Burgoa* and *Minimedusa*. *Canadian Journal of Botany* **49**: 2203-2213.

WHIPPS, J. M. (1987a). Effect of media on growth and interactions between a range of soil-borne glasshouse pathogens and antagonistic fungi. *New Phytologist* **107**, 127-142.

WHIPPS, J. M. (1987b). Behaviour of fungi antagonistic to *Sclerotinia sclerotiorum* on plant tissue segments. *Journal of General Microbiology* **133**, 1495-1501.

WHIPPS, J. M., BUDGE, S. P. & McQUILKEN, M. P. (1992). Use of *Coniothyrium minitans* and *Pythium oligandrum* as disease biocontrol agents. *Phytoparasitica* **20** (supplement), 107-111.

WHIPPS, J. M., LEWIS, K. & COOKE, R. C. (1988). Mycoparasitism and plant disease control. In *Fungi in Biological Control Systems*, pp.161-187. Edited by M. N. Burge. Manchester: Manchester University Press.

WHIPPS, J. M. & LUMSDEN, R. D. (1991). Biological control of *Pythium* species. *Biocontrol science and technology* **1**, 75-90.

WHITE, J. G., HENN, A. J. & PETCH, G. M. (1992). Deleterious effect of soil-applied metalaxyl and mancozeb on the mycoparasite *Pythium oligandrum*. *Biocontrol Science and Technology* **2**, 335-340.

WHITE, J. G., STANGHELLINI, M. E. & AYOUBI, L. M. (1988). Variation in the sensitivity to metalaxyl of *Pythium* spp. isolated from carrot and other sources. *Annals of Applied Biology* **113**, 269-277.

ZHOU, T. & REELEDER, R. D. (1990). Selection of strains of *Epicoccum purpurascens* for tolerance to fungicides and improved biocontrol of *Sclerotinia sclerotiorum*. *Canadian Journal of Microbiology* **36**, 754-759.

PUBLICATIONS

Mycol. Res. **96** (7): 605–608 (1992) *Printed in Great Britain* 91/242

Detection of presumptive mycoparasites in soil placed on host-colonized agar plates

D. F. C. MULLIGAN AND J. W. DEACON

Institute of Cell and Molecular Biology, University of Edinburgh, Rutherford Building, Mayfield Road, Edinburgh EH9 3JH, U.K.

The presumptive mycoparasites *Pythium oligandrum*, *Gliocladium roseum* (and related species), *Trichoderma* spp. and *Papulaspora* sp. were detected in, respectively, 18, 28, 24 and 21 of a total 28 British soils when samples were placed on sectors of agar colonized by appropriate host fungi. Most (25) of the soils contained three or more mycoparasites, but the frequency of detection on replicate host sectors suggested that only *P. oligandrum* and *G. roseum* were abundant in all soils in which they occurred.

The type of host fungus markedly influenced the efficiency of detection of different mycoparasites: *Fusarium culmorum* was most efficient for *P. oligandrum*, *Rhizoctonia solani* for *Trichoderma*, *Botrytis cinerea* for *Papulaspora*, and *Trichoderma aureoviride*, *R. solani* and *B. cinerea* were equally efficient for detection of *G. roseum*. But no single host was suitable for consistent detection of any single mycoparasite; several hosts may therefore be needed to determine the mycoparasite spectrum of a soil.

Mycoparasites are fungi that parasitize other fungi. In practice the term is used broadly to include presumptive parasites that coil around other fungal hyphae or overgrow other colonies on agar. This may involve penetration of living 'host' hyphae or antagonism by antibiotics (e.g. Dennis & Webster, 1971), wall lytic enzymes (Chet, 1986) or toxic radicals (Kim, Fravel & Papavizas, 1990). Mycoparasites and presumptive mycoparasites have biocontrol potential. Some are responsible for natural suppressiveness of soils to plant pathogens, examples being *Pythium nunn* Lifshitz *et al.* and *P. oligandrum* Drechsl. in *Pythium*-suppressive soils (Lifshitz, Sneh & Baker, 1984; Martin & Hancock, 1986) and *Trichoderma hamatum* (Bon.) Bain. in *Rhizoctonia*-suppressive soil (Liu & Baker, 1980). Some exert control when their natural populations are enhanced by management practices such as partial soil sterilization with fumigants or heat (reviewed by Cook & Baker, 1983). Also, some can be applied inundatively to seeds or soil to achieve control – at least in experimental conditions (e.g. Harman & Taylor, 1990).

Knowledge of the natural occurrence of mycoparasites should aid the development of biocontrol strategies. For example, it could be used to identify soils that are ecologically suited to the mycoparasites used for inundative release (Deacon, 1991), and it could facilitate the registration of mycoparasitic biocontrol agents, by identifying indigenous species. But there is no current method for determining the mycoparasite spectrum of a soil, so we have tried to develop a method, using agar precolonized by appropriate host fungi. This approach is an extension of that used to detect bacteriophage and *Bdellovibrio bacteriovorus* Stolp & Starr by plating of soil on to bacterial 'lawns' (e.g. Stolp & Starr, 1963) and subsequently used (with fungal 'hosts') to detect the mycoparasites *Verticillium biguttatum* Gams (Boogert, Jager & Velvis, 1990), *P. oligandrum* (Deacon & Henry, 1978; Foley & Deacon, 1985) and *P. acanthophoron* Sideris (Lodha & Webster, 1990).

MATERIALS AND METHODS

Soils

Twenty-eight soils were collected from arable fields (18 soils), garden sites (5) and woodlands (5) in northern Britain (Table 1). Most were loams or clay-loams, but soil 13 was a silty loam and soils 22, 24 and 25 were woodland humus. Each sample was bulked from usually 2 or 3 subsamples from the top 10 cm of the profile, then air-dried, sieved (2 mm mesh), mixed thoroughly and stored for up to 1 week in a polyethylene bag. Soil pH was measured in a soil:water mixture (2:1, v/v).

Precolonized agar plates

Plates of Difco potato-dextrose agar (PDA, 15 ml per 9 cm diam. Petri dish) were inoculated centrally with different 'host' fungi and incubated at 25 °C for different times, until the colony margin just reached the edge of the agar. Then each plate was cut into six equal sectors, which were placed separately in washed plastic Petri dishes, recycled from experiments that did not involve fungi. A sample of soil (0·4 ml, *ca* 0·4 g), was placed on the oldest part of each host sector. The sectors were incubated at 25° and examined (× 100 magnification) after 7, 14 and 21 days. Mycoparasites were detected by their sporulation or other structures on the

Table 1. Occurrence of different mycoparasites in 28 soils as determined by detection on agar previously colonized by appropriate 'host' fungi

Soil	Location	Cropping/vegetation	pH	Mycoparasites*			
1	Buil, Midlothian	Arable (oilseed rape)	6·5	**Po**	**G**	**T**	Pap
2	Penicuik, Midlothian	Garden (herbaceous)	6·8	**Po**	**G**	T	Pap
3	Burdiehouse, Midlothian	Arable (cereal)	7·7	**Po**	**G**	T	**Pap**
4	Liberton, Midlothian	Arable (cereal)	7·6	**Po**	**G**	T	**Pap**
5	Lasswade, Midlothian	Arable (cereal)	6·2	**Po**	**G**	T	**Pap**
6	Inchinnan, Renfrewshire	Arable (cereal)	6·0		**G**	T	
7	Paisley, Renfrewshire	Arable (cereal)	6·5	**Po**	**G**	T	Pap
8	Bathgate, W. Lothian	Arable (cereal)	6·6		**G**	T	Pap
9	Bathgate, W. Lothian	Arable (grass)	7·0	**Po**	**G**	T	
10	Bathgate, W. Lothian	Garden (herbaceous)	5·1		**G**	**T**	Pap
11	Edinburgh	Garden (herbaceous)	6·0	**Po**	**G**	T	**Pap**
12	Longside, Aberdeen	Arable (potatoes)	6·6	**Po**	**G**	T	Pap
13	York, Yorkshire	Arable (peas)	5·7	**Po**	**G**		**Pap**
14	York, Yorkshire	Arable (cereal)	7·7	**Po**	**G**		Pap
15	York, Yorkshire	Arable (potatoes)	7·1	**Po**	**G**	T	Pap
16	York, Yorkshire	Arable (oilseed rape)	6·7	**Po**	**G**		Pap
17	York, Yorkshire	Arable (cereal)	7·6	**Po**	**G**		
18	York, Yorkshire	Arable (cereal)	6·0	**Po**	**G**	T	
19	Roslin, Midlothian	Arable (cereal)	6·0	**Po**	**G**	T	**Pap**
20	Liberton, Midlothian	Woodland (deciduous)	4·8		**G**	**T**	**Pap**
21	Penicuik, Midlothian	Woodland (deciduous)	5·1		**G**	**T**	**Pap**
22	Bathgate, W. Lothian	Woodland (deciduous)	3·7		**G**	**T**	**Pap**
23	Roslin, Midlothian	Woodland (deciduous)	6·7		**G**	T	**Pap**
24	Edinburgh	Woodland (deciduous)	4·3		**G**	T	
25	Edinburgh	Parkland trees	4·9	**Po**	**G**	**T**	
26	Edinburgh	Garden (herbaceous)	5·7	**Po**	**G**	T	
27	Penicuik, Midlothian	Arable (strawberries)	5·6		**G**	**T**	**Pap**
28	Penicuik, Midlothian	Arable (strawberries)	6·2		**G**	T	**Pap**
Total occurrence				18	28	24	21
Total 'abundant'				18	28	10	12

* Po, *Pythium oligandrum*; G, *Gliocladium roseum* group; T, *Trichoderma* spp.; Pap, *Papulaspora* sp.; bold symbols denote occurrence in at least half the replicate soil samples on any one host type, a measure of 'abundance'.

Table 2. Incidence of detection of different mycoparasites in 28 soils when 6 or, occasionally, 10 replicate subsamples of each soil were placed on agar colonized by different host fungi

Mycoparasite	Host	No. soils/subsamples in which mycoparasite was detected: Soils (max. 28)	Subsamples (max. 192)
Pythium oligandrum	*Fusarium culmorum*	18	121
	Trichoderma aureoviride	3	17
	Rhizoctonia solani	0	0
	Botrytis cinerea	12	72
Gliocladium roseum	*Fusarium culmorum*	20	90
	Trichoderma aureoviride	27	140
	Rhizoctonia solani	26	133
	Botrytis cinerea	25	129
Trichoderma sp.	*Fusarium culmorum*	2	5
	Trichoderma aureoviride	0	0
	Rhizoctonia solani	22	60
	Botrytis cinerea	13	40
Papulaspora sp.	*Fusarium culmorum*	0	0
	Trichoderma aureoviride	11	35
	Rhizoctonia solani	3	3
	Botrytis cinerea	19	55

host colony. Identifications were confirmed with pure cultures, obtained by subculturing the mycoparasites from representative host sectors on to plates of fresh PDA.

Experimental design

Initial studies (not described) involved comparisons of fresh and air-dried soils and many potential host fungi. Then 7 hosts were selected to compare the mycoparasite populations in 12 soils. These hosts were *Fusarium culmorum* (W.G.Sm.) Sacc. (isolate CD 9), *Trichoderma aureoviride* Rifai (isolate TA 1), *Rhizoctonia solani* Kühn (isolate GM 1, anastomosis group 5), *Botrytis cinerea* Pers.: Pers. (isolate BC 1), *Phialophora* sp. (IMI 187786), *Pythium aphanidermatum* (Edson) Fitzp. (CBS 634.70) and *Gaeumannomyces graminis* (Sacc.) Arx & Olivier var. *tritici* Walker (isolate GGT 21). A further 16 soils were tested on only four hosts – *F. culmorum*, *T. aureoviride*, *B. cinerea* and *R. solani*. The presented results are for 28 soils on these four hosts, with six (or occasionally ten) replicate subsamples of each soil on each host. The host sectors were randomized, so for any one soil they were usually from different colonies.

RESULTS

Presumptive mycoparasites detected

Four types of mycoparasites were detected (Table 1). *Gliocladium* spp. formed slow-growing, sporulating colonies near the soil inoculum after 7 days; the incidence of detection increased at 14 days and again at 21 days. Most colonies (> 450) were typical of *G. roseum* Bain. (Morquer *et al.*, 1963), as confirmed by isolation in pure culture. But a few colonies on host sectors were identified as *G. atrum* Gilman & Abbott (10 cases), *G. catenulatum* Gilman & Abbott (9 cases) or *G. fimbriatum* Gilman & Abbott (3 cases), also confirmed in pure culture. These species may sometimes have been overlooked but were uncommon, and *G. roseum* was always found with them in a soil.

Pythium oligandrum, identified by its spiny-walled oogonia, was seen over the host sectors after usually 7 days. Smooth-walled oogonia were sometimes seen on host sectors, suggesting the presence of *P. mycoparasiticum* Deacon, Laing & Berry (1991). But only *P. oligandrum* could be isolated into pure culture, perhaps because it outgrew *P. mycoparasiticum* on PDA or because the smooth-walled oogonia were immature forms of *P. oligandrum*.

Trichoderma was detected by its characteristic sporing structures on the soil surface or at the periphery of host sectors and extending across the base of the Petri dish at 7 days; the incidence of this fungus did not increase with time. The isolates identified in pure culture were usually *T. harzianum* Rifai, but occasionally *T. hamatum* and *T. koningii* Oud.

Papulaspora sp. was detected as brown bulbils (Weresub & LeClair, 1971) across host sectors after 7–14 days. All pure cultures were identical in growth form and features of the bulbils. They were not identified further but did not correspond to *Minimedusa polyspora* (Hotson) Weresub & LeClair, a

Papulaspora-like fungus that antagonizes *Fusarium oxysporum* Schlecht. (Beale & Pitt, 1990).

Differential effects of host fungi

Of the seven hosts used for the first 12 soils, *P. aphanidermatum* was effective only for detecting *Gliocladium* and *Trichoderma*; *G. graminis* was effective only for *Gliocladium*, and *Phialophora* sp. only for *Gliocladium* and *P. oligandrum*. These three hosts were no better than others in these respects, and they were inconvenient to use owing to their relatively slow or erratic growth or (*P. aphanidermatum*) collapse of the aerial mycelia.

F. culmorum, *T. aureoviride*, *R. solani* and *B. cinerea* were used for all 28 soils and gave different patterns of detection of mycoparasites (Table 2). *Gliocladium* was detected on all four hosts, but at lower frequency on *F. culmorum* than on the other three. *P. oligandrum* was seen mostly on *F. culmorum*, less often on *B. cinerea*, infrequently on *T. aureoviride* and never on *R. solani*. *Trichoderma* was seen mostly on *R. solani* and *B. cinerea*, rarely on *F. culmorum* and never on *T. aureoviride*. *Papulaspora* was detected mostly on *B. cinerea* and *T. aureoviride*, seldom on *R. solani* and never on *F. culmorum*. Comparison of Tables 1 and 2 shows that only *P. oligandrum* was detected by the use of a single host (*F. culmorum*) in every soil in which it occurred. Also, no single host consistently detected all the mycoparasites; although *B. cinerea* was the best host in this respect, if it had been used alone it would have failed to detect *P. oligandrum* or *Gliocladium* in 6 soils, *Trichoderma* in 13 soils and *Papulaspora* in 2 soils. These failures cannot be ascribed to chance, because in several soils a mycoparasite that was not detected on *B. cinerea* was recorded as abundant (i.e. on at least half of the replicates) on another host. Table 2 shows that the greatest likelihood of detecting all the mycoparasites in any one soil was by the use of *F. culmorum* for *P. oligandrum*, *T. aureoviride* for *Gliocladium*, *R. solani* for *Trichoderma* and *B. cinerea* for *Papulaspora*.

Occurrence of mycoparasites in different soils

Gliocladium was detected in all 28 soils, *Trichoderma* in 24 soils, *Papulaspora* in 21 soils and *P. oligandrum* in 18 soils (Table 1). Moreover, all soils had more than one mycoparasite: 3 soils contained two types, 15 contained 3 types and 10 contained all 4 types. But these records include several instances in which a mycoparasite was detected on only one of six (or ten) replicate host sectors. As an estimate of the 'abundance' of each mycoparasite in each soil, Table 1 shows (bold type) the cases in which a mycoparasite was detected on at least half of the replicate sectors of any one host. Judged by this criterion, one soil (soil 8) contained a single 'abundant' mycoparasite, 14 soils had two 'abundant' mycoparasites (often a combination of *G. roseum* and *P. oligandrum*), 13 soils had three, and no soil had all four mycoparasites present in abundance. It is also seen that *G. roseum* and *P. oligandrum* were abundant in every soil in which they occurred, whereas *Trichoderma* and *Papulaspora* were abundant in only about half the soils in which they were detected. Overall, *P. oligandrum* was detected in 27% of the subsamples of soil placed on host colonies (but 63% of subsamples on its 'preferred' host) and *G. roseum* in 64% of all subsamples (73% on its preferred host). In contrast, strains of *Trichoderma* that can overgrow other fungi were detected in only 14% of subsamples (30% on the preferred host) and *Papulaspora* in 12% of all subsamples (29% on the preferred host).

DISCUSSION

This work confirms previous reports (Deacon & Henry, 1978; Foley & Deacon, 1985) that presumptive mycoparasites are common and often occur together in British soils. But the detection of different mycoparasites on precolonized agar was strongly influenced by the 'host' fungus. All previous studies have used a single host and thus probably underestimated the presence of mycoparasites in soils. For example, *Papulaspora* was common in our survey but was recorded infrequently in similar soils by Foley & Deacon (1985), who used only *Phialophora*-colonized agar. *Papulaspora* has received relatively little attention as a common potential mycoparasite or antagonist (Warren, 1948; Beale & Pitt, 1990).

Our method was adapted from Foley & Deacon (1985) for routine use in soil surveys. Economy was achieved by using recycled Petri dishes and sectors rather than whole host colonies. Also, mycoparasites could be detected and identified to at least generic level without the need for subculturing. Initial studies (unpublished) showed that air-drying of soils minimized the occurrence of nematodes, bacteria and mites on the host sectors and thus facilitated the growth of mycoparasites and enabled these to be isolated, if necessary, without antibacterial supplements. Air-drying might be detrimental to some mycoparasites, but we have found no more mycoparasites in moist than in air-dried soils placed on host-colonized agar.

The hosts in this study were selected for rapid, uniform growth on PDA and for the known abilities of mycoparasites to grow across them in culture (Laing & Deacon, 1990). These abilities were precisely matched by the results of the soil survey (compare Table 2 with table 4 in Laing & Deacon (1990)). The hosts for future surveys could be selected on this basis, or specific pathogens could be used to detect potential biocontrol agents (e.g. Boogert *et al.*, 1990). But the best host for detection of a mycoparasite may not be the best for its isolation into pure culture. For example, we detected *P. oligandrum* equally on sectors of *Phialophora* sp. and *F. culmorum* but isolated it more easily from *Phialophora*, probably because this is the more susceptible to parasitism (Laing & Deacon, 1991) and also grows more slowly than *P. oligandrum* on PDA.

Several soil-borne mycoparasites were not detected in this survey; they include *G. virens* Miller *et al.*, *Talaromyces flavus* (Klocker) Stolk & Samson, *Pythium acanthicum* Drechsl., *P. periplocum* Drechsl. and *P. mycoparasiticum*. We probably used the wrong hosts for *P. mycoparasiticum*, because it (as '*Pythium* SWO') grew only on colonies of *Phialophora* sp. in culture (Laing & Deacon, 1990). But this explanation cannot apply to the other 'undetected' mycoparasites, which could have grown on some of the hosts used here (Boosalis, 1956; Henry

& Deacon, 1978; Foley & Deacon, 1985). They may be uncommon in British soils, because a total 192 soils of different types were tested by us or Foley & Deacon (1985). Or they may differ in habitat requirements from the mycoparasites detected here, as seems to be the case for *P. acanthicum* in comparison with *P. oligandrum* (Dick & Ali-Shtayeh, 1986). Further work with different hosts and a broader geographical or ecological range of sites should resolve these issues.

D.F.C.M. is supported by a Studentship from the Natural Environment Research Council, which we gratefully acknowledge. We thank L. A. Berry for assistance, supported by the Agricultural and Food Research Council.

REFERENCES

Beale, R. E. & Pitt, D. (1990). Biological and integrated control of fusarium basal rot of *Narcissus* using *Minimedusa polyspora* and other micro-organisms. *Plant Pathology* **39**, 477–488.

Boogert, P. H. J. F. van den, Jager, G. & Velvis, H. (1990). *Verticillium biguttatum*, an important mycoparasite for the control of *Rhizoctonia solani* in potato. In *Biological Control of Soil-borne Plant Pathogens* (ed. D. Hornby), pp. 77–91. CAB International: Wallingford.

Boosalis, M. G. (1956). Effect of soil temperature and green-manure amendment of unsterilized soil on parasitism of *Rhizoctonia solani* by *Penicillium vermiculatum* and *Trichoderma* sp. *Phytopathology* **46**, 473–478.

Chet, I. (1986). *Trichoderma*-application, mode of action, and potential as a biocontrol agent of soil-borne plant pathogenic fungi. In *Innovative Approaches to Plant Disease Control* (ed. I. Chet), pp. 137–160. Wiley: New York.

Cook, R. J. & Baker, K. F. (1983). *The Nature and Practice of Biological Control of Plant Pathogens*. St Paul: American Phytopathological Society.

Deacon, J. W. (1991). Significance of ecology in the development of biocontrol agents against soil-borne plant pathogens. *Biocontrol Science and Technology* **1**, 5–20.

Deacon, J. W. & Henry, C. M. (1978). Mycoparasitism by *Pythium oligandrum* and *P. acanthicum*. *Soil Biology and Biochemistry* **10**, 409–415.

(*Accepted 10 December 1991*)

Deacon, J. W., Laing, S. A. K. & Berry, L. A. (1991). *Pythium mycoparasiticum* sp. nov., an aggressive mycoparasite from British soils. *Mycotaxon* **42**, 1–8.

Dennis, C. & Webster, J. (1971). Antagonistic properties of species-groups of *Trichoderma*. I. Production of non-volatile antibiotics. *Transactions of the British Mycological Society* **57**, 363–369.

Dick, M. W. & Ali-Shtayeh, M. S. (1986). Distribution and frequency of *Pythium* species in parkland and farmland soils. *Transactions of the British Mycological Society* **86**, 49–62.

Foley, M. F. & Deacon, J. W. (1985). Isolation of *Pythium oligandrum* and other necrotrophic mycoparasites from soil. *Transactions of the British Mycological Society* **85**, 631–639.

Harman, G. E. & Taylor, A. G. (1990). Development of an effective biological seed treatment system. In *Biological Control of Soil-borne Plant Pathogens* (ed. D. Hornby), pp. 415–426. CAB International: Wallingford.

Kim, K. K., Fravel, D. R. & Papavizas, G. C. (1990). Glucose oxidase as the antifungal principle of talaron from *Talaromyces flavus*. *Canadian Journal of Microbiology* **36**, 760–764.

Laing, S. A. K. & Deacon, J. W. (1990). Aggressiveness and fungal host ranges of mycoparasitic *Pythium* species. *Soil Biology and Biochemistry* **22**, 905–911.

Laing, S. A. K. & Deacon, J. W. (1991). Video microscopical comparison of mycoparasitism by *Pythium oligandrum*, *P. nunn* and an unnamed *Pythium* species. *Mycological Research* **95**, 469–479.

Lifshitz, R., Sneh, B. & Baker, R. (1984). Soil suppressiveness to *Pythium ultimum* induced by antagonistic *Pythium* species. *Phytopathology* **74**, 1054–1061.

Liu, S. & Baker, R. R. (1980). Mechanism of biological control in soil suppressive to *Rhizoctonia solani*. *Phytopathology* **70**, 404–412.

Lodha, B. C. & Webster, J. (1990). *Pythium acanthophoron*, a mycoparasi' rediscovered in India and Britain. *Mycological Research* **94**, 1006–1008.

Martin, F. N. & Hancock, J. G. (1986). Association of chemical and biologi factors in soil suppressive to *Pythium ultimum*. *Phytopathology* : 1221–1231.

Morquer, R., Viala, G., Rouch, J., Fayret, J. & Berge, G. (1963). Contribution à l'étude morphogénique du genre *Gliocladium*. *Bulletin de la Société Mycologique de France* **79**, 137–241.

Stolp, H. & Starr, M. P. (1963). *Bdellovibrio bacteriovorus* gen. et sp. n., a predatory ectoparasitic and bacteriolytic microorganism. *Antonie van Leeuwenhoek* **29**, 217–248.

Warren, J. R. (1948). An undescribed species of *Papulaspora* parasitic on *Rhizoctonia solani* Kühn. *Mycologia* **40**, 391–401.

Weresub, L. K. & LeClair, P. M. (1971). On *Papulaspora* and bulbilliferous basidiomycetes *Burgoa* and *Minimedusa*. *Canadian Journal of Botany* **49**, 2203–2213.