COMPARATIVE BEHAVIOUR OF MYCOPARASITIC <u>PYTHIUM</u> SPECIES

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DECLARATION

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

Eirian Jones

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I wish to express my sincere gratitude to Jim Deacon for his supervision and guidance during my PhD. Now that I'll be going he can now start converting the lab back into a male dominated zone.

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Diolch im rhieni, Mair a John, ag im chwaer, Nerys am ei cefnogaeth trwy'n ngyrfa academaidd, hwyrach gai swydd iawn or diwedd, mae'n hen bryd yn tydi dad!

ABSTRACT

The nutrition and physiology of the mycoparasites *Pythium oligandrum*, *P. mycoparasiticum* and *P. acanthophoron* differed from that of plant pathogenic *Pythium* spp., but with minor variations between strains and species of the mycoparasites. *P. oligandrum*, *P. mycoparasiticum* and *P. acanthophoron* did not use inorganic nitrogen. *P. oligandrum* and *P. mycoparasiticum* required exogenous thiamine, whereas *P. acanthophoron* was self-sufficient for thiamine. The mycoparasites grew on mannitol as a carbon source in the presence of cholesterol, calcium or both, but the effects of calcium and cholesterol differed between the mycoparasites, and isolates of *P. mycoparasiticum* were intolerant of ethanol, used as the solvent for cholesterol. *P. oligandrum*, *P. acanthophoron* and *P. mycoparasiticum*, tested as single strains, showed different growth responses to ergosterol, cholesterol and β -sitosterol. These mycoparasites were less tolerant of elevated NaCl concentrations than were *P. ultimum* and *P. aphanidermatum*, and grew much better in complex undefined media than in defined media.

Of various media tested, 3% molasses gave maximum oospore production. Oospores of *P. oligandrum* and *P. acanthophoron* showed maximum 17 - 19% germination after 18 h incubation on agar at 25°C, when oospores were harvested from 5-week cultures on molasses medium. The apparent viability of oospores, determined by tetrazolium bromide stain, was 75% and 90% respectively, contrasting with the low germinability. Oospores required an ageing period of 35 days, which was nutrient-independent, before they showed maximum germination. Storage of oospores in air-dried culture biomass reduced their germinability. Of many treatments used, the maximum percentage germination (*c.*30%) was obtained after treatment with 0.001% potassium permanganate. However, culture-produced oospores of *P. mycoparasiticum* consistently failed to germinate and seemed to be non-viable when stained with tetrazolium bromide. The low germinability of oospores of mycoparasitic *Pythium* spp. in general, especially after dry storage, could be a major limitation to their use as biocontrol agents of plant pathogens.

Plating of soils on agar precolonised by susceptible "host" fungi (*Botrytis cinerea*, *Fusarium culmorum* and *Phialophora* sp.), enabled *P. oligandrum* and *P. mycoparasiticum* to be detected in, and isolated from, soils. Isolation of *P. mycoparasiticum* was favoured by soil dilution when *P. oligandrum* was also present, but not when *P. oligandrum* was absent from the soil. Dilution of soil with sand also

increased the isolation of *P. mycoparasiticum* relative to *P. oligandrum* when host-precolonised cellulose film was buried in the soils and subsequently placed on host-precolonised agar. *P. oligandrum* and *P. mycoparasiticum* were also isolated from buried root pieces that were precolonised by *P. ultimum* or *P. aphanidermatum*. All such experiments suggested that the activities of *P. mycoparasiticum* in soils might be limited by competition from *P. oligandrum*.

Fungal host ranges of *P. oligandrum*, *P. acanthophoron* and *P. mycoparasiticum* were compared by ability to grow across agar precolonised by other fungi. *P. acanthophoron* colonised a wider range of hosts than did *P. mycoparasiticum,* but both had narrower ranges than for *P. oligandrum*. The mechanism of mycoparasitism, studied by video microscopy of interhyphal interactions, was similar for *P. acanthophoron* and *P. oligandrum*. Both these parasites caused coagulation or lysis of susceptible hosts such as *Trichoderma aureoviride* and *Fusarium oxysporum* less than 5 min after contact, but coiled round hyphae of the phytopathogenic *Pythium* spp. and caused coagulation only after 60 min. The plant pathogen *P. aphanidermatum* was resistant to the mycoparasites and was the aggressor in some interactions, causing coagulation of the mycoparasite hyphae and occasionally penetrating them. *P. aphanidermatum* also caused coagulation of *Trichoderma* hyphae 30 min after contact and penetrated them after 50 min. It coiled around and penetrated the hyphae of other phytopathogenic *Pythium* spp. but the incidence of this was highest for *P. ultimum* and low for *P. graminicola,* suggesting differences in susceptibility of *Pythium* spp. to damage by *P. aphanidermatum*. The mode of antagonism of *P. aphanidermatum* was broadly similar to that of mycoparasitic *Pythium* spp., although it acted more slowly.

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CHAPTER 1

INTRODUCTION

There is increasing interest in the potential use of antagonistic fungi as inoculants for control of plant pathogens. Of special interest are the mycoparasitic fungi, and this thesis is concerned with one such group, the mycoparasitic *Pythium* species. In particular, the thesis focuses on three members of this group - *Pythium oligandrum* Drechsler, *Pythium mycoparasiticum* Deacon, Laing & Berry and *P. acanthophoron* Sideris. Published work relating to various aspects of mycoparasitism by these fungi and to the development of biological control agents will be discussed here.

1.1 The genus Pythium

The genus *Pythium* was formed by Pringsheim (1858) as part of the family Saprolegniaceae but Schröter (1897) transferred it to a new family, Pythiaceae (Hendrix & Campbell, 1973). *Pythium* species were seen to be associated predominantly with root diseases, so the genus *Pythium* has long been established as an important genus of soil-borne plant pathogens. However, not all *Pythium* species are plant pathogens, and one group in particular can be set apart from the majority because of its ability to parasitise other fungi. These mycoparasites include *P. acanthicum* (Drechsler, 1946; Hoch & Fuller, 1977), *P. acanthophoron* (Lodha & Webster, 1990), *P. mycoparasiticum* (Foley & Deacon, 1985; Deacon, Laing & Berry, 1991), *P. nunn* (Lifshitz, Stanghellini & Baker, 1984a), *P. oligandrum* (Drechsler, 1946; Foley & Deacon, 1985) and *P. periplocum* (Drechsler, 1946). These mycoparasites are not necessarily closely related to one another, although it has been proposed that *P. oligandrum* and *P. acanthicum* can, for practical purposes, be grouped as a complex (Hendrix & Campbell, 1970, 1973). However, all the mycoparasitic *Pythium* spp. do have two general features in common: first, their propensity to parasitise other fungi *in vitro*, and second their general lack of phytopathogenic activity.

1.2 The mycoparasitic Pythium species

Drechsler (1943a, 1943b, 1946) first observed mycoparasitism within the genus *Pythium*, in that three species- *P. acanthicum*, *P. oligandrum* and *P. periplocum*- were found to coil extensively round the hyphae of phytopathogenic *Pythium* spp. in dual-membered culture. A fourth mycoparasitic species, *P. nunn*, was isolated by Lifshitz, *et al.* (1984a) in the United States. Deacon & Henry (1978) and subsequently Foley & Deacon (1985) isolated another mycoparasitic *Pythium* species, recently named as *P. mycoparasiticum*. More recently an isolate identified as *P. acanthophoron* was shown by Lodha & Webster (1990) to have mycoparasitic properties. Of the six *Pythium* species suggested to have mycoparasitic properties, four have echinulate oogonia- *P. acanthicum*, *P. acanthophoron*, *P. periplocum* and *P. oligandrum*- whilst the other two have smooth-walled oogonia (*P. nunn* and *P. mycoparasiticum*). Most of the information on behaviour of mycoparasitic *Pythium* spp. concerns only three species- *P. acanthicum*, *P. oligandrum* and *P. periplocum*, which behave almost identically to one another.

P. oligandrum, *P. acanthicum* and *P. periplocum* are periodically reported from diseased plants but, upon inoculation, are not aggressively phytopathogenic. Instead they are frequently found in association with known plant pathogens; for example, Drechsler (1943a), who originally isolated *P. oligandrum* from damaged pea roots, reported it to occur with three well-known phytopathogens, *P. ultimum* Trow, *P. debaryanum* Hesse and *P. irregulare* Buisman. This, and the observation that, in culture, hyphae of *P. oligandrum*, *P. periplocum* and *P. acanthicum* coiled around the hyphae of phytopathogenic *Pythium* species, led Drechsler to propose that the three species were not usually primary plant parasites but were secondary invaders of diseased tissues, obtaining their nutrition both from mycelia of the primary invaders and from plant tissue killed by the primary invaders. However the role of these fungi as plant parasites cannot be completely discounted since isolates of *P. acanthicum* and *P. periplocum* have been shown to cause blossom end-rot of watermelon (Drechsler, 1930, 1946).

Tribe (1961) observed *P. oligandrum* on cellulose film that was buried in soil and noted that this was unexpected as *P. oligandrum* is not regarded as cellulolytic. Mycelium of another fungus, identified as *Botryotrichum piluliferum* Sacchardo & Marchal, was found growing on the film near *P. oligandrum*. As *B. piluliferum* was known to be cellulolytic, Tribe (1966) suggested that *P. oligandrum* grew on the cellulose film by utilizing small molecules, perhaps sugars and organic acids, released

by *B. piluliferum* or by its action on the cellulose substrate. The growth of a non-cellulolytic fungus through utilisation of cellulose-breakdown products released by the actions of extrahyphal enzymes of a cellulolytic fungus has been termed secondary sugar saprophytism (Garrett, 1970). Tribe (1966) supported this proposed mode of growth by *P. oligandrum* by studies of interactions of fungi on cellulose film *in vitro*. He saw no microscopical evidence of mycoparasitism and also found no detrimental effect of *P. oligandrum* on the cellulolytic activity of *B. piluliferum*. However, Deacon (1976) and Deacon & Henry (1978), in similar studies, concluded that *P. oligandrum*, *P. periplocum* and *P. acanthicum* are aggressive mycoparasites. They were found to be non-cellulolytic and yet could grow on cellulose in the presence of some other fungi, significantly reducing the growth and cellulolytic activities of the fungi with which they grew best. Differences in the level of susceptibility of the cellulolytic fungi to mycoparasitism by *P. oligandrum* and the other *Pythium* spp. were established, by recording the degrees to which the cellulolytic activities of various "host" fungi were reduced by co-inoculation with *P. oligandrum*, *P. acanthicum* or *P. periplocum*. It was also established that these three mycoparasites had similar host ranges and similar degrees of mycoparasitic aggressiveness to one another. Most recently Laing & Deacon (1990, 1991) used the same method, and videomicroscopy of inter-hyphal interactions, to compare the host ranges and aggressiveness of *P. oligandrum*, *P. mycoparasiticum* and *P. nunn*.

1.3 The mycoparasitic Pythium spp. in relation to other mycoparasites

A mycoparasite can be defined as a fungus that parasitises other fungi. The definition and scope of this term has been addressed in several papers and reviews (e.g. Boosalis, 1964; Barnett & Binder, 1973; Jeffries, 1985). In the older literature, the term hyperparasite was used instead of mycoparasite but in present usage a hyperparasite is considered to be a fungus that parasitises another parasite (of plants or animals) and the term is thus largely redundant because it is encompassed by the more general term, mycoparasite.

Strictly speaking, a fungus can be described as a parasite if it is shown to grow in intimate association with another organism, from the functional tissues of which it obtains all or part of its nutrient requirements (Federation of British Plant Pathologists, 1973). Deacon (1976) was the first to apply this strictly to a mycoparasite, *P. oligandrum*, and to demonstrate nutritional dependency in certain conditions. Whipps, Lewis & Cooke (1988) adopted another definition of parasitism in Federation of British Plant Pathologists (1973) and defined a mycoparasite as "A

fungus existing in intimate association with another fungus from which it derives some or all of its nutrients while conferring to benefit to return".

Mycoparasites can be separated into two groups based on the nature of the host-parasite interaction (Barnett & Binder, 1973). These are termed biotrophic and necrotrophic mycoparasites. The biotrophic mycoparasities obtain nutrients from living cells with, at least initially, little or no harm to the host. They tend to have a restricted host range, and obtain their nutrients in various ways, reviewed by Jeffries (1985). In some cases the mycoparasite may only contact the host cell, without penetration, and obtain nutrients through "buffer" or adsorptive cells. In other cases it may penetrate the host with a peg from an appressorium-like swelling and form haustoria within the host cell. In yet other cases it may penetrate the host hypha and grow internally from cell to cell without an apparent effect in the early stages of development (Lumsden, 1981).

In contrast to these forms of behaviour, necrotrophic mycoparasites characteristically kill the host cells, early in the parasitic process, by production of enzymes or toxins or simply by penetrating and disrupting cellular membranes. They then utilise nutrients from the dying or dead cells. The host range of nectrotrophic mycoparasites is usually broad (Barnett & Binder, 1973). All the known mycoparasitic *Pythium* spp. are in this category.

1.4 Isolation of Pythium species

Pythium species are seldom isolated from soil and plant material using dilution plating on agar media, since this tends to favour the more competitive, widespread saprophytes (Hendrix & Campbell, 1973). Most techniques used to isolate pythiaceous fungi from soil either use selective media, or baiting methods. Many selective media for the isolation of *Pythium* species have been reported (Schmitthenner, 1962; Vaartaja & Bumbieris, 1964; Vaartaja, 1968; Flowers & Hendrix, 1969; Mircetich, 1971; Robertson, 1973; Pieczarka & Abawi, 1978; Ali-Shtayeh, 1985; Dick & Ali-Shtayeh, 1986; Liddell *et al.*, 1989; Cother & Gilbert, 1993). Often they involve the incorporation of polyene antibiotics, such as pimaricin or endomycin, which are not strongly inhibitory to the *Pythium* spp. but inhibit the growth of other fungi. These antifungal agents can be combined with the use of other selective chemicals such as other antibiotics, gallic acid, pentachloronitrobenzene (PCNB) and rose bengal, to suppress non-pythiaceous fungi and bacteria.

Baiting techniques often involve placing pieces of soft (non-lignified) living plant material into soil; these select for phytopathogenic *Pythium* species and exclude saprophytic organisms. The baits are then retrieved and placed on agar media for isolation. Baiting techniques, however, only give qualitative results on the occurrence of *Pythium* species in soils and, as stated by Hendrix & Campbell (1970, 1973), no one baiting technique is suitable for isolation of all *Pythium* species. For example, Stanghellini & Kromland (1985) developed a potato-baiting technique specifically for detecting *P. aphanidermatum* (Edson) Fitzpatrick in soil. Davison & Bumbieris (1973) used a pear-baiting technique to isolate *Pythium* spp. from pine plantation soil, and found that it was selective for some *Pythium* species, such as *P. mamillatum* Meurs, *P. anandrum* Drechsler, *P. mastophorum* Drechsler and *P. irregulare*. *P. mamillatum* was also selectively isolated from cultivated soils using turnip seedlings as baits (Barton, 1958). Other baits that have been used include pineapple root and leaf baits (Klemmer & Nakano, 1964), apple (Hendrix & Campbell, 1970) and cellulose for the cellulolytic *Pythium* spp. in freshwater habitats (Park, 1977).

Hendrix & Campbell (1970) compared two selective media and a baiting technique for isolation of a range of *Pythium* species from soils. The two selective media were Kerr's modified medium which contained PCNB, rose bengal, streptomycin sulphate and mycostatin (Hendrix & Kuhlman, 1965) and the gallic acid medium described by Flowers & Hendrix (1969). The baiting technique involved placing soil samples into holes cut into apples, and isolations were made from the rot that developed around these holes. The three methods were found to differ in efficiency for isolation of the *Pythium* species. The *"P. irregulare - P. debaryanum* group", *"P. dissotocum* Drechsler - *P. perniciosum* Serbinow group" and *P. spinosum* Sawada were isolated infrequently by the apple-baiting technique, but were readily isolated by the selective media. The opposite was true for *P. splendens* Braun, *P. ultimum* and *P. helicoides* Drechsler. The two selective media were also seen to differ in ability to isolate different *Pythium* species. *P. afertile* Kanouse & Humphrey, *P. aphanidermatum* and the *"P. dissotocum - P. perniciosum* group" were detected more frequently using the modified Kerr's medium, whilst *P. vexans* de Bary, *"P. oligandrum - P. acanthicum*" group and *P. periplocum* were more frequently isolated on the gallic acid medium. Since different isolation techniques are required for the isolation of different *Pythium* species, it can be expected that the different mycoparasitic *Pythium* species would probably require different techniques from those employed for other *Pythium* species. Those used to date are outlined below.

1.4.1 Isolation of mycoparasitic Pythium *species*

Many of the selection media described for other *Pythium* species will also enable mycoparasitic *Pythium* species to be isolated. This is true for *P. oligandrum* and *P. acanthicum* in particular (Schmitthenner, 1962; Vaartaja & Bumbieris, 1964; Vaartaja, 1968; Flowers & Hendrix, 1969; Hendrix & Campbell, 1970; Mircetich, 1971; Robertson, 1973; Pieczarka & Abawi, 1978; Ali-Shtayeh, 1985; Dick & Ali-Shtayeh, 1986; Liddell *et al.*, 1989; Cother & Gilbert, 1993). As a refinement on these media, Martin & Hancock (1986) developed a selective medium consisting of cornmeal agar with pimaricin, vancomycin, penicillin G, benomyl and rose bengal to determine the propagule density of *P. oligandrum* in soil. White, Henn and Petch (1993) used a much simpler medium, consisting only of 0.1% glucose in 1.5% water agar to isolate *P. oligandrum* from air-dried soil. According to these authors, *P. oligandrum* was the one of few *Pythium* spp. that grew on this medium.

Mycoparasitic *Pythium* species have also been isolated from soil using baiting techniques (Hendrix & Campbell, 1970; Davison & Bumbieris, 1973). For example, Quimio & Abilay (1977) isolated *P. acanthophoron* by baiting soil with sorghum seeds. Ribeiro & Butler (1992), used sclerotia of *Sclerotinia sclerotiorum* (Lib.) de Bary as bait to isolate *P. oligandrum*, *P. acanthicum* and *P. periplocum* from soil, and compared this technique to a dilution plate method using a selective medium containing pimaricin, penicillin and polymyxin B (Liddell *et al.*, 1989). Whilst only *P. oligandrum* was isolated using the dilution plate technique, the sclerotia baiting technique isolated *P. acanthicum*, *P. periplocum* and *P. oligandrum*.

An alternative approach was developed by Deacon & Henry (1978) who placed soil samples on plates of potato-dextrose agar previously colonised by *Phialophora* sp. This method was found to be highly selective for isolation of mycoparasitic *Pythium* species, especially *P. oligandrum* and a *Pythium* sp. forming smooth-walled oogonia (*P. mycoparasiticum*). However, the use of hemp-seed baits, incubated in soil and then placed on the precolonised agar plates, was much less selective for mycoparasitic *Pythium* spp.; it allowed, instead, a range of phytopathogens such as *P. intermedium* de Bary and *P. sylvaticum* Campbell & Hendrix to grow on the plates. Foley & Deacon (1985) also used agar plates precolonised by *Phialophora* sp. to isolate *P. oligandrum*, *P. acanthicum* and *P. mycoparasiticum* from a range of soils. They compared the efficiency of this technique with selective media based on Schmitthenner's (1962) basal synthetic medium but supplemented with various antifungal or antibacterial agents. They found that whilst the precolonised plate technique consistently yielded mycoparasitic *Pythium* spp. from soil, none of the

Pythium species that grew on the different selective media was a mycoparasite. Mulligan & Deacon (1992) reported that other fungi could be used in place of *Phialophora* sp. on precolonised agar plates. *Fusarium culmorum* (W. G. Sm) Sacc. was found to be as efficient for isolation of *P. oligandrum* as was *Phialophora* sp.

By means of these techniques it has been possible to study the ecology and distribution of the mycoparasitic *Pythium* spp.

1.5 Ecology of mycoparasitic Pythium species

Pythium spp. have been reported from a wide range of habitats worldwide (Table 1). They tend to be found most abundantly and commonly in cultivated soils compared to non-cultivated soils, and in conditions of neutral or near-neutral pH, rather than highly acidic soils (Barton, 1958; Van der Plaats-Niterink, 1975).

Table 1 Habitats from which *Pythium* species have been isolated.

Habitat	Selected references
Cultivated soils	Chesters & Hickman (1944). Robertson (1973). Van der Plaats-Niterink (1975).
Rice fields	Cother & Gilbert (1993)
Rivers	Park (1975, 1977).
River forelands	Van der Plaats-Niterink (1975).
Forest soils	Campbell & Hendrix (1967). Hendrix *et al.* (1971). Van der Plaats-Niterink (1975).
Forest nursery soil	Vaartaja (1968).
Sands and pumices	Robertson (1973).
Recreational turfgrass soil	Hendrix *et al.* (1970).
Marshes, swamp and wet pasture soil	Apinis (1964).

The available information for mycoparasitic *Pythium* spp. suggests that they occur in habitats favoured by *Pythium* spp. in general (Van der Plaats-Niterink,

1975). They are isolated more frequently from cultivated soils compared to undisturbed soils (Van der Plaats-Niterink, 1975; Deacon & Henry, 1978; Foley & Deacon, 1985) and are abundant in appropriate sites. For example, Deacon & Henry (1978) isolated mycoparasitic *Pythium* spp. from 9 of 16 British soils, taken at random and placed on precolonised agar plates. Of the isolates studied, 6 were identified as *P. oligandrum* and 3 had smooth-walled oogonia and were designated "*Pythium* SWO". This fungus has since been described as *P. mycoparasiticum* (Deacon *et al.*, 1991). However, neither *P. acanthicum* nor *P. periplocum* was isolated in that study by Deacon & Henry (1978). A more widespread survey using precolonised agar plates was carried out by Foley & Deacon (1985) who assessed 164 soils or other materials for presence of mycoparasitic *Pythium* species. *P. oligandrum* was detected in 47 samples, *Pythium* SWO (*P. mycoparasiticum*) in 28 samples and *P. acanthicum* in 2 samples; again *P. periplocum* was not isolated. *Pythium* spp. were most frequently isolated from samples of pH 5.5 - 6.5 and seldom from samples of pH less than 4.5. Similarly Mulligan & Deacon (1992) detected *P. oligandrum* in 18 of 28 arable, garden and woodland soils placed on *F. culmorum*-colonised agar, but none of the other mycoparasitic *Pythium* spp. was detected. White (1992) also showed *P. oligandrum* to be widespread in soils used for agricultural and horticultural crops. He found that the common fungicides metalaxyl and mancozeb used agriculturally as a mixture against plant-pathogenic *Pythium* spp., reduced the population of *P. oligandrum* in soil. The occurrence of mycoparasitic *Pythium* spp. in 93 Californian soils was investigated by Ribeiro & Butler (1992) using the sclerotium baiting technique. *P. oligandrum* was the most frequently isolated, being found in 74 of these soils which represented a wide range of soil textures, and pH values from 4.1 to 8.0. *P. acanthicum* was the next most commonly isolated mycoparasitic species, detected in 25 soils. Whilst being present in soils of all pH ranges (4.1 to 8.0) it was not isolated from all the different soil types. *P. acanthicum* was isolated only from clay loam, loam, sandy loam or silt loam soils, being most common in silt loam soils. *P. periplocum* was only isolated from 12 of the 93 soils, which were similar to those from which *P. acanthicum* was isolated. It was isolated from soils of pH range 5.1 to 8.0. In this study it was also reported that there was no correlation between the presence of *P. acanthicum*, *P. oligandrum* or *P. periplocum* and the crop grown in the soil at the time when the samples were taken.

The first recorded isolation of *P. oligandrum* from Britain was by Chesters & Hickman (1944), who obtained it from diseased stems and roots of viola, along with *P. violae* Chesters & Hickman- a known pathogen of viola. Among other records, worldwide, Vaartaja (1968) isolated *P. oligandrum* from 3 and *P. acanthicum* from 2

of the 7 forest nursery soils tested in Ontario, Canada. In the same study *P. oligandrum* was isolated from 1, and *P. acanthicum* from 2, of the 3 agricultural soils tested. *P. oligandrum* was isolated from 1 of 25 rice fields in Southern Australia by Cother & Gilbert (1993), and from 20 of the 39 carrot fields in the San Joaquin Valley of California sampled by Liddell *et al.* (1989). Two of the seven Ohio soils tested by Schmitthenner (1962) were found to contain *P. oligandrum* while *P. acanthicum* was isolated from 1 of the 7 soils. *P. oligandrum* was found by Ali-Shtayeh (1985) to be present in 31% of cultivated soils sampled from the West Bank and the Gaza Strip. It was, however, found to be more common in non-irrigated soils, in contrast with *P. ultimum* which was associated more with irrigated soils. Dick & Ali-Shtayeh (1986) suggested that *P. oligandrum* and *P. acanthicum* had different habitats, explaining why often one is isolated with high frequency from one particular soil where the other is not. They used statistical analysis of community structure to analyse populations of *Pythium* spp and found that *P. oligandrum* and *P. acanthicum* fell into different blocks representing different ecological associations. *P. acanthicum* was isolated by Apinis (1964) from pasture and initial stages of marshes in Britain; and by Campbell & Hendrix (1967) from forest soils in Southeastern USA. Neither *P. oligandrum* nor *P. periplocum* was isolated in either case. Van der Plaats-Niterink (1975) observed that in the Netherlands *P. oligandrum* was the second commonest *Pythium* sp. in cultivated soils; *P. acanthicum* and *P. periplocum* were found much less often. Of the 10 *Pythium* spp. isolated by Pratt & Janke (1980) from plants with root rot in Texas, USA, 8 were identified as *P. periplocum* and 2 as *P. myriotylum* Drechsler. *P. periplocum* was, however, not observed to cause damping-off of the plants upon inoculation.

P. nunn has, so far, been isolated only from soil in Colorado, U.S.A. (Lifshitz *et al.*, 1984a). The other mycoparasite, *P. acanthophoron*, was first isolated by Sideris (1932) from diseased pineapple leaves in Hawaii. It was subsequently isolated from Philippine soil by Quimio & Abilay (1977), from soil around a healthy rhizome of ginger collected from India (Lodha & Webster, 1990) and from a site at Dawlish Warren, Devon by Lodha & Webster (1990).

In summary of these and other reports, it is clear that mycoparasitic *Pythium* spp. are common and widely distributed in sites that favour *Pythium* spp. in general. However the most common of them seems to be *P. oligandrum*, while others such as *P. mycoparasiticum*, *P. nunn* and *P. acanthophoron* may have more restricted distributions. It will only be possible to obtain full comparative data when a range of selective isolation methods are compared across a range of geographical regions. For

example, *P. mycoparasiticum* has only been detected in British soils at present, by the use of precolonised agar plates; apparently this method has not been used in attempts to detect it elsewhere. Similarly, *P. nunn* is only known from Colorado, USA, but this may reflect the lack of attempts to detect it elsewhere.

1.6 Nutrition of the mycoparasitic Pythium species

Foley & Deacon (1986a) investigated the physiological differences between four mycoparasitic *Pythium* spp. (*P. oligandrum*, *P. acanthicum*, *P. periplocum* and *P. mycoparasiticum*) and some phytopathogenic *Pythium* spp. All the *Pythium* spp. tested by Foley & Deacon (1986a) were able to utilise glucose, cellobiose and trehalose to at least some degree, but only the mycoparasitic *Pythium* spp. utilised mannitol, though to different degrees. This extended the findings of Child, Défago & Haskins (1969a), that one isolate of *P. acanthicum* grew well on mannitol if cholesterol was present in the medium. McQuilken, Whipps & Cooke (1992a) also reported that *P. oligandrum* utilised mannitol in the presence of cholesterol and calcium. Foley & Deacon (1986a), however, did not incorporate cholesterol or calcium into their medium. Deacon (1979) reported that *P. oligandrum*, *P. acanthicum* and *P. periplocum* could not utilise cellulose, but several of the non-mycoparasitic *Pythium* spp. were able to do so. *P. oligandrum* was also reported to be non-cellulolytic by Tribe (1966), and *P. mycoparasiticum* and *P. nunn* were shown to be non-cellulolytic by Laing (1989) and Laing & Deacon (1990) respectively.

Foley & Deacon (1986a) reported that, unlike most phytopathogenic *Pythium* species, the mycoparasitic *Pythium* species cannot utilise inorganic nitrogen sources; instead an organic nitrogen source such as amino acids is required. This was also demonstrated for *P. acanthicum* by Child *et al.* (1969a) and for *P. oligandrum* by Leonian & Lilly (1938) and McQuilken *et al.* (1992a). Laing & Deacon (1990) however found that *P. nunn* differs from other mycoparasitic *Pythium* spp. because it does not require organic nitrogen. *P. acanthophoron* has not been used in comparative tests of this type. An exogenous source of thiamine or its pyrimidine moiety was required by the mycoparasitic *Pythium* spp., in contrast to most phytopathogenic *Pythium* spp. (Foley & Deacon, 1986a). Leonian & Lilly (1938) also demonstrated this for *P. oligandrum*, whilst Ridings, Gallegly & Lilly (1969) showed this requirement for *P. acanthicum* and *P. periplocum* as well as for *P. oligandrum*. However, Ridings *et al.* (1969) reported *P. acanthophoron* to be self-sufficient for thiamine; a nutritional characteristic of non-mycoparasitic *Pythium* species. Laing (1989) showed that *P. nunn* had a partial requirement for thiamine, it could grow

without thiamine, but supplementation of the medium with thiamine improved its growth. From all these findings, it seems that there are relatively minor differences between the mycoparasitic *Pythium* spp. in nutritional requirements, but that the mycoparasitic species, as a group, often have nutritional features that separate them from the majority of phytopathogenic *Pythium* spp. In particular, the mycoparasitic *Pythium* spp. often lack cellulolytic activity, can utilise polyols such as mannitol (at least in the presence of sterols and calcium), require organic nitrogen sources and thiamine or its pyrimidine moiety.

Compared to phytopathogenic *Pythium* spp., agar containing rose bengal and gallic acid was reported by Foley & Deacon (1986a) to inhibit the growth of mycoparasitic *Pythium* spp. They observed that whereas the hyphae of almost all the non-mycoparasitic *Pythium* isolates tested were white and the medium had changed from the original plum-purple colour to green, the hyphae of *P. oligandrum*, *P. acanthicum* and *P. periplocum* were pink and the colour of the medium was unchanged. The reason for this is unknown. A component in commercial potato extract was also observed by Foley & Deacon (1986a) to reduce the radial growth of mycoparasitic but not non-mycoparasitic *Pythium* spp.

1.7 Fungal hosts of the mycoparasitic Pythium species

Mycoparasitic *Pythium* spp. have been reported to parasitise a wide range of hosts. Haskins (1963) investigated the host range of an isolate of *P. acanthicum*. Of the 98 species of fungi tested as potential hosts *in vitro*, *P. acanthicum* parasitised 68 resulting in the production of oogonia and 10 without oogonium production; 9 fungi were not parasitised, and 10 inhibited the growth of *P. acanthicum* on agar. The fungi that supported growth of *P. acanthicum* included members of the Oomycetes, Ascomycotina, Basidiomycotina, Deuteromycotina and Zygomycotina. The criteria for mycoparasitism in this work included the ability of *P. acanthicum* to overgrow the host fungal colony and to coil around the host hyphae. This broad definition might account for the wide range of hosts reported to be parasitised. Veselý & Hejdanek (1984) used a similar criterion to assess the host range of *P. oligandrum*. Of the 16 species tested, 8 were seen to be strongly antagonised by *P. oligandrum*, and the mycoparasite overgrew their colonies and produced abundant oogonia, 3 were weakly inhibited by the mycoparasite, 3 were weakly inhibitory to the mycoparasite, and 2 were strongly inhibitory to it, forming large inhibition zones resulting in the death of the mycoparasite.

Prior to this work on *P. oligandrum*, Deacon (1976) had reported a similar range of responses between *P. oligandrum* and other fungi, ranging from susceptibility to resistance and inhibition by potential hosts. He also used a stricter criterion of host susceptibility whereby *P. oligandrum* must be shown to be able to grow in the presence of a host in conditions that would not support its growth in the absence of a host fungus, for example, when cellulose was the sole carbon source, and that such growth must be to the detriment of the host fungus. Indeed *P. oligandrum* was shown to grow with susceptible hosts even when cellulose was used as sole carbon source, nitrate as sole nitrogen source and no vitamins or sterols were supplied in the medium. Since oogonium production by *P. oligandrum* requires an exogenous source of sterols (Haskins, Tulloch & Micetich 1964; Hendrix & Campbell, 1973) any oogonium production by the mycoparasite was supported by host-derived sterols, while growth *per se* was supported by carbon, nitrogen and vitamins derived from the host fungus. *P. oligandrum*, like *P. acanthicum*, was observed to have a wide host range, with the fungi exhibiting varying degrees of susceptibility to it. Whipps (1987) tested several mycoparasites, including *P. oligandrum*, on three different media, to test for different antagonistic responses towards a range of "host" fungi. He showed that interactions between the different fungi varied significantly under different cultural conditions. This was suggested to be related to both the inherent properties of the host or mycoparasite fungus, and to the effect of the environment on host susceptibility and mycoparasite aggressiveness. It was also found that *P. oligandrum* was consistently mycoparasitic on all the media tested, which indicated that it is likely to behave the same *in vivo* as it does *in vitro*. Bradshaw-Smith, Whalley & Craig (1991), however, showed that the ability of *P. oligandrum* to overgrow and parasitise three fungal plant pathogens in dual cultures varied depending on the agar medium used. On cornmeal agar, *P. oligandrum* was seen to overgrow and parasitise all three fungi, but on tap water agar the hosts supported less parasitism by *P. oligandrum*. Differences in the susceptibility of host fungi can also be dependent on other growth conditions. Foley & Deacon (1986b) found that, in liquid media of defined composition containing nitrate as a sole nitrogen-source and no thiamine, *P. oligandrum* could not grow on undisturbed colonies of *F. culmorum* although it grows well across colonies of this fungus on potato-dextrose agar (Laing & Deacon 1990). In contrast, another susceptible host fungus, *Phialophora* sp., supported good growth by *P. oligandrum* in both liquid and agar culture. In further analysis of such effects, Foley & Deacon (1986b) found that macerated liquid cultures of *F. culmorum* supported good growth of *P. oligandrum*, and even the filtered culture medium from undisturbed culture (the mycelia of *F.*

culmorum having been removed) supported growth by *P. oligandrum*. So this host fungus released the necessary nutrients for *P. oligandrum* into the medium, but the active mycelia of *F. culmorum*, in some way, prevent the mycoparasite from utilising these nutrients in liquid culture media.

Lutchmeah & Cooke (1984) reported that interactions between *P. oligandrum* and three plant pathogenic fungi (*P. ultimum*, *Mycocentrospora acerina* (Hartig) Deighton and *Rhizoctonia solani* Kühn) resembled hyphal interference. The hosts were also seen to vary in susceptibility to *P. oligandrum*, *M. acerina* was seen to be the most susceptible, where loss of opacity of the hyphae was seen 5 min post-contact. With *P. ultimum* and *R. solani* this process took 30 min post-contact, but whilst the hyphae of both *M. acerina* and *R. solani* were seen to be penetrated by hyphae of *P. oligandrum*, *P. oligandrum* was never seen to penetrate *P. ultimum*.

Deacon & Henry (1978) compared the host range of *P. oligandrum*, *P. acanthicum* and *P. periplocum*. They found that not only were their host ranges similar but also the degrees of susceptibility of the host fungi to the mycoparasites were similar. Laing & Deacon (1990, 1991) compared the host ranges of three mycoparasitic *Pythium* spp.- *P. oligandrum*, *P. nunn* and *P. mycoparasiticum*- by videomicroscopy of inter-hyphal interactions and by the ability of the mycoparasites to overgrow agar colonies of other fungi. They found that *P. oligandrum* was the most aggressive mycoparasite of the three, with the widest host range. *P. mycoparasiticum* affected a similar host range but was less aggressive on any one of them and *P.nunn* only affected a few hosts and was much less aggressive than either *P. mycoparasiticum* or *P. oligandrum* on these. It was also found that the hosts could be ranked in the same order of susceptibility to each mycoparasite.

Several workers have analysed, specifically, the susceptibility of phytopathogenic *Pythium* spp. to parasitism by *P. oligandrum* and the other mycoparasitic pythiums. These studies date from the original observations of Drechsler (1943a, 1943b, 1946), that *P. oligandrum*, *P. acanthicum* and *P. periplocum* overgrow other *Pythium* spp. in culture and coil conspicuously round their hyphae. In some studies this conspicuous coiling has been taken as evidence of parasitism and even host susceptibility (Lifshitz *et al.*, 1984c). However, Deacon (1976) considered the *Pythium* spp. in general to be among the most resistant fungi to parasitism by *P. oligandrum*. He argued that hyphal coiling is an indication of host resistance, not susceptibility, because the host hyphae persisted long enough for the mycoparasite to coil extensively round them, whereas hyphae of the most susceptible

fungi were rapidly antagonised, lysed and destroyed. Laing & Deacon (1991) also found, by videomicroscopy, that *P. vexans* and *P. ultimum* were among the most resistant host fungi to the mycoparasitic *Pythium* spp. Hoch & Fuller (1977) found that *P. aphanidermatum* was resistant to *P. acanthicum*, and that *P. aphanidermatum* might even be the aggressor in inter-hyphal interactions, coiling round hyphae of the mycoparasite. Conversely, Hockenhull, Jensen & Yudiarti (1992) reported *P. aphanidermatum* to be susceptible to *P. periplocum*. Foley & Deacon (1986b) reported that *Pythium* spp. are among the most resistant host fungi to mycoparasitism by *P. oligandrum*, but that different *Pythium* spp. differed in resistance as evidenced by their ability to support growth of *P. oligandrum* across their colonies on agar and by their abilities to degrade cellulose in the presence of *P. oligandrum*. Berry, Jones & Deacon (1993) reported that the production of oogonia of *P. oligandrum* when coinoculated with each of four *Pythium* species on sunflower seed agar varied depending on the *Pythium* host. *P. vexans* enabled more oogonia of *P. oligandrum* to be produced than when *P. oligandrum* was grown alone, whilst *P. graminicola* Subram. and *P. aphanidermatum* reduced the propagule production by *P. oligandrum*. *P. ultimum* did not change the number of propagules produced by *P. oligandrum* relative to that by *P. oligandrum* alone on the medium. Sunflower seed agar was used to supply sterols for reproduction, since *Pythium* species do not supply the sterol requirement of *P. oligandrum*. Berry *et al.* (1993) also showed that *P. oligandrum* varied in its ability to overgrow plates precolonised with the various *Pythium* hosts. Again *P. vexans* supported best growth of *P. oligandrum*, whilst *P. graminicola* and *P. aphanidermatum* supported poor or no growth of *P. oligandrum*.

Little information is available on the host range of *P. acanthophoron*, recently suggested to be a mycoparasite by Lodha & Webster (1990). They reported it to be a mycoparasite of two fungal pathogens, *Fusarium solani* Mart (Sacc.) and *Pythium myriotylum*. It was also observed to coil around the hyphae of four other fungi.

1.8 Mode of mycoparasitic action

Whipps *et al.* (1988) divided the events involved in mycoparasitism into four stages, namely location, recognition, contact and penetration, and nutrient acquisition.

P. oligandrum was observed by Lewis, Whipps & Cooke (1989) to be able to detect host hyphae over ranges of up to 100μm, with *P. oligandrum* forming lateral branches towards the host. However, it is not clear whether such tropic responses would be observed to all or only to susceptible host hyphae since only susceptible

hosts were tested. Bradshaw-Smith *et al.* (1991) noted that directed growth of *P. oligandrum* towards host fungi occurred occasionally. When it was seen, it seemed that *P. oligandrum* was only able to detect host hyphae over very short distances, as little as 10 - 20μm. Similarly Lutchmeah & Cooke (1984) reported that there was some indication of directed growth of *P. oligandrum* towards hyphal apices of *Mycocentrospora acerina*. This was, however, not so evident when *P. oligandrum* attacked *Rhizoctonia solani* or *P. ultimum*; these fungi are not so susceptible to attack by *P. oligandrum*. Tropism of *P. acanthicum* towards host hyphae was also reported by Hoch & Fuller (1977); the parasite was stimulated to grow towards the host, even when they were as much as 100μm apart. Laing & Deacon (1991), in contrast, found no convincing evidence of tropism in 148 videotaped interactions between mycoparasitic *Pythium* spp. and ten host fungi on films of water agar. They reported that contact seemed to be a chance event, and this was supported by the high incidence of "near misses" in predicted contacts that were videotaped. They gave a possible explanation for this discrepancy, in that their interactions were observed on water agar, whilst most other workers used nutrient-based media or, at least, cellulose films. It might be expected that mycoparasities would be most responsive to tropic factors on nutrient-poor media, such as water agar, but Laing & Deacon (1991) suggested that the hosts might not produce them in such conditions.

Post-contact recognition by other mycoparasites, such as *Trichoderma* spp., was postulated by Elad, Barak & Chet (1983) to be lectin mediated. Barak *et al.* (1985) showed that the aggressiveness of three isolates *Trichoderma aureoviride* Rifai towards *Sclerotium rolfsii* Sacc. correlated with both the amount of agglutination of their conidia by crude agglutinin of *S. rolfsii*, and the ability of their conidia to attach to the hyphae of *S. rolfsii*. They suggested that the agglutinin of *S. rolfsii* was involved in the recognition of *S. rolfsii* by the *Trichoderma* species. This lectin was purified by Barak & Chet (1990). Using this purified *S. rolfsii* lectin, Inbar & Chet (1992) observed that nylon fibres, of hyphal diameter, coated with it or concanavalin A induced coiling and hook formation by *T. harzianum* Rifai, which coiled round the nylon fibres, as round host hyphae. It would therefore seem that the lectin is involved in the recognition event which leads to the next steps of mycoparasitism. Similarly, Manocha, Chein & Rao (1990) showed that lectins were involved in the recognition and attachment of the biotrophic haustorial mycoparasite *Piptocephalis virginiana* Leadbeater & Mercer to host fungi. No work, however, has been carried out on this aspect for mycoparasitic *Pythium* spp.

Coiling of mycoparasite hyphae around those of their hosts may involve surface-recognition, but could depend also on topographic features or host diffusates. Coiling, as reported by Deacon (1976), is thought to be associated with host resistance rather than susceptibility. It may, however, still be to some degree specific, since Dennis & Webster (1971b) reported that *Trichoderma* spp. do not coil round nylon threads of hyphal diameter. The hyphae of mycoparasitic *Pythium* spp. often branch at points of contact with the host hyphae before penetration, while the main hypha involved in the initial contact grows on (Lewis *et al.*, 1989; Laing & Deacon, 1991; Berry *et al.,* 1993). The branch usually arises from the precise region where the first contact occurs between host and parasite. Laing & Deacon (1991) noted that *P. oligandrum* branched at the point of contact in 95% of videotaped interactions when a mycoparasite tip contacted the lateral wall of a host hypha, and in 69% of cases when the mycoparasite was contacted subapically by a host tip. *P. mycoparasiticum* branched in 68% of all interactions and *P. nunn* in only 42%, reflecting a difference in "responsiveness" of these mycoparasites that perhaps relates to mycoparasitic aggressiveness. The cause of branching is not known.

The mode of host penetration by mycoparasites is thought to be enzymatic. Transmission electron micrographs by Hoch & Fuller (1977) show evidence of enzymatic degradation of host walls in areas of parasitism by *P. acanthicum*. These workers also observed that penetration appeared to be partly mechanical, where enzymatically weakened host tissue was penetrated by force of the growing *P. acanthicum* hypha. Elad, Lifshitz & Baker (1985) showed that *P. nunn* was able to produce the enzymes β-1,3-glucanase, cellulase and chitinase in different amounts in response to the differing wall compositions of host hyphae. In the same study, *P. oligandrum* produced fewer enzymes (for example, no chitinase) and lesser amounts of those that it did produce. Lewis *et al.* (1989) reported that *P. oligandrum* produces extracellular β-1,3-glucanase and protease but no chitinase. The enzymes seemed to be inducible, and their production was subject to catabolite repression by glucose, and also by asparagine and $MgSO_4$ in the case of the protease. A clear relationship between degree of mycoparasitism and inducible enzyme synthesis was reported by Elad *et al.* (1985) insofar as *P. nunn* produced more enzymes, and in higher amounts, than did *P. oligandrum*, and *P. nunn* was assessed as being the more aggressive in interhyphal interactions. Also, these workers based their suggestion on the fact that *P. nunn* produced most wall-lytic wall enzymes in the presence of *Rhizoctonia solani*, *Sclerotium rolfsii* and *Pythium* spp. and little or no production with "non-hosts" such as *Fusarium oxysporum* Schlecht. But this categorisation of host susceptibility is the exact opposite of that reported by Laing & Deacon (1990, 1991), who found *F.*

oxysporum to be the most susceptible and *R. solani* among the most resistant potential hosts. Elad *et al.* (1985) may have used the intensity of hyphal coiling as a criterion of host susceptibility (the paper by Elad *et al.* (1985) gives no details on the criterion used). There is thus much confusion in this area. In the most detailed comparative analysis of hyphal interaction to date, Laing & Deacon (1991) found that *P. oligandrum* was significantly more aggressive than *P. nunn* and yet *P. nunn* is reported by Elad *et al.* (1985) to produce most wall-lytic enzymes. Similarly Laing & Deacon (1991) found that the potential host fungi that were susceptible to parasitism were the opposite of those reported by Elad *et al.* (1985), and yet these susceptible fungi (according to Laing & Deacon, 1991) were reported by Elad *et al.* (1985) to induce least production of wall-lytic enzymes by *P. nunn*.

Apart from these points, Elad *et al.* (1985) reported that a component of the hyphal surface of *F. oxysporum* influenced the mycoparasitic interaction. *F. oxysporum* was found to be resisitant to parasitism (? absence of hyphal coiling) in its native state, but could be rendered susceptible (? coiling) by enzymatic removal of a surface polysaccharide on its hyphae. These workers also reported that intense fluorescence was observed around coiling and penetration sites of *P. nunn* on either *Pythium* species or *R. solani*, and also on regions where the hyphae of the mycoparasite were in close association with the plant pathogens, when stained with Calcofluor White M2R New. This was taken as evidence for enzymatic degradation of cell walls, since Calcofluor White M2R New binds to β-glucans and *N*-acetyl-D-glucosamine oligomers in regions of incomplete cell wall polymers.

Enzymes synthesised by mycoparasites are usually assayed in culture filtrates 24 - 72 h after induction. This, however, is difficult to relate to the rapidity of mycoparasitic events, whereby hyphal lysis can occur within 55 sec. after contact of *P. oligandrum* with the most susceptible host hyphae (Laing & Deacon, 1991). Laing & Deacon (1991) thus proposed that the enzymatic degradation of the host hyphal wall may be brought about by activation of the host's own enzymes, which would satisfy the temporal requirement for mycoparasitism. This has been shown for another mycoparasite - host interaction by Adams & Ayers (1983) where the sclerotial mycoparasite *Sporidesmium sclerotivorum* Uecker *et al.* was unable to release detectable wall-lytic enzymes but was suggested to induce the production of these enzymes by the fungal hosts, *Sclerotinia minor* Jagger or *S. sclerotiorum*, which thus degraded themselves, providing nutrients for the mycoparasitic growth. The inducible enzymes of mycoparasites may have a more important role to play in the breakdown and utilisation of the dead host hyphae. Deacon & Berry (1992) indicated, using a

focused beam of light to inactivate either the host, *F. oxysporum*, or mycoparasite, *P. oligandrum*, that both host and mycoparasite activities are required for explosive lysis. This would agree with the idea that the mycoparasite releases a substance on or shortly after contact, leading to localised activation of host lytic enzymes. However, this does not rule out the possibility of other mechanisms for parasitism.

Elad *et al.* (1985) showed that non-volatile substances produced by *P. nunn* inhibit mycelial growth of *R. solani* and *Pythium* spp. in culture and in soil. Inhibitory volatile compounds were not detected. Foley & Deacon (1986b), found no evidence that *P. oligandrum* produces diffusible inhibitors. However, Whipps (1987) observed that *P. oligandrum*, grown on cellophane overlying agar plates, was able to reduce the subsequent growth by host species inoculated onto the plates. No volatile growth-inhibiting compounds were found, but the production of non-volatile inhibitory compounds was suspected. Bradshaw-Smith *et al.* (1991) using the same *P. oligandrum* isolate, showed the opposite: there was evidence to suggest the production of a volatile inhibitor effective against *Phoma medicaginis* Malbr. & Roum. var *pinodella* (Jones) Boerema and *Mycosphaerella pinodes* (Berk. & Blox.) Vestgr. but not against *Fusarium solani* (Mart.) Sacc. f.sp. *pisi* (Jones) Snyd. & Hans., and no evidence to suggest the production of a non-volatile inhibitor by *P. oligandrum*.

Competition has been implicated as a mechanism of biocontrol by *P. oligandrum* (Martin & Hancock, 1986). Whereas *P. oligandrum* has been observed to control other *Pythium* spp. on seeds (Veselý, 1978a; Al-Hamandi, Lutchmeah & Cooke, 1983; Martin & Hancock, 1987; McQuilken, Whipps & Cooke, 1990b), several reports have found *Pythium* hosts to be among the least susceptible fungi to mycoparasitism *in vitro* (Deacon, 1976; Foley & Deacon, 1986b; Laing & Deacon, 1990, 1991). Berry *et al.* (1993) found that, even though interactions with *Pythium* hosts differ from the faster interactions (often less than 10 min. post-contact) seen with several other fungi, *P. oligandrum* does eventually penetrate the *Pythium* hosts and causes damage to the hyphae. This sequence of events took up to 1-2 h and was preceded by much coiling of the mycoparasite around the hyphae of the host *Pythium* species. *P. aphanidermatum*, however, responded differently to the other *Pythium* species: it often was the aggressor in such interactions, resulting in parasitism of the *P. oligandrum* hyphae. Similarly Hoch & Fuller (1977) reported that *P. aphanidermatum* seemed to be the aggressor in interactions with *P. acanthicum*, unlike both Hockenhull *et al.* (1992) and Lifshitz *et al.* (1985) who reported that *P. periplocum* and *P. nunn* respectively parasitised *P. aphanidermatum*. Berry *et al.*

(1993) suggested that mycoparasitism could be significant in biological control of some phytopathogenic *Pythium* species, but not of others.

1.9 Use of mycoparasitic Pythium species as biological control agents

Mycoparasitic *Pythium* spp. have two specific advantages in relation to their potential use as biological control agents of soil-borne plant pathogens. They are active against a wide range of soil-borne fungi, at least *in vitro*, and they are generally non-pathogenic to plants (Kilpatrick, 1968; Van der Plaats-Niterink, 1975; Deacon & Henry, 1978; Martin & Hancock, 1987).

P. oligandrum has been shown to be effective against a number of important soil-borne plant pathogens, both in the laboratory and in the field. Notably, when applied on or around seeds it has been shown to reduce the incidence of *P. ultimum*-induced damping-off of sugar beet (Veselý, 1978a, 1979; Veselý & Hejdanek, 1984; Lutchmeah & Cooke, 1985; Martin & Hancock, 1984, 1987; Walther & Gindrat, 1987a; McQuilken *et al.*, 1990b; Whipps, Budge & McQuilken, 1992), cress (Al-Hamdani *et al.*, 1983; Lutchmeah & Cooke, 1985; McQuilken *et al.*, 1990b, 1992c) and chickpeas (Trapero-Casas, Kaiser & Ingram, 1990); it can control *Mycocentrospora acerina* on carrots (Lutchmeah & Cooke, 1985), *Phoma betae* Frank on sugar beet (Walther & Gindrat, 1987a) and *Aphanomyces cochlioides* Drechsler on sugar beet (Whipps *et al.*, 1992). In most of the above studies oospore preparations of *P. oligandrum* were applied as seed treatments before sowing. Most treatments involved the incorporation of the oospores into a carboxymethyl cellulose base for seed coating (Al-Hamdani *et al.*, 1983; Martin & Hancock, 1987; Trapero-Casas *et al.*,1990; McQuilken, Whipps & Cooke, 1992c). More recently, McQuilken *et al.* (1990b) used a commercial seed-pelleting system or a film-coating technique and showed that the oospores survived the rigours of the coating process.

Paulitz, Ahmad & Baker (1990) employed integrated control using *P. nunn* and *T. harzianum* isolate T-95 to control *P. ultimum*-induced damping-off of cucumber. *T. harzianum* isolate T-95 was used to coat the cucumber seeds, whilst *P. nunn* was applied to the soil. They suggested that the use of two biocontrol agents with the ability to function under different environmental conditions might increase the environmental range over which biological control is successful.

Hydroponics, whereby plants are grown in circulating nutrient solutions, is increasingly being used for crop production in glasshouses. One commercial method

using rockwool, an inert fibrous material produced from dibas and basalt, is used extensively for the production of cucumbers and is increasingly being used for tomatoes (M.A.F.F., 1984). Control of plant pathogens is a problem in such systems. Thinggaard, Larsen & Hockenhull (1988) reported the control of *Pythium splendens* on cucumber roots by *P. oligandrum* in rockwool culture.

Lifshitz, Sneh and Baker (1984b) observed that the addition of dried bean leaves to soil infested with *P. nunn* resulted in an increase in the population of *P. nunn* and a corresponding decrease in the levels of plant-pathogenic *Pythium* species in soil. They suggested that organic amendment could be a means of inducing suppressiveness in soil by increasing propagule level of the biocontrol agent in soil. The use of rolled oats rather than bean leaves as an organic substrate for the added *P. nunn* resulted in the suppression of *P. ultimum*, but other substrates such as cotton leaves, alfalfa or wheat straw did not significantly influence disease incidence (Paulitz & Baker 1987b). Paulitz & Baker (1987a) however, suggested that the timing of the organic amendment was important for inducing suppression. Addition of organic substrates, thereby increasing the population of *P. nunn*, when population densities of *P. ultimum* are relatively low was favourable for disease suppression. Paulitz & Baker (1987b) also reported that temperature, pH and soil matric potential may influence disease suppression by *P. nunn*. Paulitz & Baker (1988b) studied the colonisation of bean leaf fragments by *P. nunn* and *P. ultimum* in naturally and artificially infested soils. They reported that *P. nunn* not only colonised bean leaves previously colonised by *P. ultimum* but also was able to displace *P. ultimum* from the substrate. This was unlike the *P. oligandrum* - *P. ultimum* system reported by Martin & Hancock (1986), where organic substrates were colonised by *P. oligandrum*, thus preventing subsequent establishment of *P. ultimum*. Martin & Hancock (1986) also demonstrated the natural role of *P. oligandrum* in the control of *P. ultimum* in soils of high chloride content in California. They reported that *P. oligandrum* was more tolerant of high chloride levels than was *P. ultimum*, and therefore competes more successfully with *P. ultimum* for organic substrate in soils with higher chloride concentration. *P. oligandrum*, like *P. nunn*, might therefore be used to control plant pathogen populations in soil as well as being used as a seed inoculant. In the only report of the use of *P. periplocum* as a biocontol agent (Hockenhull *et al.*, 1992), damping-off of cucumber seedlings caused by *P. aphanidermatum* was controlled using potting compost infested with the mycoparasite. Nevertheless, a major potential limitation to the commercial development of this technology lies in the often poor germinability of oospores, which are the most appropriate form of inoculum of *P. oligandrum* or other mycoparasitic *Pythium* spp. for biocontrol.

1.10 Oospores of Pythium species and their germination

Pythium spp. produce two types of resting structures- sporangia which yield zoospores and are mainly involved in short term survival (Stanghellini, 1974), and thick-walled oospores which are considered to be the primary propagules of *Pythium* capable of long-term survival in soil. Only oospores will be discussed here due to their present and future potential use as biological control inocula (Veselý, 1979; Veselý & Hejdanek, 1984; Lutchmeah & Cooke, 1985; Martin & Hancock, 1987; Walther & Gindrat, 1987a; McQuilken *et al.*, 1990b, 1992c; Trapero-Casas *et al.*, 1990).

Until, recently most of the information on oospores has related to their taxonomic significance (e.g. Dreschler, 1930) and not their behaviour. Much of the information available on the behaviour of oospores is concerned with two phytopathogenic species, *P. aphanidermatum* and *P. ultimum*, largely to determine their role in initiating infection of plants. In contrast, attention on the oospores of *P. oligandrum* is concerned mostly with formulation and efficiacy of these spores as biological control inocula.

1.10.1 Germination of oospores of phytopathogenic Pythium *species*

Adams (1971), in one of the earliest investigations into the factors affecting oospore germination, examined the effects of temperature, pH and incubation period on the germination of *P. aphanidermatum* in autoclaved soil. The optimum temperature of 30°C for oospore germination was the same as that for mycelial growth of *P. aphanidermatum*. Some oospores germinated after 2 h, and most germinated within 10 h incubation, consistent with the rapid growth rate of *P. aphanidermatum*. The pH optimum of 7.4 for oospore germination seemed unusually high, especially since the pathogen can cause extensive damage of crops over a wide range of pH values. He observed maximum germination levels of between 50 - 60%. With the addition of supplements such as casein and gallic acid to the germination medium and with an optimum pH of 6.0, Flowers & Littrell (1972) increased the germination level to 95%. Stanghellini & Russell (1973), however, observed higher germination with the use of other carbohydrate sources such as galactose, lactose, dextrose, dextrin and sucrose. This was irrespective of whether the oospores were produced *in vivo* (on infected oat roots) or *in vitro* on V8 juice agar. They also reported that the pH optimum for germination was between 6.0 and 8.0, with the

optimum temperature range between 32 and 37°C. Ruben, Frank & Chet (1980) reported that lecithin, a natural lipid source, stimulated germination of oospores of *P. aphanidermatum*. This effect could not be achieved if the lecithin was replaced by other organic lipid-like compounds or precursors such as cholesterol and glycerol. Lecithin has also been reported to increase germination of oospores of *Phytophthora parasitica* Dastur (Ann & Ko, 1987). Addition of glucose to the lecithin medium resulted in direct germination of the oospores to produce mycelium; however in its absence the oospores germinated indirectly to produce zoospores. The effect of glucose could be imitated by a few other carbon sources, such as ethanol, asparagine, glycine or sodium acetate. A similar effect was observed by Stanghellini & Burr (1973a), whereby oospores of *P. aphanidermatum* were seen to be capable of either direct or indirect germination in field soils. The mode of germination was again controlled by the absence or presence of an exogenous source of nutrients, and also the presence or absence of free surface water in saturated soils.

Barton (1957) demonstrated that the germination of oospores of *P. mamillatum* increased in response to exudates from living seedlings. A similar response was also observed by Stanghellini & Burr (1973a), whereby addition of either bean seed exudates or single nutrients, such as glucose or asparagine, increased the germination of oospores of *P. aphanidermatum*. They also observed that oospore germination was maximum in the immediate vicinity of various host organs. The rate of germination of oospores of *P. ultimum* was reported by Johnson & Arroyo (1983) to be greatest with oospores that were close to roots (within 1.5 mm of the root surface), which suggested a stimulatory effect of root exudates on germination. Exudates from ryegrass and creeping bentgrass were found by Han & Nelson (1993) to stimulate germination of both *P. graminicola* and *P. torulosum* Coker & Patterson. As oospores aged, they became more responsive to exudates. Germination of oospores of *P. torulosum*, but not *P. graminicola*, was found to be stimulated by the amino acid serine (E. B. Nelson & D. Y. Han, pers. comm.).

Stanghellini & Russell (1973) suggested that oospore germination in *P. aphanidermatum* consisted of two distinct stages, each with a different exogenous nutrient requirement. Firstly the pregermination stage involving the adsorption of the endospore wall or the conversion of the oospore from being thick- to thin-walled and a reduction in the size of the central reserve globule. This stage was dependent on an exogenous calcium source. The next stage, germination by production of a germ tube, required an exogenous carbohydrate source. The lipid nature of the central reserve globule was shown by Ruben & Stanghellini (1978) and they suggested that the

reduction in size of the central reserve globule was due to the metabolism of the storage reserve lipids. The vacuoles that were observed to be closely associated with the central globule were indicated to be localised sites of enzymes hydrolysing the storage reserves and they may act as lysosomes. The oospore wall was digested during the course of germination.

The effect of matric potential- that is, the availability of water controlled by adsorption, surface tension and capillary effects in a system- on germination of oospores of *P. aphanidermatum* was investigated by Stangellini & Burr (1973b). Maximum germination was reported over the matric potential range -0.01 to -0.1 bars (-0.001 to -0.01 MPa), with both the percentage and speed of germination decreasing at matric potentials higher than this. They suggested that high moisture conditions (= low matric potentials) improved germination by increasing the available nutrients necessary to overcome fungistasis in soil. This nutrient requirement for germination led Burr & Stanghellini (1973) to postulate that oospores in naturally infested soils are exogenously and not constitutively dormant; that is, the dormancy is not an innate property of the oospore itself but results from unfavourable chemical or physical environmental conditions (Sussman & Halvorson, 1966).

Burr & Stanghellini (1973) postulated that enzymatic degredation of constitutively dormant oospores in colonised host tissues resulted in release of oospores from dormancy, so that they have an exogenous nutrient requirement for germination. Germination of oospores of *P. aphanidermatum* was shown by Stanghellini & Russell (1973) to be enzymatically induced. The snail gut enzyme used was suggested to increase the permeability of the oospore wall, allowing diffusion of nutrients necessary for germination. Ruben *et al.* (1980) observed that oospores were also activated by sequential treatment involving desiccation, high temperature and addition of dilute potassium permanganate; only oospores that had been previously desiccated were activated further by the other two treatments. Desiccation was suggested to bring about enhanced permeability of oospores by weakening the cell wall, as a consequence of contraction of wall polymers during desiccation and expansion during rehydration. Dilute potassium permangante and high heat treatment were also found by El-Hamalawi & Erwin (1986) to activate oospores of *Phytophthora megasperma* Drechsler f. sp. *medigaginis* Kuan & Erwin. Unlike Ruben *et al.* (1980) these workers observed that desiccation of oospores decreased the percentage germination. Drying was also reported by Lumsden & Ayers (1975) to decrease percentage germination of oospores of *P. ultimum*. Activation of spores by heat or chemical treatment was suggested to be caused by conformational changes in

the constituent proteins or lipids of membranes (Sussman, 1976) bringing about an increase in the permeability of the oospore.

Schmitthenner (1972) observed that light appeared to increase the germination of oospores of *P. aphanidermatum*. However, Johnson (1988) showed that even though conversion of oospores of *P. ultimum* from thick- to thin-walled was inhibited in darkness, thin-walled oospores germinated equally well in darkness or light. Rosendahl & Olsen (1991), on the other hand, showed that oospores of *P. ultimum* produced in the light germinated better that those produced in the dark, and both types of oospore germinated better when subsequently incubated in the light than in darkness. Rosendahl & Olsen (1991) noted that for light-produced oospores, soaking in sterile dilute salt solution in the light increased both the rate and percentage germination. However, for oospores produced in the dark, the same treatment inhibited germination. This led Rosendahl & Olsen (1991) to suggest that oospores produced in light and darkness are physiologically different, and therefore require different environmental factors to trigger germination.

Significant differences were found by Ayers & Lumsden (1975) between the germination of oospores of *P. aphanidermatum, P. ultimum* and *P. myriotylum*. They concluded that this may be due to different optimum conditions for germination in the three *Pythium* species. The variation seemed to be in the pre-germination stage because oospores of *P. aphanidermatum* converted to the germinable state relatively rapidly (1 to 2 days) in favourable conditions whereas oospores of *P. ultimum* converted at a much slower rate (2 - 6 weeks). From this, and studies into the greater environmental stress-tolerance exhibited by thick-compared to thin-walled oospores of *P. ultimum* (Stasz & Martin, 1988), Lumsden & Ayers (1975) concluded that thick-walled oospores are constitutively dormant. Johnson & Arroyo (1983), however, observed that in both non-rhizosphere and cotton rhizosphere soil 30% of thick-walled oospores of *P. ultimum* were converted to thin-walled oospores in 48 h. This led Johnson (1988) to suggest that both thick- and thin-walled oospores are exogenously dormant. He was able rapidly to convert oospores of *P. ultimum* from being thick- to thin-walled (69% in 2 days) when they were subjected to appropriate conditions of full aeration, optimum pH and light conditions. Nutrients were apparently not required for this change. Qian & Johnson (1987) confirmed this, noting that the rate of conversion of oospores of *P. ultimum* from thick- to thin-walled was not related to the concentration of plant nutrients in soils, nor to pH, organic matter content or soil texture. However, pH was reported by Lumsden & Ayers (1975) to influence the conversion of oospores from thick- to thin-walled, with the

optimum pH for conversion being pH 7.0. Johnson, Qian & Ferriss (1990) investigated the effect of moisture levels on the conversion rate of oospores of *P. ultimum*. In soils with matric potentials of -0.03 to -0.3 MPa, almost all of the thick-walled oospores were converted to thin-walled; however in soils near saturation (0 MPa) and in drier soils (-1.5 MPa), most of the oospores remained thick-walled. Since the conversion of the oospores from thick- to thin-walled seems to be influenced by both chemical and physical conditions of the environment, it can be suggested that oospores of most *Pythium* species are exogenously dormant and optimum germination levels will result from exposure to the appropriate conditions.

1.10.2 Germination of oospores of P. oligandrum

Drechsler (1946), in the first report of germination by *P. oligandrum*, observed that after resting for 40 - 50 days a small proportion of oospores germinated when immersed in a shallow layer of water; practically all oospores germinated after aging for 150 - 200 days. The process of germination was described, whereby the reserve globule changed from a spherical to an irregular shape, the refringement bodies became less distinct, while radial markings appeared in the dark inner layer of the oospore wall, which eventually broke down leaving a thin-walled spherical oospore. This thin-walled oospore then "budded", producing a germ hypha and eventually a vesicle yielding biflagellate zoospores in most instances. Only in some cases did oospores germinate to form mycelia. Oospores of *P. periplocum* were reported by Drechsler (1946) to germinate in a similar manner to those of *P. oligandrum*: after storage for 165 days, a third of them germinating when immersed in a shallow layer of water, and nearly all germinated when stored for a further 45 days. Again, most oospores germinated indirectly to produce zoospores. There are no reports on oospore germination, or the use of oospores as biocontrol inocula, for the remaining mycoparasitic *Pythium* species, namely *P. nunn*, *P. acanthicum*, *P. acanthophoron* and *P. mycoparasiticum*. Therefore the remainder of this section will be concerned with germination of oospores of *P. oligandrum*.

Oospores of *P. oligandrum* have been widely used as seed inocula for biological control of soil-borne fungal pathogens, as noted earlier. However, these studies have rarely investigated oospore germination. McQuilken, Whipps & Cooke (1990a) observed germination levels of between 16 and 23% for oospores freshly produced in culture, after 16 h incubation on corn meal agar. This was similar to the 15% germination obtained by Al-Hamdani *et al.* (1983). Veselý (1987), however, reported that 83% of oospores germinated after 24 h on nutrient agar medium. Walther &

Gindrat (1987a) reported that different isolates of *P. oligandrum* showed varying germination levels. They observed germination levels of 5 to 40%, depending on the isolate, for oospores obtained from 20 to 27 - day-old cultures grown in a carrot-based liquid medium. Yet 22 to 64% germination was obtained if the oospores were subsequently aged for 10 days in sterile distilled water. Walther & Gindrat (1987a), unlike Drechsler (1946), did not observe any thinning of the oospore wall in germinating oospores.

Laing (1989) observed that acetaldehyde (0.25 mM) or ethanol (0.5 mM) stimulated germination of *P. oligandrum* oospores in water suspension. Bacteriological peptone (0.5%) and malt extract (0.5%) was also seen to increase percentage germination.

A most probable number technique was used by Foley & Deacon (1985) to estimate the recovery of *P. oligandrum* from soil artificially infested with a known concentration of oospores of *P. oligandrum*. Between 48 and 60% of oospores were shown to be capable of establishing colonies on agar plates precolonized by a susceptible host fungus. By plating oospore preparations onto malt extract agar, Foley & Deacon (1985) in the same study reported maximum germination of 68% after 5 days incubation.

The effect of water potential on oospore germination is important in relation to inoculum production and subsequent activity in the field. Water potential has osmotic and matric components (Papendick & Campbell, 1981). McQuilken *et al.* (1992b) reported that germination of oospores of *P. oligandrum* was affected more by matric than by osmotic potentials. The matric potential range for oospore germination was markedly narrower than the osmotic range. They found that oospores germinated maximally (19 - 26%) on unamended soil extract agar or on osmotically-adjusted agar, using NaCl or KCl over the range -0.7 to -2.0 MPa. However, below -2.0 MPa germination declined markedly with decreasing osmotic potential and was completely inhibited at -3.5 MPa. On the other hand, matric potentials below -0.8 MPa markedly reduced germination, with germination completely inhibited at below -2.0 MPa. They also reported that mycelial growth was more sensitive to decreasing osmotic potential than was oospore germination.

Storage of *P. oligandrum* oospores has been reported to decrease their germinability (McQuilken *et al.*, 1990a; Al-Hamdani *et al.*, 1983). McQuilken *et al.* (1990a) observed that the decrease in germinability was more pronounced when oospores were stored at 20, 25 and 30°C compared to 5 and 15°C over a 16 week

period. Storage under desiccation at 15°C was also observed by Al-Hamdani *et al.* (1983) to decrease both germinability and speed of germination, over a 5 month period. Initially 15% germinated within 5 - 7 h of being placed on cornmeal agar; however after 5 months storage only 5% germinated within 12 - 14 h of being placed on agar. Treatment of oospores with myo-inositol during storage was shown by Walther & Gindrat (1987a) to increase oospore germination. Myo-inositol has been postulated (Webb, Cormack & Morrison, 1964) to protect spores from desiccation by increasing the number of substitutvent hydroxyl groups in the microbial cell wall.

Both McQuilken *et al.* (1990b) and Lutchmeah & Cooke (1985) reported that oospores of *P. oligandrum* survived the extremes of commercial seed coating processes. Lutchmeah & Cooke (1985) used a clay-based carrier to pellet seeds with oospores, whereas McQuilken *et al.* (1990b) used both a film-coating binder system and one based on an organic filler material. McQuilken *et al.* (1990b) showed that oospores from treated seeds had a similar germination, 9 - 19%, to that of those in the inoculum used to coat the seeds, 14 - 23%.

1.11 Aims and objectives of the work this thesis

The overall aim of the project was to compare various characteristics of mycoparasitic *Pythium* species, especially *P. oligandrum*, *P. mycoparasiticum* and *P. acanthophoron*, with a view to the potential use of these fungi as biological control agents. The work has involved 4 major lines of investigation.

1. Comparison of the physiology of the different mycoparasitic *Pythium* spp., especially their nutrition. This concentrated mainly on *P. acanthophoron* since little was known about the physiology of this fungus compared to that of the other mycoparasitic *Pythium* spp.

2. Attempts to maximise production of oospores of the three species as biocontrol inocula, and to enhance their naturally low germinability.

3. Comparative ecology of the different species, with particular reference to *P. mycoparasiticum*, by developing effective methods for their isolation from soil.

4. Comparative modes of mycoparasitic action against plant pathogens including phytopathogenic *Pythium* spp. which may be among the major targets for biocontrol in practice.

CHAPTER 2

MATERIALS AND METHODS

2.1 Culture media

Distilled water agar (WA). Agar (Oxoid No 3), 20g; distilled water,1 litre.

Potato dextrose agar (PDA). Potato dextrose agar (Oxoid), 39g; distilled water, 1 litre.

Sunflower seed extract (SSE). Sunflower seeds, 60g, boiled in distilled water for 1 h, then homogenised, filtered through three layers of muslin and diluted to 20% v/v in distilled water.

Sunflower seed agar (SSA). Sunflower seed extract, 10ml; distilled water, 1 litre; agar (Oxoid No 3), 20g.

0.1% Glucose Agar. Glucose, 1g; agar (Oxoid no 3), 15g; distilled water, 1 litre.

Carrot extract (CE). Carrots (50g) boiled in 100 ml distilled water until soft then homogenised, filtered through 3 layers of muslin and the filtrate was diluted to 7.5 % v/v in distilled water.

Potato dextrose broth (PDB). Peeled potatoes (200g) were boiled in 100 ml distilled water until soft, homogenised, filtered through 3 layers of muslin and the filtrate diluted to 20% v/v with distilled water; dextrose (20g/l) was added.

3% Molasses medium. Cane molasses, 30g; distilled water 1 litre.

3% Molasses agar. Cane molasses, 30g; Agar (Oxoid No 3), 20g; distilled water, 1 litre.

Mineral nutrient solution (MNS). KH_2PO_4, 1.23g; K_2HPO_4, 0.17g; $MgSO_4.7H_2O$, 0.5g; $FeCl_3.6H_2O$, 1.0mg; $ZnSO_4.7H_2O$, 0.9mg; $MnSO_4. 4H_2O$, 0.4mg; distilled water, 1 litre.

Malt extract agar (MEA). Malt extract agar (Oxoid), 50g; distilled water, 1 litre.

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

2.2 Fungal Cultures

The fungi used are shown in Table 2.1

Table 2.1 Origins of fungal cultures

Fungal species	Isolate	Origin
MYCOPARASITIC *PYTHIUM* SPP.		
Pythium acanthophoron Sideris	IMI 330 382	Sand dune colonised by *Ammophila arenaria*, Devon. Supplied by J. Webster
	CBS 337.29	Diseased leaves of *Ananas sativus*, Hawaii Supplied by J. Webster
Pythium mycoparasiticum Deacon, Laing & Berry	AR7A	Arable field, Straiton, Lothian
	AR5A	Arable field, Lasswade, Lothian
Pythium oligandrum Drechsler	CGH1	Wasteland soil, Edinburgh (Laing & Deacon 1991)
	P14.1.2	Garden soil, Edinburgh (E. E. Jones)
	F9.2.3	Hay field, Bathgate, Lothian (E. E. Jones)
Pythium periplocum Drechsler	P9.8.4	Hay field, Bathgate, Lothian (E. E. Jones)
OTHER *PYTHIUM* SPP.		
Pythium aphanidermatum (Edson) Fitzpatrick	CBS 634.70	From *Lycopersicon esculentum*, Israel
	IMI 332 885	From *Cucumis sativus* U.K. supplied by J. G. White
	IMI 335 084	From *Cymbopogan winterianus*, India, supplied by J. G. White
	IMI 334 887	From *Brassica* seedlings, Malaysia, supplied by J. G. White
	PA4	Supplied by J. G. White, source unknown
	CBS 287. 79	Soil Bulgaria, supplied by J. G. White

Table 2.1 continued.

Pythium catenulatum Matthews	MS1	From spinach, supplied by M. E. Stanghellini
Pythium dissotocum Drechsler	MS2	From lettuce, supplied by M. E. Stanghellini
Pythium graminicola Subram.	IMI 91329	From sugar cane root, Jamaica
Pythium sylvaticum Campbell & Hendrix	S1	Supplied by J. G. White
Pythium ultimum Trow	G39	Greece, supplied by D. Lascaris
Pythium vexans de Bary	CBS 270.38	From alfalfa root
OTHER FUNGAL ISOLATES		
Botryotrichum piluliferum Sacchardo & Marchal	145 A	ICMB Culture Collection
Botrytis cinerea Sardina	BC1	ICMB Culture Collection
Fusarium culmorum (W.G.Sm) Sacc.	CD9	From wheat, ICMB Culture Collection
Fusarium oxysporum Schlecht f. sp. *lycopersici*	ESCA SCC	From tomatoes, ICMB Culture Collection
Humicola grisea Traaen	A14	From garden soil, Kings Buildings, Edinburgh; supplied by D. Mulligan
Phialophora sp. (lobed hyphopodia)	IMI 187786 (ATCC22834)	From maize, Essex, U. K. ICMB Culture Collection
Phytophthora cinnamomi Rands	C9	Kiwi fruit, A. Stewart, New Zealand.
Trichoderma aureoviride Rifai	TA1	ICMB Culture Collection
Rhizoctonia solani Kühn	GMI, T125	Anastomosis group 5 from wheat, supplied by R. T. Sherwood (see Deacon & Scott 1985)
Zygorhynchus moelleri Vuill.	ZM1	Supplied by A. E. Brown, U.K.

The cultures were maintained by subculturing fortnightly onto plates of PDA and incubated at 25°C in the dark. PDA plates were inoculated at the plate margins, using inoculum from the youngest part of the previous colonies, to enable young mycelium to grow for longer than if placed centrally. The fungi were also stored on slopes of SSA at 4°C and also as inoculum discs from PDA-grown cultures under sterilised distilled water in universal bottles at 4°C.

2.3 Plant pathogenicity tests

Plant pathogenicity tests were carried out on each of the mycoparasite species and the plant pathogens; the plant hosts were tomato (cv. Moneymaker, Suttons seeds), cucumber (cv. Ridge Perfection, Mr Fothergills seeds) and capsicum (cv. Californian Wonder, Mr Fothergills seeds). Plant pots (75mm diameter and 80mm height) were three-quarters filled with perlite and 3 discs (10 mm diameter) of the fungus colony on PDA were placed on the surface. Four replicate pots (12 seeds) were set up per fungal isolate and for each plant species. The seeds were surface sterilised using 10% sodium hypochlorite solution (1% active chlorine) and washed three times in sterile distilled water. One seed was placed on top of each agar disc and covered with a layer of perlite, carefully placed so as not to the disturb the position of the seeds. The pots were then watered, incubated at room temperature *c.*22°C, and the seeds allowed to germinate. The emergence was assessed every 5 days up to a total of 25 days and the pots watered as necessary. Controls were prepared similarly but with uninoculated agar discs.

2.4 Nutritional Studies

2.4.1 Liquid Culture

The method of Foley & Deacon (1986a) was used in these studies with medical flats of 300ml capacity (200mm length) containing 12ml of culture medium. The basal mineral nutrient solution (MNS; as in section 2.1) lacked a carbon and nitrogen source and other growth factors; these were added as required and autoclaved in the bottles at 121°C for 15 min.

The bottles were inoculated with a plug (8mm diameter) cut from the margin of a colony on water agar plates and placed centrally on one of the broad sides of each flat. The bottles were then placed horizontally in the final position for incubation such that the inoculum was partially immersed in liquid; flats were incubated in a growth

room at 25 ± 1°C. After different times, the fungal growth was assessed visually or as mycelial dry weight. For dry weight determinations, the contents of the flats were filtered under suction onto cellulose nitrate filters (0.45μm pore size) using a Buchner funnel (membrane filter holder) and washed with 100ml distilled water. The filters with fungal mycelium were placed on squares of aluminium foil and dried at 80°C to a constant weight. The dry weight of the mycelium was found by subtracting the final weight from the weight of cellulose filters dried alone, taking into account the dry weight of the agar inoculum disc (*c.*3mg).

For oospore counts, the mycelium was homogenised in 1ml of distilled water using an Ultraturrax T25 blender at 20,500 rpm for 60 seconds and the oospore number determined using a haemocytometer.

2.4.2 Radial growth on agar plates

Plates of PDA, half-strength PDA, SSA and WA (section 2.1) were inoculated centrally with a disc (8mm diameter) cut from the margin of a colony on water agar plates. Two lines were marked across the bottom of the plate such that a cross was drawn with the centre marking the inoculation point. The position of the colony margin was marked on these lines every 24 h during incubation at 25°C until the fungus reached the edge of the plate. The mean growth rate (mm per day) was calculated.

2.4.3 Colonisation of vermiculite

Samples of vermiculite (10g; sieved through a 2.86 mm mesh) were placed into 250ml conical flasks, moistened with 40ml of 3% molasses medium (section 2.1) and autoclaved at 121°C for 15 min. The flasks were inoculated with a single fungal disc (diameter 10mm) obtained from the margin of a fungus colony on PDA. Flasks were incubated at 25°C for 70 days, with 5 replicate flasks for each fungus. The flasks were vigorously shaken by hand each day to disperse the fungal growth. Samples were taken after 1, 3, 7 days and every subsequent 7 days up to 70 days. At each time 6 particles from each flask were placed on each of 5 PDA plates supplemented with Penicillin G (100μg/ml), a total of 30 particles per flask per sampling time. The plates were incubated at 25°C and the number of particles yielding colonies of the inoculated fungus was recorded.

2.4.4 Cellulose utilisation

Unlaquered cellulose film (Rayophane PU 525, supplied by British Sidac Ltd, Merseyside, UK) was cut into 6cm x 2cm strips and autoclaved in distilled water for 15 minutes at 121°C. Two strips were placed in parallel on mineral nutrient agar plates, the agar containing 1.2g asparagine, 100μg thiamine and 20g agar (Oxoid No 3) per litre of mineral nutrient solution (Section 2.1). The cellulose strips were inoculated at one end with a disc (5mm diameter) from the margin of a fungus colony on WA. The plates were then incubated at 25°C until the colony margin had reached the far edge of the cellulose strip.

Cellulolysis was assessed using a needle penetrometer which comprised of dissecting needle with a small petri dish attached to the top. A length of glass tubing held in a clamp stand supported the shaft of the needle. The needle point was lowered onto the cellulose film and raised if it did not puncture the film. Weights were progressively added to the dish until the film was punctured, with the needle being lowered to an adjacent point on the film each time, if the weight used was unsufficient to cause puncturing (Deacon & Henry, 1978). In this way it was hoped to reduce the weakening of the film either by leaving the penetrometer in position for too long or by repeatedly probing the same position. The weight required to puncture the film was recorded for five points spaced 10mm apart along the length of the strip, with the first point being next to the inoculation point (Figure 2.1). 70g was required to puncture uninoculated film.

2.5 Production and germination of oospores

A similar method to that of McQuilken *et al.* (1990a) was employed for the production of oospores. Medical flats (300ml capacity) containing 12ml of 3% molasses medium were autoclaved 121°C for 15 min then inoculated as in section 2.4.1 on the broad side with a 10mm diameter disc of a mycoparasite colony grown on PDA. The bottles were incubated at 25°C in the dark for 5 weeks unless otherwise stated.

After the given incubation period, the mycelium of 5 replicate bottles was harvested, the agar inoculum discs removed and the mycelium dried overnight (16 h) on a sterile laminar flow bench. This was reported by Al-Hamdani *et al.* (1983) to kill vegetative hyphae, and also might help break the constitutive dormancy of the oospores (R. P. Bradshaw-Smith, pres. comm.). The dried mycelium was then

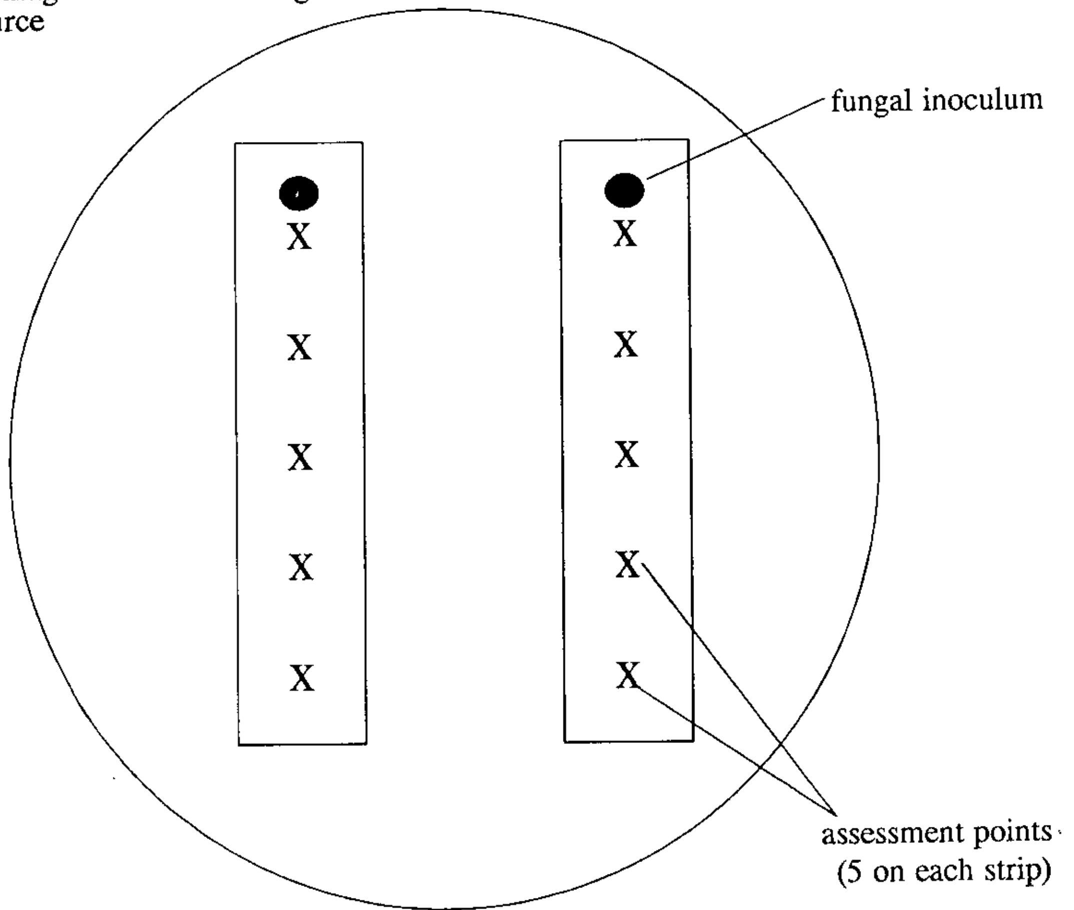


Figure 2.1 Diagramatic representation of the assessment of cellulose breakdown by test fungi.

resuspended in 1ml sterile distilled water (SDW) and homogenised using an Ultraturrax T-25 blender at 20,500 rpm for 60 seconds. The oospore suspension was then washed 3 times with centrifugation at 3,600 rpm (2333g) for 30 seconds. The supernatent was discarded and the pellet resuspended in SDW to achieve an oospore concentration of 10^5 oospores ml^{-1}. Aliquots (0.5ml) of the oospore suspension were spread on each of 4 replicate PDA plates containing 200μg vancomycin HCl per ml and 100μg penicillin G per ml (McQuilken *et al.*, 1990b). Dishes were placed in sealed plastic bags and incubated for 18 h for *P. oligandrum* and 19 h for *P. acanthophoron* at 25°C in the dark. Then germination of 500 oospores per plate were assessed microscopically.

2.6 Soil experiments

2.6.1 Soils

The soils used are shown in Table 2.2. Each sample was bulked from usually 2 or 3 subsamples from the top 10cm of the profile, then air-dried and sieved (2mm mesh). Soil pH was measured in a soil:water mixture (2:1; v:v).

Table 2.2 Origin and pH of the soils used.

Soil	Origin	pH
A	Hay field, Bathgate, Lothian	7.0
B	Potato field, Longside, Aberdeen	6.6
C	Winter wheat field, Hermitage, Lothian	5.9
D	Winter Barley field, Abergele, N. Wales	5.4

2.6.2 Isolation of Pythium *mycoparasites from soil*

2.6.2.1 Precolonised agar plates

A method similar to that of Deacon & Henry (1978), Foley & Deacon (1985) and Mulligan & Deacon (1992) was used. Plates of PDA were inoculated centrally with different host fungi and incubated at 25°C until the host colony margin just reached the edge of the agar. Each plate was then cut into 6 equal sectors which were

placed singly into clean nonsterile plastic Petri dishes that had been recycled from experiments that did not involve fungi. Samples of air-dried sieved soil (0.25mm mesh) were serially diluted with sieved sterile washed sand (0.25mm mesh). Equal volumes of soil and sand were mixed thoroughly and half of this mixture added to an equal volume of sand and the process repeated, down a 2-fold dilution series. Aliquots (0.4ml; *c*.0.4mg) of the soil/sand mixture for each dilution were placed on the youngest part of each of 5 replicate host sectors. The sectors were incubated at room temperature and examined microscopically after 7, 10 and 14 days for the characteristic oogonia of the *Pythium* mycoparasites. Pure cultures of the mycoparasites were obtained by cutting small squares of agar, not immediately adjacent to the soil inoculum, and placing these on fresh PDA plates. Identification was confirmed by subculturing the mycoparasites onto SSA to allow production of the oogonia.

2.6.2.2 Glucose plates

The method of White, Henn & Petch (1993) was used to try to isolate *Pythium* mycoparasites from soil, using Petri dishes containing 0.1% glucose agar (Section 2.1). A sample from each soil, air-dried, was spread thinly on paper and a glass rod was surface sterilised by flaming and allowed to cool before one end was moistened with agar on the plate to receive the soil. Samples of soil were collected by touching the moistened rod to the soil sample and transferring to the isolation plate. Five soil samples were placed on each plate. The plates were then incubated at room temperature and inspected 2 to 4 days later. Any colonies that grew from the soil samples were subcultured onto SSA for identification.

2.6.3 Precolonised cellulose strips

Pieces (2.5cm x 2.0cm) of cellulose film (Rayophone PU 525) were autoclaved in distilled water at 121°C for 15 min. Two strips were then placed in parallel on plates of PDA, a nutrient-rich medium which should suppress the production of cellulase enzymes by fungi. The centre of each strip was inoculated with one of the host fungi, and plates incubated at 25°C until the host fungus had colonised the whole strip. Square Petri dishes (120 x 120 x 13mm, supplied by Gibco/BRL) were half-filled with air-dried soil (sieved 2mm mesh) or a soil dilution where soil was mixed with sand (sieved 2mm mesh) as in section 2.6.2.1. Colonised cellulose strips were then placed face down on the soil and a square of nylon mesh placed on top so that the mesh covered the soil and hung over the edge of the box. The box was then filled with soil or soil/sand dilution. The soils were then brought to 50% saturation

with water and incubated at room temperature for 3 or 7 days. After this, the cellulose strips were removed by carefully lifting the overlying gauze, any soil attached to the strip was removed and each strip was cut into four equal sized portions, which were placed singly on host-precolonised PDA sectors as in section 2.6.2.1. After incubation at room temperature for 7, 10 or 14 days the sectors were observed microscopically for the presence of the characteristic oogonia of mycoparasites.

2.6.4 Precolonised plant roots

Seeds of pea (cv. Kelvedon Wonder, Mr Fothergills seeds) and broad bean (cv. Bunyards Exhibition, Mr Fothergills seeds) were allowed to germinate on damp tissue paper in the dark. From the seedlings, 2cm-long pieces of root were cut, starting just behind the apical meristem, and placed on plates of PDA colonised by *P. aphanidermatum* or *P. ultimum*. These were incubated for 24 h at 25°C, to allow colonisation of the root pieces by the fungi. As in the previous section, square plastic dishes were half-filled with sieved soil (2mm mesh), a square of nylon mesh was placed on top of the soil so that it overhung the edge of each box, and the precolonised root pieces were placed on top of this. Another layer of nylon mesh was then placed over the roots and the boxes were filled with soil. The soil was then brought to 50% saturation with water and the boxes were incubated at room temperature for 5 days. Then the root pieces were retrieved, each cut in half, and one half was placed on agar precolonised by *F. culmorum*, while the other half was placed on agar colonised by *Phialophora* sp. These plates were incubated at room temperature and examined microscopically after 7, 10 and 14 days to detect outgrowth of mycoparasites from the root pieces.

2.7 Mycoparasitic Interactions

2.7.1 Growth on precolonised agar plates

Plates of potato dextrose agar were inoculated at the margin with a PDA disc (8mm diameter) of a host fungus and incubated at 25°C until the colony margin just reached the far edge of the dish (Deacon & Henry, 1978; Laing & Deacon, 1990). Then a PDA disc (8mm diameter) of a mycoparasite was placed on the colony margin and the plates were reincubated at 25°C. Four parallel lines, 5mm apart, were marked on the base of each plate such that three strips were marked, the centre strip joining the host and mycoparasite inoculum discs. After 7 days the centre strip was cut and

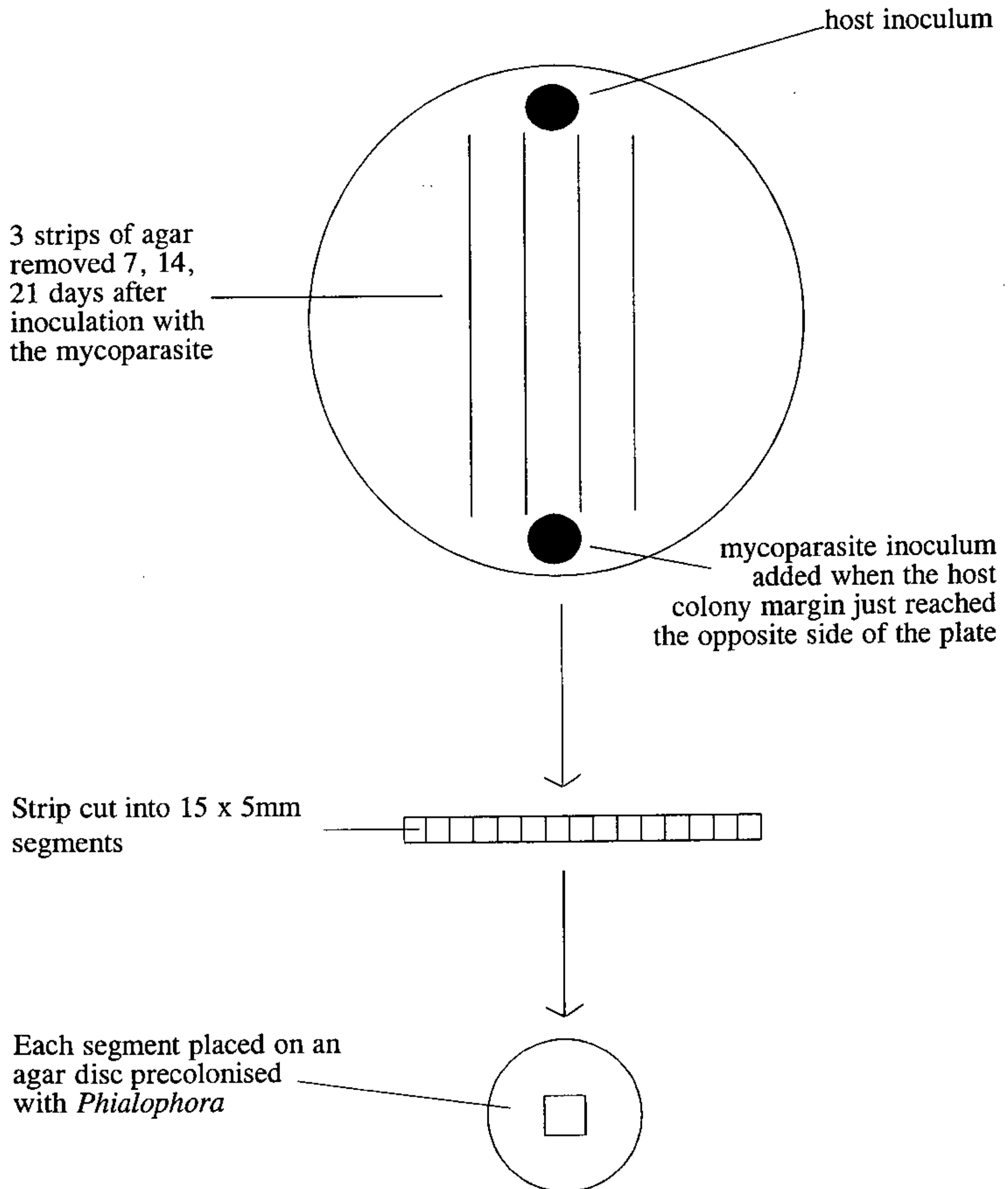


Figure 2.2 Method used to assess growth of mycoparasites across agar precolonised by different host fungi.

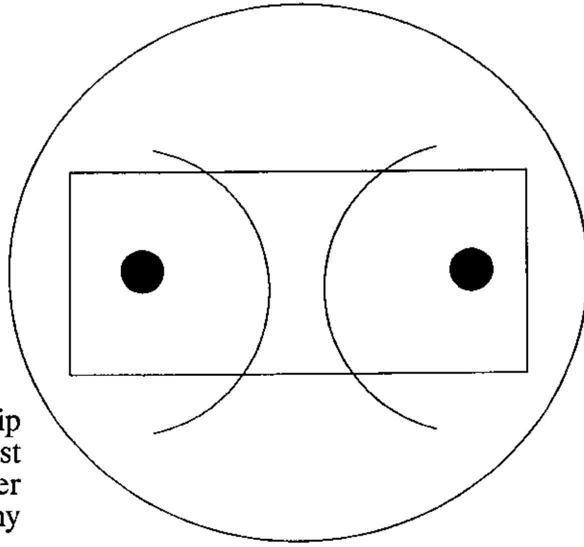
removed, then cut into successive 5 mm pieces, starting from the host inoculum disc. These 5 mm squares were placed on discs (12 mm diameter) of *Phialophora* sp., removed from a colonised plate of PDA. After 7 days the *Phialophora* discs were examined microscopically for presence of oogonia of mycoparasites (Figure 2.2). Thereby, the extent of growth of the mycoparasite on the original agar strip could be assessed to the nearest 5 mm. A second and third strip on either side of the first were removed and treated similarly after 14 and 21 days respectively.

2.7.2 Videotaped interactions

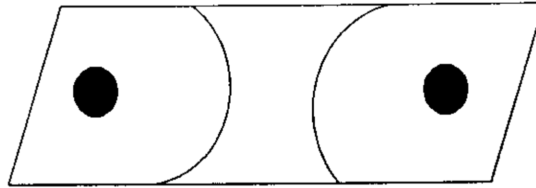
A method similar to that of Laing & Deacon (1991) was used. Glass coverslips (46 x 64 mm) were sterilised by autoclaving at 121°C for 15 minutes. The coverslips were coated with a thin film of water agar by dipping them in sterile molten agar (Difco Bacto, 20g/litre), and allowing excess agar to drain off. They were then placed on solidified water agar plates. Once the agar on the coverslips had set, they were inoculated with opposing agar discs (4 mm diameter) of hosts and mycoparasites, spaced approximately 3.5 cm apart; the discs were from the margins of colonies grown on PDA plates. The plates bearing the coverslips were then incubated overnight at 25°C. For the different host-mycoparasite combinations, the distance between the inoculum discs was adjusted such that contact between the margins of the two colonies occurred after overnight incubation at 25°C.

When the host and mycoparasite colonies had almost touched, the coverslips were removed from the agar plates and inverted, without removing the inoculum blocks, onto observation chambers (Figure 2.3). The observation chambers consisted of a rectangle of glass spacers, 2 mm high, glued onto a large microscope slide. The coverslips were sealed to the observation chamber with vaseline to prevent drying out of the coverslips.

The hyphal interactions were observed by bright field microscopy, using a Leitz Orthoplan microscope with x70 oil-immersion objective. The interactions between individual hyphae were usually recorded for 1-2 h, using a Panasonic S-VHS F15 colour video camera attached to the microscope. The camera was attached to a video recorder (Panasonic S-VHS AG 6720) with time lapse facility, which was attached to a colour video monitor (Panasonic S-VHS BT M1420PY). Interactions were recorded on Master Broadcast S-VHS videotapes. After recording, the videotapes were replayed so as to enable the interactions to be analysed. Photographs of the events that



Agar coated coverslip inoculated with the test fungi, incubated on water agar plate until the colony margins almost touched



Coverslip carefully removed, inverted and placed on observation chamber

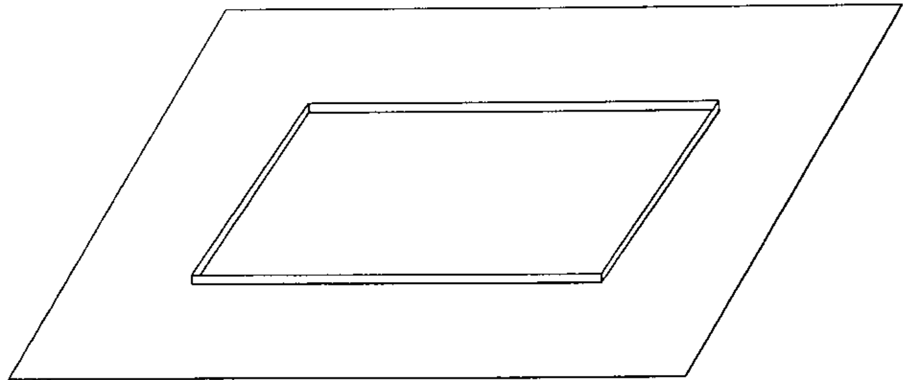


Figure 2.3 Diagram showing the method used to produce interactions for video analysis

occurred were taken using a Mitsubishi P61-8 video copy processor, attached to the video recorder.

CHAPTER 3

PHYSIOLOGY AND NUTRITION OF MYCOPARASITIC PYTHIUM SPECIES

The genus *Pythium*, as stated in the introduction, includes six mycoparasitic species:- *P. oligandrum*, *P. mycoparasiticum*, *P. nunn*, *P. acanthicum*, *P. periplocum* and, most recently recognised as a mycoparasitic species, *P. acanthophoron*. Even though work on the physiology and nutrition of *P. oligandrum* has been widely reported, little is known about these aspects for the other mycoparasitic species, especially *P. acanthophoron*, compared to *P. oligandrum*. Any differences in the physiology of the mycoparasitic pythia might be exploitable for biocontrol purposes.

Included in this chapter are studies on the physiology of *P. acanthophoron* in comparison with other mycoparasitic pythia, work to increase the available information on physiology and nutrition of *P. mycoparasiticum* compared to *P. oligandrum*, and also attempts to optimise the production of oospores of these fungi.

3.1 Nitrogen and thiamine requirements

Leonian & Lilly (1938) and Riding *et al.* (1969) investigated the nutritional requirements of several *Pythium* spp. including several mycoparasitic pythia. *P. oligandrum* (Leonian & Lilly, 1938) and *P. acanthicum* (Child *et al.*, 1969a) were reported to require an organic nitrogen source. Foley & Deacon (1986a) reported differences between the mycoparasitic and non-mycoparasitic *Pythium* species with regards to their nutrition. Mycoparasitic *Pythium* spp. required an organic nitrogen source and an exogenous thiamine source or one of its components, whilst the non-mycoparasites were able to utilise an inorganic nitrogen source and were self-sufficient for thiamine. Ridings *et al.* (1969) found that whilst *P. oligandrum*, *P. acanthicum* and *P. periplocum* required the pyrimidine moiety of thiamine, *P. acanthophoron* was self-sufficient for thiamine. The nutritional requirement of *P. nunn* was reported by Laing (1989) to be more characteristic of the phytopathogenic pythia than the mycoparasitic *Pythium* spp., in that it is able to utilise inorganic nitrogen such as nitrate and was self sufficient for thiamine.

3.1.1 Nitrogen requirement in liquid culture

The method of Foley & Deacon (1986a) was employed to test the nitrogen requirements of *P. acanthophoron* IMI 330 382 compared to two known mycoparasites, *P. oligandrum* (isolate P14.1.2) and *P. mycoparasiticum* (isolate AR7A) and one known plant pathogen, *P. ultimum* (isolate G39). Medical flats (capacity 300 ml) contained 12 ml of the nitrogen-free basal medium containing salts but no vitamins (Section 2.1), supplemented with glucose (20 gl^{-1}) as a carbon-source and $NaNO_3$ (2 gl^{-1}), $(NH_4)_2SO_4$ (1.51 gl^{-1}), L-asparagine (1.51 gl^{-1}) or Oxoid Yeast Extract (0.5 gl^{-1}) as potential nitrogen sources. The flats were inoculated with the fungi and incubated at 25°C as explained in Section 2.4.1. The heat-labile supplement, L-asparagine, was filter-sterilised and added to the autoclaved basal medium. The mycelial dry weights after 14 days incubation are shown in Table 3.1. A one-way analysis of variance was carried out on the results for each isolate.

None of the isolates grew in the nitrogen-free control, whilst all the isolates grew in the medium containing yeast extract. *P. ultimum* grew well with either sodium nitrate or L-asparagine as a nitrogen source, confirming the findings of Foley & Deacon (1986a) that it was able to utilise inorganic nitrogen as its sole nitrogen source. For this fungus there was no significant difference in the mycelial dry weight between sodium nitrate, L-asparagine or yeast extract. *P. ultimum* did not however utilise $(NH_4)_2SO_4$ as its nitrogen source, unlike the findings of Foley (1983) who reported that *P. ultimum* was able to grow in the presence of all inorganic nitrogen sources tested including ammonium sulphate. None of the three mycoparasites was able to utilise the inorganic nitrogen sources, sodium nitrate or ammonium sulphate. Unlike *P. oligandrum* and *P. mycoparasiticum*, *P. acanthophoron* was able to grow using L-asparagine as a nitrogen source without the addition of any other growth factors or vitamins. The addition of sodium nitrate to the yeast extract did not increase the growth of any mycoparasite, indicating that either they could not utilise inorganic nitrogen or yeast extract provided an optimal nitrogen source.

3.1.2 Thiamine requirement in liquid culture

The thiamine requirements of the mycoparasites *P. acanthophoron* (IMI 330 382), *P. oligandrum* (P14.1.2) and *P. mycoparasiticum* (AR7A) were compared to that of *P. ultimum* (G39) using a similar method to that above. The basal nitrogen-free medium was variously supplemented with glucose (20 gl^{-1}) as a carbon source, L-asparagine (1.51 gl^{-1}) as the nitrogen source and either thiamine

Table 3.1 Comparison of the mycelial dry weight (mg) of *Pythium* species after 14 days in glucose-mineral salts liquid culture with different nitrogen sources (mean and SEM for 5 replicates *).

Supplements to basal medium 2*	*P.oligandrum* (P14.1.2)	*P.acanthophoron* (IMI 330 382)	*P.mycoparasiticum* (AR7A)	*P.ultimum* (G39)
Control	1.0 ± 0.3^{a}	0.5 ± 0.4^{a}	0.6 ± 0.3^{a}	0.5 ± 0.5^{a}
$NaNO_3$	1.2 ± 0.5^{a}	0.4 ± 0.4^{a}	0.3 ± 0.3^{a}	28 ± 4.3^{b}
$(NH_4)_2SO_4$	1.2 ± 0.4^{a}	0.4 ± 0.7^{a}	0.4 ± 0.5^{a}	1.3 ± 0.8^{a}
Asparagine	1.0 ± 0.3^{a}	19.2 ± 3.1^{c}	0.6 ± 0.5^{a}	33.1 ± 2.6^{b}
Yeast extract	15.9 ± 0.8^{b}	18.7 ± 0.8^{c}	17.0 ± 1.9^{b}	24.3 ± 1.3^{b}
Yeast extract & $NaNO_3$	14.5 ± 0.6^{b}	12.0 ± 2.5^{b}	5.2 ± 1.9^{a}	32.7 ± 2.4^{b}

* Values, within a column, not followed by the same letter differ significantly ($P < 0.01$) by one way analysis of variance.

*2 The basal medium contained glucose and mineral salts without nitrogen or vitamins.

hydrochloride (100 μgl^{-1}) or yeast extract (0.5 gl^{-1}). The supplements, L-asparagine and thiamine, were filter-sterilised and added to the autoclaved basal medium. A one-way analysis of variance was carried out on the results, shown in Table 3.2.

No fungus grew in the presence of glucose alone (nitrogen-free medium) or glucose and thiamine alone. Also, the fungi grew poorly, if at all, in the presence of L-asparagine and thiamine (glucose-free), indicating that L-asparagine could not supply a suitable carbon-source for growth. In contrast, all grew well in the presence of glucose plus yeast extract, and often equally well in the presence of glucose, asparagine and thiamine. Omission of thiamine (glucose and asparagine medium) enabled only *P. ultimum* and *P. acanthophoron* to grow. Thus it seems that *P. oligandum* and *P. mycoparasiticum* require thiamine, whereas *P. acanthophoron* is self-sufficient for thiamine.

3.1.3 Comparison of the nitrogen and thiamine requirement of another P. acanthophoron *isolate*

To test if the nutritional requirements of isolate IMI 330 382 of *P. acanthophoron* were similar to those of the original (Sideris's) isolate of *P. acanthophoron* (CMI 337.29), the same method as above was used, with glucose (20 gl^{-1}) as a carbon source, either sodium nitrate (2 gl^{-1}) or L-asparagine (1.51 gl^{-1}) as the nitrogen source, and in medium with or without thiamine hydrochloride (100 μgl^{-1}). The results are shown in Table 3.3. Again, a one-way analysis of variance was carried out on the results.

Neither isolate grew to any significant degree in the presence of nitrate as sole nitrogen source. Both isolates could utilise L-asparagine as sole nitrogen source, and their growth in asparagine supplemented medium was not significantly enhanced by addition of thiamine.

Thus both isolates of *P. acanthophoron* seem to require an organic nitrogen source, but to be self-sufficient for thiamine.

3.1.4 Nitrogen and thiamine requirements of a range of isolates of P. oligandrum

A range of soil-derived isolates identified as *P. oligandrum* were tested for their nitrogen and thiamine requirements compared with *P. oligandrum* (P14.1.2) and *P. acanthophoron* (IMI 330 382). The same method as above was used, with the basal medium supplemented with glucose (20 gl^{-1}), sodium nitrate (2 gl^{-1}) or L-asparagine

Table 3.2 Comparison of the mycelial dry weight (mg) of *Pythium* species after 14 days in mineral-salts medium containing different supplements (mean and SEM for 5 replicates*).

Supplements to basal medium *2	*P.oligandrum* (P14.1.2)	*P.acanthophoron* (IMI 330 382)	*P.mycoparasiticum* (AR7A)	*P.ultimum* (G39)
Glucose	0.8 ± 0.5^{a}	2.0 ± 0.5^{a}	1.0 ± 0.4^{a}	1.0 ± 0.2^{a}
Glucose & thiamine	0.2 ± 0.2^{a}	1.6 ± 0.4^{a}	1.2 ± 0.4^{a}	0.2 ± 0.2^{a}
Glucose & asparagine	2.6 ± 0.9^{a}	26.6 ± 5.5^{b}	1.2 ± 1.2^{a}	47 ± 7.7^{c}
Glucose, asparagine & thiamine	12.0 ± 0.5^{b}	32.6 ± 3.4^{b}	11.2 ± 2.2^{b}	50 ± 4.5^{c}
Asparagine & thiamine	1.2 ± 0.4^{a}	6.4 ± 0.6^{a}	0.8 ± 0.2^{a}	4.4 ± 1.5^{a}
Yeast extract & glucose	17.4 ± 2.1^{c}	34.0 ± 3.9^{b}	15.4 ± 2.4^{b}	31 ± 0.4^{b}
Yeast extract	1.2 ± 0.4^{a}	4.8 ± 1.3^{a}	2.0 ± 1.0^{a}	9.2 ± 2.2^{a}

* Values, within a column, not followed by the same letter differ significantly ($P < 0.01$) by one way analysis of variance.

*2 The basal medium contained mineral salts without a nitrogen or carbon source.

Table 3.3 Comparison of the mycelial dry weight (mg) of two isolates of *P. acanthophoron* after 14 days in glucose-mineral salts liquid culture supplemented with different nitrogen sources and thiamine (mean and SEM of 5 replicates*).

Supplements to basal medium *2	Isolate IMI 330 382	Isolate CBS 337.29
Control	0.6 ± 0.4^a	0.6 ± 0.4^a
$NaNO_3$	0.3 ± 0.2^a	0.6 ± 0.3^a
Asparagine	21.6 ± 2.1^b	34.6 ± 4.0^b
Asparagine & thiamine	31.0 ± 7.8^b	36.6 ± 6.2^b

* Values, within a column, not followed by the same letter differ significantly ($P < 0.01$) by one way analysis of variance.

*2 The basal medium contained glucose and mineral salts, without a nitrogen source.

(1.51 gl^{-1}) as a nitrogen source, and with or without thiamine hydrochloride (100 μgl^{-1}). The growth, for each isolate, after 14 days incubation at 25°C is summarised in Table 3.4.

None of the soil isolates, *P. acanthophoron* (IMI 330 382) or *P. oligandrum* (P14.1.2) grew in the presence of sodium nitrate as a sole nitrogen source, whilst all isolates grew in the presence of L-asparagine supplemented with thiamine. Only *P. acanthophoron* (IMI 330 382) grew without the addition of thiamine. Thus all the soil isolates identified as *P. oligandrum* behaved in the same way as the *P. oligandrum* isolate P14.1.2.

3.2 Utilisation of mannitol as a carbon source

A major physiological difference between mycoparasitic and non-mycoparasitic pythia is reported to be the ability of mycoparasitic *Pythium* spp. to utilise mannitol as a carbon source (Foley & Deacon, 1986a; McQuilken *et al.*, 1992a). Child *et al.* (1969a) found that a mycoparasitic isolate of *P. acanthicum* grew well on mannitol if cholesterol was present in the medium, although cholesterol was not incorporated into the medium used by Foley & Deacon (1986a). Addition of sterols has also been reported to stimulate the growth of both mycoparasitic and non-mycoparasitic *Pythium* species in artificial medium with glucose as a carbon source (Hendrix, 1964, 1965; Lenny & Klemmer, 1966; Child *et al.*, 1969a). Calcium alone or in combination with cholesterol has also been reported to increase the utilisation of glucose by a non-mycoparasitic species, *Pythium graminicola* (Lenny & Klemmer, 1966). Some of these factors were investigated here.

3.2.1 Utilisation of mannitol compared to glucose as a carbon source

A similar method to that the previous section was used to investigate the utilisation of mannitol compared to glucose by three mycoparasitic *Pythium* spp. - *P. oligandrum* (P14.1.2), *P. mycoparasiticum* (AR7A) and *P. acanthophoron* (IMI 330 382)- and by the plant pathogen *P. ultimum* (G39). The mineral salts basal medium was supplemented with either glucose (20 gl^{-1}) or mannitol (20 gl^{-1}), with L-asparagine (1.51 gl^{-1}) as nitrogen source and thiamine hydrochloride (100 μgl^{-1}). The mycelial dry weights after 14 days are shown in Table 3.5. Data were analysed by one-way analysis of variance.

Table 3.4 Summary of nitrogen and thiamine requirements of a range of *P. oligandrum* isolates compared to *P. oligandrum* (P14.1.2) and *P. acanthophoron* (IMI 330 382).*

Isolate	$NaNO_3$	L-Asparagine	L-Asparagine & Thiamine
P. acanthophoron IMI 330 382	0	++	+++
P. oligandrum P14.1.2	0	0	+++
P. oligandrum FcB.2.4	0	-/+	+++
P. oligandrum P9.1.3	0	-/+	+++
P. oligandrum Fc9.2.3	0	-/+	+++
P. oligandrum Bc3.4.1	0	-/+	+++
P. oligandrum Bp9.2.2	0	-/+	+++
P. oligandrum BcB.8.3	0	-/+	+++

* 0, No growth; -/+, very slight growth; ++, considerable growth; +++, abundant growth.

Table 3.5 Comparison of the growth of four *Pythium* spp. in asparagine-mineral salts liquid culture, with glucose or mannitol as a carbon source (means of 5 replicates ± SEM).

Supplements to basal medium *2	Mycelial dry weight (mg) *			
	P.oligandrum (P14.1.2)	*P. acanthophoron* (IMI 330 382)	*P mycoparasiticum* (AR7A)	*P. ultimum* (G39)
None	2.2 ± 0.3^{a}	6.0 ± 1.3^{a}	5.3 ± 1.1^{a}	5.2 ± 0.9^{a}
Glucose	12.0 ± 0.9^{b}	15.0 ± 2.3^{b}	16.2 ± 1.6^{c}	56.2 ± 1.5^{b}
Mannitol	2.9 ± 0.8^{a}	8.6 ± 1.5^{a}	10.8 ± 1.6^{b}	3.3 ± 1.2^{a}

* Values, within a column not followed by the same letter differ significantly ($P < 0.05$) by one way analysis of variance.

*2 Basal medium contained mineral salts, L-asparagine and thiamine without a sugar source.

Compared with the small amount of growth in the unsupplemented medium, only *P. mycoparasiticum* made significantly more growth using mannitol as a carbon source. However, the growth with mannitol was significantly less than with glucose. These results differ from the findings of Foley & Deacon (1986a), that *P. oligandrum*, *P. acanthicum* and *P. periplocum* all grew on mannitol as sole carbon source.

3.2.2. The effects of cholesterol and calcium on the utilisation of mannitol and glucose

Effects of calcium and cholesterol on utilisation of mannitol and glucose were investigated by similar methods to those above, using two isolates of *P. oligandrum* (P14.1.2 and F9.2.3), two of *P. acanthophoron* (IMI 330 382 and CBS 337.29) two of *P. mycoparasiticum* (AR7A and AR5A), and one of *P. ultimum* (G39). The basal medium was supplemented with either glucose (20 gl^{-1}) or mannitol (20 gl^{-1}), and with L-asparagine (1.41 gl^{-1}) and thiamine hydrochloride (100 μgl^{-1}). Treatments included the addition of calcium chloride (55 mgl^{-1}) and cholesterol (60 mgl^{-1}), alone or in combination. Cholesterol was dissolved in hot ethanol and added to hot medium immediately after sterilising. The maximum amount of alcohol was never greater than 0.5 ml per 100 ml of medium. The mycelial dry weights after 14 days incubation at 25°C for glucose are shown in Table 3.6 and for mannitol in Table 3.7. For each isolate a one-way analysis of variance was carried out.

All isolates grew significantly better with glucose than in glucose-free medium (Table 3.6), and calcium supplementation had no effect on growth in the glucose medium by any of the fungi. The addition of cholesterol, or cholesterol plus calcium, also had little effect on several isolates (*P. oligandrum* P14.1.2, *P. acanthophoron* IMI 330 382 and CBS 337.29 and *P. ultimum* G39) but variable effects were found with the other isolates. Growth of *P. oligandrum* (F9.2.3) was slightly but significantly enhanced by the addition of cholesterol, but not by addition of cholesterol and calcium. The growth of both *P. mycoparasiticum* isolates was significantly decreased by the addition of cholesterol or cholesterol plus calcium to the glucose-containing medium, such that there was no significant increase in growth compared with that in the glucose-free control. Since cholesterol was dissolved in ethanol before addition to the medium, the effect of ethanol alone was tested. Even at the low level used (0.5 ml 100 ml^{-1} medium), ethanol was found to suppress growth of *P. mycoparasiticum* (isolates AR7A and AR5A) in glucose-containing medium, but had no effect on the isolates of *P. oligandrum, P. acanthophoron* or *P. ultimum* (results not tabulated).

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Table 3.6 Comparison of the effect of calcium and cholesterol on the growth of *Pythium* spp. in asparagine-salts-thiamine liquid medium with glucose as carbon-source (means of 5 replicates with SEM in parentheses).

Supplements to basal medium *2	Mycelial dry weight (mg) *						
	P. oligandrum		*P. acanthophoron*		*P mycoparasiticum*		*P. ultimum*
	(P14.1.2)	(F.9.2.3)	(IMI 330 382)	(CBS 337.29)	(AR7A)	(AR5A)	(G39)
None	2.4^{a} (0.6)	6.4^{a} (0.7)	7.2^{a} (1.2)	7.8^{a} (0.5)	6.8^{a} (1.3)	1.6^{a} (0.6)	5.0^{a} (0.5)
Glucose	13.6^{b} (1.1)	24.6^{b} (0.9)	14.6^{b} (0.7)	31.2^{b} (4.2)	17.0^{b} (1.6)	15.4^{b} (1.6)	52.0^{b} (7.2)
Glucose & calcium	12.8^{b} (0.5)	26.0^{b} (2.3)	16.4^{b} (1.4)	30.4^{b} (4.3)	18.0^{b} (2.5)	17.0^{b} (2.1)	62.8^{b} (7.0)
Glucose & cholesterol	13.0^{b} (3.2)	34.8^{c} (1.2)	17.4^{b} (0.8)	29.0^{b} (2.4)	10.0^{a} (0.5)	5.6^{a} (1.1)	63.6^{b} (7.3)
Glucose, calcium & cholesterol	15.4^{b} (1.4)	26.4^{b} (2.1)	15.8^{b} (0.6)	36.0^{b} (3.6)	9.8^{a} (1.4)	4.6^{a} (1.0)	67.4^{b} (6.5)

* Values, within a column not followed by the same letter differ significantly ($P < 0.01$) by one way analysis of variance.

*2 Basal medium contained mineral salts, L-asparagine and thiamine.

Table 3.7 Comparison of the effects of calcium and cholesterol on the growth of *Pythium* spp. in asparagine-salts-thiamine liquid medium with mannitol as carbon source (mean of 5 replicates with SEM in parentheses).

Supplements to basal medium *2	Mycelial dry weight (mg)*						
	P. oligandrum		*P. acanthophoron*		*P mycoparasiticum*		*P. ultimum*
	(P14.1.2)	(F.9.2.3)	(IMI 330 382)	(CBS 337.29)	(AR7A)	(AR5A)	(G39)
None	1.6[a] (0.3)	4.2[a] (1.9)	7.6[a] (0.7)	7.4[a] (1.2)	6.2[a] (0.7)	2.6[a] (2.3)	4.8[a] (0.7)
Mannitol	2.2[a] (0.5)	10.6[b] (2.3)	8.2[a] (1.1)	9.0[ab] (1.0)	11.4[b] (1.0)	10.0[b] (1.3)	5.0[a] (1.3)
Mannitol & calcium	2.8[ab] (1.0)	15.0[b] (1.2)	13.4[b] (1.6)	13.4[ab] (1.8)	15.8[c] (1.4)	16.2[c] (0.8)	5.6[a] (1.7)
Mannitol & cholesterol	6.0[bc] (1.6)	12.0[b] (0.4)	7.2[a] (1.5)	12.0[ab] (2.2)	5.2[a] (1.3)	10.2[b] (1.2)	9.6[ab] (1.4)
Mannitol, calcium & cholesterol	7.0[c] (0.8)	12.8[b] (1.8)	13.6[b] (1.4)	14.4[b] (1.0)	16.2[c] (1.0)	14.0[bc] (1.0)	15.2[b] (0.9)

* Values, within a column not followed by the same letter differ significantly ($P < 0.01$) by one way analysis of variance.

*2 Basal medium contained mineral salts, L-asparagine and thiamine.

In a parallel experiment to that above, mannitol alone was a less satisfactory carbon source for all fungi (Table 3.7) than was glucose (Table 3.6). Only *P. oligandrum* (F9.2.3), *P. mycoparasiticum* (AR7A) and *P. mycoparasiticum* (AR5A) showed significantly more growth in the presence of mannitol alone than in the mannitol-free medium. The addition of calcium further increased the growth of the isolates on mannitol and also promoted growth of *P. acanthophoron* (IMI 330 382 and CBS 337.29) but not of *P. oligandrum* (P14.1.2) or *P. ultimum* (G39). Addition of cholesterol enabled *P. oligandrum* (P14.1.2) to make significant growth on mannitol, but suppressed the growth of *P. mycoparasiticum* (AR7A) on mannitol, as it did in the glucose-supplemented medium. Addition of both calcium and cholesterol also enabled *P. oligandrum* (P14.1.2) to grow on mannitol. It is difficult to summarise all the effects because of the variations in responses of the *Pythium* spp. or even of isolates within the species. However, the following general points (with the noted exceptions) were observed.

1. All the isolates of all species utilised glucose as a major or sole carbon source (a slight amount of growth sometimes being found in glucose-free medium, perhaps occurring at the expense of asparagine as a combined carbon and nitrogen source).

2. Growth on glucose was seldom significantly enhanced by supplements of calcium, cholesterol or calcium plus cholesterol. On the contrary, growth of some isolates (*P.mycoparasiticum* AR7A and AR5A) on glucose was sometimes suppressed by addition of cholesterol. This effect was, at least partly, attributable to the use of ethanol as a solvent for cholesterol.

3. All fungi made conspicuously poorer growth on mannitol alone compared with glucose alone, and no significant growth on mannitol alone was observed for *P. oligandrum* (P14.1.2), *P. acanthophoron* (IMI 330 382 and CBS 337.29) and *P. ultimum*.

4. The only significant growth of *P. ultimum* on mannitol was observed in the presence of calcium plus cholesterol, and even then this fungus grew poorly compared with on glucose.

5. In contrast to *P. ultimum*, all isolates of the mycoparasites utilised mannitol to some degree when it was supplied alone or in the presence of a single supplement of calcium or cholesterol. In several cases a calcium supplement significantly enhanced the growth on mannitol, and the addition of cholesterol plus calcium had no

greater effect than addition of calcium alone. In some cases (*P. oligandrum* isolate P14.1.2) cholesterol was necessary for utilisation of mannitol, but in other cases (*P. acanthophoron* IMI 330 382, *P. mycoparasiticum* AR7A) cholesterol tended to be inhibitory to growth, presumably owing to the effect of ethanol mentioned above.

3.2.3 Effects of different sterols on the utilisation of mannitol

The same method as above was used, with the basal medium supplemented with mannitol (20 gl^{-1}), L-asparagine (1.4 gl^{-1}), and thiamine hydrochloride (100 μgl^{-1}), and with one of the following sterol sources: cholesterol (60 mgl^{-1}), ergosterol (60 mgl^{-1}) or β-sitosterol (60 mgl^{-1}). The sterols were dissolved in hot ethanol and added to the hot medium immediately after sterilising. The mycelial dry weights after 14 days are shown in Table 3.8. A one-way analysis of variance was carried out for each isolate.

For *P. oligandrum* (P14.1.2) only ergosterol gave a significant increase in growth compared to the sterol-free control. For *P. acanthophoron* (IMI 330 382) no sterol gave significantly higher growth than in the control. For *P. mycoparasiticum* (AR7A) only β-sitosterol gave a significant increase in growth, but both ergosterol and cholesterol significantly decreased the growth of this fungus. Thus the different sterols had different effects on growth and utilisation of mannitol by the mycoparasitic isolates tested.

3.2.4 Effect of calcium and cholesterol supplements on the growth rate of P. oligandrum *in glucose mineral medium*

The effect of the addition of calcium and (or) cholesterol on the growth rate of *P. oligandrum* isolate P14.1.2 in glucose mineral medium was tested using a similar method to the above. The basal medium was supplemented with glucose (20 gl^{-1}), L-asparagine (1.41 gl^{-1}) and thiamine hydrochloride (100 μgl^{-1}); in different treatments it was supplemented with (1) calcium chloride (55 mgl^{-1}), (2) cholesterol (60 mgl^{-1}) or (3) both calcium chloride and cholesterol at the stated concentrations. The mycelial dry weight was assessed after 1, 2, 3, 7, 10 and 14 days (Figure 3.1) and a one-way analysis of variance was used to compare the different treatments at each time.

From Figure 3.1 (data tabulated in Appendix 1), it is seen that calcium and, to a lesser extent, cholesterol significantly stimulated the early growth rate of *P. oligandrum* compared to that in the glucose-asparagine-mineral medium control. The

Table 3.8 Comparison of the effect of different sterols on the growth of mycoparasites in mineral nutrient liquid culture with mannitol as carbon source (mean of 5 replicates ± SEM).

Supplements to basal medium *2	Mycelial dry weight (mg)*		
	Pythium mycoparasiticum (AR7A)	*Pythium acanthophoron* (IMI 330 382)	*Pythium oligandrum* (P14.1.2)
None	12.0 ± 0.9^{a}	9.6 ± 1.0^{ab}	4.6 ± 0.9^{a}
Ergosterol	3.0 ± 0.9^{b}	8.8 ± 1.0^{a}	15.0 ± 2.3^{b}
β-sitosterol	22.8 ± 0.7^{c}	14.0 ± 1.8^{b}	9.6 ± 1.4^{ab}
Cholesterol	6.0 ± 1.1^{b}	8.6 ± 1.0^{a}	9.2 ± 2.1^{ab}

* Values, within a column not followed by the same letter differ significantly ($P < 0.01$) by one way analysis of variance.

*2 Basal medium contained mineral salts, mannitol, L-asparagine and thiamine.

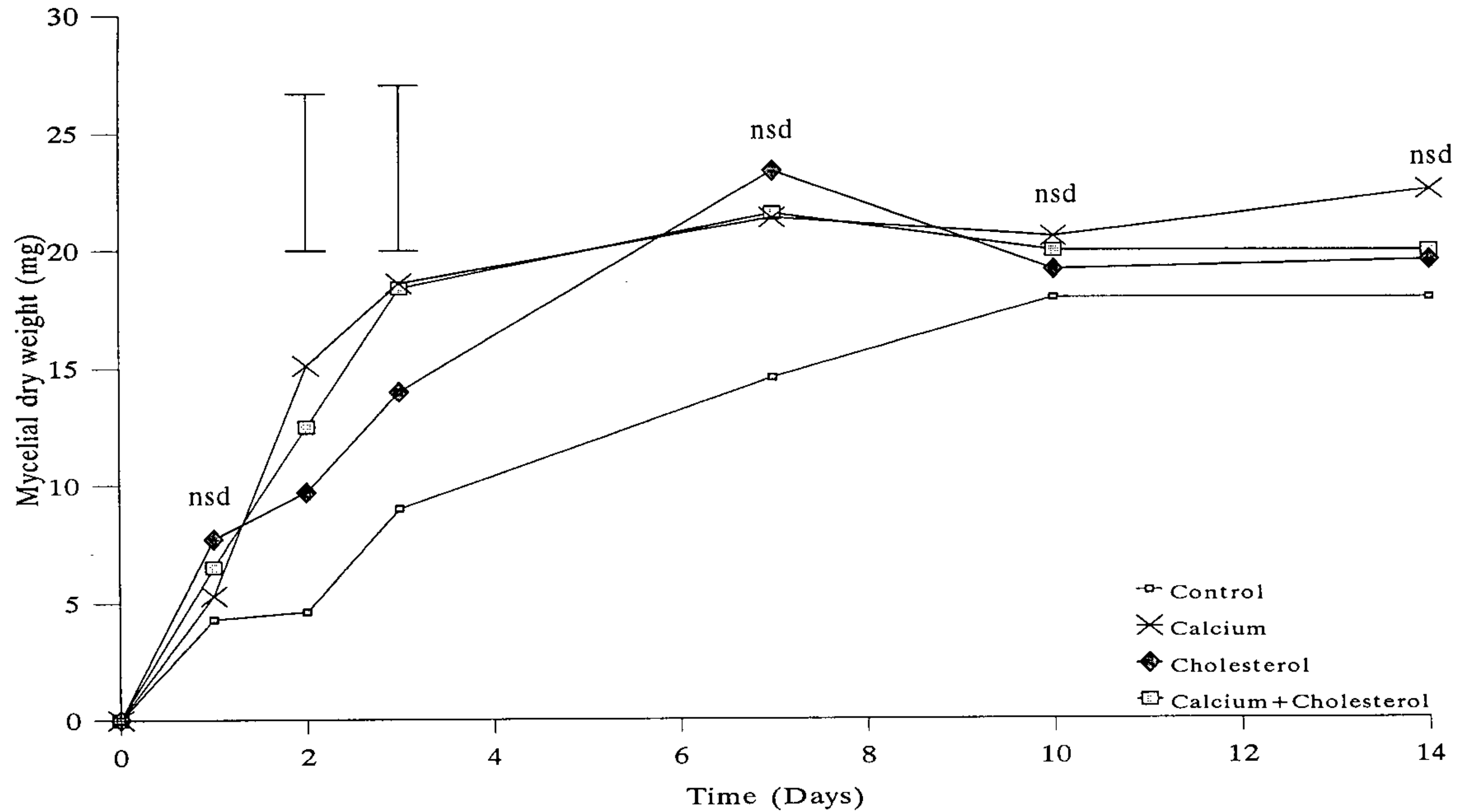


Figure 3.1 The effect of calcium and cholesterol on the mycelial dry weight (mg) of *P. oligandrum* grown in glucose-asparagine mineral medium after various times; means of 5 replicates with the bars representing L. S. D. at $P < 0.01$ (nsd = no significant difference).

addition of calcium with cholesterol had no greater effect than calcium alone. But there was evidently a limit on the amount of growth in the conditions of this experiment, such that the ultimate yield of mycelium (15 - 20mg) was similar irrespective of the supplement used.

3.3 Growth of P. oligandrum and P. mycoparasiticum in undefined liquid media

In all the defined liquid media used for nutritional studies the growth of the mycoparasites was sparse. The growth and oospore production in undefined liquid media were now investigated.

P. oligandrum (P14.1.2) and *P. mycoparasiticum* (AR7A) were grown in 300 ml capacity medical flats, each containing 12 ml of the following media (Section 2.1): 3% cane molasses (McQuilken *et al.*, 1990a), carrot extract (Walther & Gindrat, 1987a), sunflower seed extract, and potato-dextrose, using the method described in Section 2.4.1. At harvest (14 days) half the flats were used for determining biomass dry weights and half for oospore counts. For oospore counts the biomass from each flat was homogenised in 1 ml of distilled water, using an Ultraturrax T25 blender at 20,500 rpm for 60 sec. The oospore number in the resulting suspension was determined using a haemocytometer, and the counts were converted to total oospore number in each flat. The biomass dry weights were determined after oven-drying.

As shown in Table 3.9, growth of both fungi was significantly greater in potato-dextrose than in the other three media. When the data for potato-dextrose were omitted and the remaining data subjected to analysis of variance then growth in 3% molasses was greater than in carrot extract or sunflower seed extract for both fungi. Negligible numbers of oospores were formed in potato-dextrose, however; for both fungi the numbers of oospores were significantly highest in molasses medium.When the oospore counts were expressed on the basis of mycelial dry weight then oospore production by both fungi was again highest in molasses medium, though sometimes only marginally so.

In similar conditions to those above, potato-dextrose was supplemented with β - sitosterol (60 mgl^{-1}), but this had no effect on mycelial dry weight nor production of oospores by *P. oligandrum* or *P. mycoparasiticum*; the data for the sterol-supplement treatment only are shown at the foot of Table 3.9.

Table 3.9 Comparison of the mycelial dry weight (mg) and oospore production (x10^5) for *P. oligandrum* and *P. mycoparasiticum* after 14 days in undefined liquid media (means for 5 replicates ± SEM).*

Medium	*P. oligandrum* (P14.1.2)			*P. mycoparasiticum* (AR7A)		
	Mycelial dry weight (mg)	Oospore number (x10^5)	Oospores (x10^5)/mg mycelial dry weight	Mycelial dry weight (mg)	Oospore number (x10^5)	Oospores (x10^5)/mg mycelial dry weight
Carrot extract	19.5 ± 2.1^{a}	3.9 ± 0.9^{b}	0.20	19.1 ± 0.9^{a}	4.3 ± 0.3^{c}	0.23
Molasses (3%)	31.0 ± 0.8^{a}	9.9 ± 0.6^{c}	0.32	27.3 ± 0.9^{b}	8.2 ± 0.3^{d}	0.30
Sunflower seed extract	16.2 ± 0.6^{a}	4.4 ± 0.6^{b}	0.27	14.7 ± 0.3^{a}	1.2 ± 0.2^{b}	0.08
Potato-dextrose	81.4 ± 11.3^{b}	0.36 ± 0.15^{a}	0.004	51.0 ± 3.3^{c}	0.04 ± 0.03^{a}	0.0008
Potato-dextrose & β-sitosterol*2	71.2 ± 1.3^{b}	0.8 ± 0.8^{a}	0.01	45.3 ± 5.2^{b}	0.04 ± 0.03^{a}	0.0009

* Values, within a column not followed by the same letter differ significantly ($P < 0.01$) by one way analysis of variance.

*2 Data from a separate experiment.

A further experiment compared growth and oospore production in sunflower seed extract, potato-dextrose, or the combination of both at full strength (Table 3.10). Good growth was obtained in both potato-extract and potato-extract supplemented with sunflower seed extract, but oospore numbers were low in both cases compared with oospore numbers in sunflower seed extract alone.

The results from this set of experiments thus indicate that molasses is the best medium of those used for oospore production. Potato-dextrose, even when supplemented with β - sitosterol or sunflower seed extract, did not support oospore production, perhaps because it supported abundant mycelial growth whereas oospore production occurs in conditions not conducive to good vegetative growth.

In order to investigate this, in the following experiments different sterols were added to potato-extract or potato-dextrose, to try to promote oospore production. Incubation times were extended until it was expected that nutrients would be depleted.

3.3.1 Effects of various supplements to potato-extract medium on oospore production and growth.

P. oligandrum (P14.1.2) was grown in potato-extract and potato-extract plus dextrose media supplemented with a range of sterols or with molasses. As before, the sterols were dissolved in hot ethanol and added to hot medium immediately after sterilising. For the molasses plus potato-extract medium 6 ml of each was used, such that both components were at half the strength normally used. Mycelia were harvested after 14 and 28 days to investigate the effect of culture time on oospore production and mycelial dry weight (Table 3.11). Statistical analysis was by one-way analysis of variance.

After 14 days, the mycelial dry weight in potato-dextrose alone or with any sterol supplement was significantly higher than that in potato-extract (dextrose-free). None of the sterol supplements increased the amount of growth in potato-dextrose, and similarly the addition of cholesterol to potato-extract did not enhance growth (no other sterol was tested in this medium). The amount of growth in potato-extract plus molasses was similar to that in potato-extract or molasses alone. The same pattern of results to those after 14 days was observed after 28 days. But, by this time the fungal dry weights had declined compared with the 14 day values in media where growth had been best (potato-dextrose with or without sterols), indicative of autolysis. The decline in weight was less in the sterol-supplemented media than in potato-dextrose

Table 3.10 Comparison of the mycelial dry weight (mg) and oospore production ($x10^5$) for *P. oligandrum* and *P. mycoparasiticum* after 14 days 25°C in potato-dextrose medium and (or) sunflower extract medium (means for 5 replicates ± SEM).*

Medium	*P. oligandrum* (P14.1.2)			*P. mycoparasiticum* (AR7A)		
	Mycelial dry weight (mg)	Oospore number ($x10^5$)	Oospores ($x10^5$)/mg mycelial dry weight	Mycelial dry weight (mg)	Oospore number ($x10^5$)	Oospores ($x10^5$)/mg mycelial dry weight
Sunflower seed extract	15.1 ± 0.4[a]	3.3 ± 0.6[b]	0.22	15.0 ± 0.4[a]	2.4 ± 0.6[b]	0.16
Potato-dextrose	79 ± 10.6[b]	0.02 ± 0.01[a]	0.0003	45.2 ± 1.6[b]	0.005 ± 0.005[a]	0.0001
Sunflower seed extract & potato dextrose	99.5 ± 6.3[b]	0.29 ± 0.07[a]	0.003	47.5 ± 0.7[b]	0.005 ± 0.005[a]	0.0001

* Values, within a column, not followed by the same letter differ significantly ($P < 0.01$) by one way analysis of variance.

Table 3.11 Comparison of the mycelial dry weight (mg) and oospore production (x10^5) for *P. oligandrum* after 14 and 28 days at 25°C in potato-extract medium with different supplements (means for 5 replicates ± SEM).*

Medium	14 days			28 days		
	Mycelial dry weight (mg)	Oospore number (x10^5)	Oospores(x10^5) /mg mycelial dry weight	Mycelial dry weight (mg)	Oospore number (x10^5)	Oospores (x10^5) /mg mycelial dry weight
Potato-dextrose	64.8 ± 8.6^{b}	0^{a}	0	44.2 ± 1.7^{cd}	0^{a}	0
Potato-dextrose & β-sitosterol	69.8 ± 5.7^{b}	0.04 ± 0.01^{a}	0.000	57.2 ± 2.1^{e}	0.06 ± 0.03^{a}	0.001
Potato-dextrose & cholesterol	82.8 ± 11.4^{b}	0.01 ± 0.06^{a}	0.0001	53.6 ± 6.9^{de}	0.07 ± 0.05^{a}	0.001
Potato-dextrose & ergosterol	71.8 ± 10.7^{b}	0.07 ± 0.03^{a}	0.001	57.0 ± 2.8^{e}	0.07 ± 0.03^{a}	0.001
Potato-extract	27.2 ± 3.4^{a}	0^{a}	0	18.6 ± 1.2^{a}	0^{a}	0
Potato-extract & cholesterol	31.6 ± 2.4^{a}	0.005 ± 0.005^{a}	0.0002	24.2 ± 2.2^{ab}	0.05 ± 0.03^{a}	0.002
Potato-extract & molasses	35.6 ± 1.7^{a}	6.0 ± 0.2^{b}	0.17	34.2 ± 1.0^{bc}	6.1 ± 0.7^{b}	0.18
Molasses	31.6 ± 0.6^{a}	8.8 ± 0.2^{c}	0.28	33.0 ± 1.8^{bc}	8.8 ± 0.3^{c}	0.27

* Values, within a column, not followed by the same letter differ significantly ($P < 0.01$) by one way analysis of variance.

alone, so that the fungal dry weights in sterol-supplemented media were, at 28 days, sometimes significantly higher than in potato-dextrose alone. In molasses plus potato-dextrose and in molasses medium there was no noticeable decline in fungal dry weight between 14 and 28 days. Oospore production was poor or non-existent in the potato-dextrose and potato-extract media, even when supplemented with sterols. In contrast, large numbers of oospores were formed in molasses or potato-extract plus molasses media. The oospore numbers in these media were large at 14 days, with no significant increase after 28 days.

The notable feature of these results is that potato-dextrose and potato-extract were unable to support oospore production by *P. oligandrum*, even in the presence of sterols and even when (potato-extract) mycelial growth was limited. Yet potato-extract did not suppress the production of oospores when combined with molasses. Significantly the largest number of oospores was produced in full-strength molasses medium, even though this supported as much fungal growth as did potato-extract and molasses. It seems that molasses provides nutrients not present in potato-extract and that are required for oospore production.

3.3.2 Growth and oospore production of mycoparasitic Pythium *species in molasses*

As 3% molasses had been found to support good growth and oospore production of *P. oligandrum* and *P. mycoparasiticum* (Table 3.9) its suitability for *P. acanthophoron* (CBS 337.29 and IMI 330 382) and *P. periplocum* (P9.8.4) was now tested, with a non-mycoparasite, *P. ultimum* (G39), for comparison. The mycelial dry weights and oospore numbers after 14 days incubation are shown in Table 3.12.

The five mycoparasitic isolates, representing four species, grew satisfactorily in 3% molasses, although yield of *P. ultimum* was significantly greater than for any mycoparasite. The yields of some of the mycoparasites differed significantly from one another. Large numbers of oospores were produced by *P. acanthophoron* (IMI 330 382), *P. oligandrum* (P14.1.2) and *P. mycoparasiticum* (AR7A), but not by the isolate of *P. periplocum* (P9.8.4), for reasons that are unknown. The isolate CBS 337.29 of *P. acanthophoron* did not produce oospores, but this is the original isolate of Sideris (1932) and is known to be sterile (Lodha & Webster, 1990). As a proportion of total dry weight, *P. oligandrum* and *P. mycoparasiticum* produced more oospores than did *P. acanthophoron* (IMI 330 382), indicating that they might be more efficient in converting nutrients into resting structures.

Table 3.12 Comparison of the mycelial dry weight (mg) and oospore number (10^5) of *Pythium* species after 14 days at 25°C in 3% molasses liquid culture (mean of 5 replicates with SEM).*

Pythium spp.	Total dry weight (mg)	Oospore number ($x10^5$)	Oospores ($x10^5$) / mg total dry weight
P ultimum (G39)	64 ± 3.9^{d}	N.D*2	N.D
P. acanthophoron (CBS 337.29)	42.4 ± 0.7^{c}	0	0
P. acanthophoron (IMI 330 382)	37.8 ± 3.4^{cb}	7.1 ± 0.6^{b}	0.19
P.oligandrum (P14.1.2)	30.6 ± 1.3^{bc}	9.3 ± 0.2^{c}	0.3
P. mycoparasiticum (AR7A)	29.6 ± 0.5^{ab}	8.1 ± 0.2^{bc}	0.27
P. periplocum (P9.8.4)	20.8 ± 1.5^{a}	0.4 ± 0.1^{a}	0.02

* Values, within a column, not followed by the same letter differ significantly ($P < 0.01$) by one way analysis of variance.

*2 N.D, not determined

3.3.3. Comparison of growth and oospore production of isolates of P. mycoparasiticum

Growth and oospore production of eight isolates of *P. mycoparasiticum* was studied in 3% molasses, using the same method as above (Table 3.13).

The type culture, AR7A, showed the greatest biomass production and also largest number of oospores. Most other isolates grew reasonably well and produced oospore numbers consistent with their biomass yields. However, there were two exceptions among the eight tested isolates. The isolate P3.1.5 grew poorly and produced even fewer oospores in proportion to its biomass yield. Isolate AR5A grew well but produced very few oospores.

The results indicate that there is substantial variation, at least within *P. mycoparasiticum*, in terms of ability to grow and produce oospores in a medium known to favour most mycoparasitic *Pythium* species.

3.3.4 Growth and oospore production of P. oligandrum *in molasses in static and shake cultures*

For commercial production of biocontrol inocula, shake cultures rather than static cultures might be preferred (McQuilken *et al.*, 1990a). The growth and oospore production of *P. oligandrum* (P14.1.2) in both static culture, using medical flats, and shake cultures in conical flasks on an orbital shaker, were compared. The medical flats were as used in the previous experiment. Conical flasks (250 ml capacity) containing 100 ml of 3% molasses were inoculated with a disc of *P. oligandrum* and incubated on an orbital shaker (110 rpm) at 25°C for 14 days. For the shaken cultures, approximately 10mg (fresh weight) biomass, the exact weight being recorded, was taken from each flask and homogenised in 1 ml distilled water for 60 seconds at 20,500 rpm, using an Ultraturrax T25 blender. The oospore counts were then determined using a haemocytometer. For the shake cultures the oospore number was calculated by finding the dry weight of the remaining mycelium and using this to calculate the total biomass. Knowing the oospore number in a known wet sample weight, and based on the finding that 1mg wet weight corresponds to 0.04mg dry weight when dried overnight at 80°C, an estimate of the oospore number in each flask was calculated.

As shown in Table 3.14, the shake cultures had the largest biomass dry weight, as would be expected from the larger culture volume (100 ml compared to 12 ml in

Table 3.13 Comparison of the total dry weight (mg) and oospore number ($x10^5$) of different isolates of *Pythium mycoparasiticum* after 14 days at 25°C in 3% molasses liquid medium (means of 5 replicates with SEM).*

Isolate	Total dry weight (mg)	Oospore number ($x10^5$)	Oospores ($x10^5$) / mg total dry weight
AR7A	19.8 ± 2.1^{c}	5.7 ± 0.4^{e}	0.29
AR5A	17.4 ± 0.9^{bc}	0.8 ± 0.1^{a}	0.05
PW.4.5	15.0 ± 1.5^{b}	4.4 ± 0.2^{d}	0.29
Bc9.2.3	14.2 ± 0.7^{b}	3.6 ± 0.3^{cd}	0.25
Bc9.8.3	15.4 ± 0.6^{b}	3.1 ± 0.1^{c}	0.20
BcB.2.3	15.6 ± 0.4^{bc}	3.2 ± 0.1^{c}	0.21
FcW.8.4	14.7 ± 0.6^{b}	3.9 ± 0.2^{cd}	0.27
P3.1.5	9.8 ± 0.9^{a}	1.9 ± 0.3^{b}	0.19

* Values, within a column, not followed by the same letter differ significantly ($P < 0.01$) by one way analysis of variance.

Table 3.14 Comparison of the mycelial dry weight (mg) and oospore count ($x10^5$) of *P. oligandrum* grown in 3% molasses liquid medium in static and shake cultures: means for 5 replicates with SEM.

	Mycelial dry weight (mg)	Oospores ($x10^5$)	Oospores ($x10^5$)/ mg dry weight
Shake	94.4 ± 2.8	245.2 ± 14.2	2.6
Static	23.4 ± 0.6	8.8 ± 0.2	0.4

the static culture system). However, the shake culture system also yielded more oospores per mg biomass dry weight compared to the static culture.

3.4 Radial growth on agar plates

3.4.1 Radial growth of mycoparasitic Pythium *species*

Radial growth rates of four of the six mycoparasitic *Pythium* spp. were recorded on six media (Section 2.4.2). Agar discs were placed centrally on plates of potato-dextrose agar (PDA), half-strength potato-dextrose agar (HS PDA), sunflower seed extract agar (SSA), water agar (WA), malt extract agar (MEA) and molasses agar (MA). Colony diameters were recorded after 24h and then at 24h intervals at 25°C to obtain mean radial growth rate (Table 3.15).

MEA supported the slowest radial growth for all the fungi tested on it. WA also supported slow growth of several isolates. For both *P. oligandrum* and *P. acanthophoron* isolates the fastest linear growth was recorded on PDA. There was no significant difference in the growth of *P. oligandrum* (P14.1.2) and *P. acanthophoron* (IMI 330 382) on HS PDA compared to PDA, but for *P. oligandrum* (CGH1) and *P. acanthophoron* (CBS 337.29) growth on PDA was significantly faster than on HS PDA. Both *P. acanthophoron* isolates grew at the same rate on SSA as on HS PDA, and in the case of *P. acanthophoron* (IMI 330 382) the same as on PDA and molasses.

The fastest growth of *P. mycoparasiticum* AR7A was recorded on SSA. The growth on WA being significantly less but still faster than that observed on PDA and HS PDA. For *P. mycoparasiticum* AR7A only, molasses agar supported significantly slower growth than on all media except MEA. Isolate AR5A also had the fastest linear growth on SSA; unlike isolate AR7A it grew faster on HS PDA than on both PDA and WA.

P. periplocum, grew fastest on molasses and SSA, and only marginally slower on all other media.

The results show that no single medium supports the fastest linear extension of all mycoparasitic *Pythium* spp. Also, even when the best medium for each isolate is considered, it is clear that the *Pythium* spp., and isolates within them, differed greatly in linear extension rates.

Table 3.15 Comparison of the linear growth (mm 24 h^{-1}) of mycoparasitic *Pythium* spp. on different agar media (means of 5 replicates; SEM in parentheses).*

Medium	Linear growth (mm 24 h^{-1}) *P.oligandrum* (P14.1.2)	*P.oligandrum* (CGH1)	*P. acanthophoron* (IMI 330 382)	*P. acanthophoron* (CBS 337.29)	*P mycoparasiticum* (AR7A)	*P mycoparasiticum* (AR5A)	*P. periplocum* (P9.8.4)
PDA	27.5^{d} (0.4)	28.5^{c} (0.4)	18.8^{b} (0.5)	7.0^{c} (0.3)	4.9^{c} (0.2)	2.8^{a} (0.1)	5.8^{ab} (0.3)
HS PDA	26.0^{d} (0.2)	25.3^{b} (0.4)	17.1^{b} (0.3)	5.3^{b} (0.1)	5.3^{c} (0.1)	4.3^{c} (0.2)	5.8^{ab} (0.3)
SSA	20.8^{b} (0.7)	21.5^{a} (0.5)	17.3^{b} (0.4)	5.5^{b} (0.3)	8.5^{e} (0.5)	5.9^{d} (0.1)	6.5^{bc} (0.2)
WA	17.4^{a} (0.3)	19.5^{a} (0.8)	13.8^{a} (0.6)	3.1^{a} (0.2)	6.1^{d} (0.3)	3.4^{b} (0.1)	5.6^{ab} (0.1)
MEA	16.5^{a} (0.5)	NT*2	12.8^{a} (0.3)	NT	0.3^{a} (0.04)	NT	4.9^{a} (0.2)
MA	24.1^{c} (0.9)	NT	17.6^{b} (0.7)	NT	2.6^{b} (0.1)	NT	7.7^{c} (0.6)

* Values, within a column, not followed by the same letter differ significantly ($P < 0.01$) by one way analysis of variance.

*2 NT Not tested

A comparison of seven isolates of *P. mycoparasiticum* (Table 3.16) confirmed that all grew fastest across SSA plates, and that all had relatively slow extension rates compared with *P. oligandrum* and isolate IMI 330 382 of *P. acanthophoron*. Further isolates of *P. periplocum* were not tested because only one isolate (P9.8.4) was obtained from soil in this study.

3.5 Colonisation of vermiculite

Previous experiments in this chapter suggested that *P. mycoparasiticum* grows much slower than *P. oligandrum* across agar media, but at a comparable rate to *P. oligandrum* in liquid media. It was thus of interest to compare the growth of these fungi in a particulate substratum. Vermiculite moistened with molasses medium was chosen for this; Lutchmeah & Cooke (1985) had used a mixture of vermiculite and cornmeal moistened with distilled water to grow *P. oligandrum* for seed-pelleting in biocontrol experiments.

The medium was prepared by autoclaving 10g vermiculite with 40 ml of 3% molasses solution in 250 ml conical flasks. The flasks were inoculated with *P. oligandrum* (P14.1.2), *P. mycoparasiticum* (AR7A), *P. ultimum* (G39) and *Fusarium culmorum* (CD9) for comparison. Five flasks were inoculated with each fungus, incubated at 25°C and shaken by hand each day. After different times of incubation 30 particles of vermiculite were removed aseptically from each flask and placed on each of five PDA plates supplemented with Penicillin G (100 μgml^{-1}), giving a total 150 vermiculite particles per treatment at each time. The numbers of these particles yielding colonies of the inoculated fungus were recorded (Table 3.17).

F. culmorum colonised the vermiculite most rapidly, but by 3 day sampling both *P. oligandrum* (P14.1.2) and *P. ultimum* (G39) were found to grow from all the sampled vermiculite particles. *P. mycoparasiticum* (AR7A), was somewhat slower to colonise, because it was not detected in all the sampled particles until day 7. Also of note is that *P. oligandrum*, *P. ultimum* and *F. culmorum* could be detected in all vermiculite particles sampled during 70 days incubation of the flasks, whereas the frequency of detection of *P. mycoparasiticum* declined progressively after day 14; by 50 or 70 days it was detectable in only half the sampled particles.

Table 3.16 Comparison of the linear growth (mm 24 h^{-1}) of different *Pythium mycoparasiticum* isolates on various agar media (mean of 5 replicates; SEM in parentheses).*

Medium	Linear growth (mm 24 h^{-1}) of *Pythium mycoparasiticum* isolates						
	AR7A	AR5A	Bc9.2.3	Bc9.8.3	BcB.2.3	FcW.8.4	PW.4.5
PDA	4.6^{a} (0.3)	2.8^{a} (0.1)	3.2^{a} (0.1)	4.4^{a} (0.1)	3.7^{a} (0.1)	4.9^{a} (0.1)	3.7^{a} (0.1)
HS PDA	5.4^{a} (0.3)	4.0^{b} (0.2)	4.2^{a} (0.1)	4.8^{ab} (0.1)	4.6^{b} (0.1)	5.3^{a} (0.1)	4.6^{b} (0.1)
SSA	7.8^{b} (0.2)	5.8^{c} (0.1)	7.7^{b} (0.1)	7.4^{c} (0.1)	6.9^{d} (0.2)	7.0^{c} (0.1)	6.6^{d} (0.04)
WA	4.6^{a} (0.1)	3.5^{b} (0.1)	4.1^{a} (0.7)	5.0^{b} (0.1)	5.7^{c} (0.1)	5.4^{b} (0.1)	5.2^{c} (0.2)

* Values, within a column, not followed by the same letter differ significantly ($P < 0.01$) by one way analysis of variance.

Table 3.17 Comparison of the number of vermiculite particles (max. 30 per plate), sampled from flasks during 70 days incubation, that gave rise to a colony when plated on potato dextrose agar (means of 5 replicates with SEM).

	Days incubation of flasks						
Fungus	1	3	7	14	28	49	70
P. oligandrum (P14.1.2)	0.2 ± 0.2	30 ± 0	30 ± 0	30 ± 0	30 ± 0	29.4 ± 0.6	30 ± 0
P. mycoparasiticum (AR7A)	0.2 ± 0.2	20 ± 0.7	29.6 ± 0.4	30 ± 0	21.4 ± 4.2	15.4 ± 5.5	14.4 ± 5.2
P. ultimum (G39)	1.4 ± 1.4	30 ± 0	30 ± 0	30 ± 0	30 ± 0	30 ± 0	28.6 ± 1.2
F. culmorum (CD9)	5.6 ± 2.9	30 ± 0	30 ± 0	30 ± 0	30 ± 0	30 ± 0	30 ± 0

3.6 Cellulose utilisation

Deacon (1976) reported that several, but not all, non-mycoparasitic *Pythium* species can utilise cellulose, but none of the mycoparasitic *Pythium* spp. are reported to do so (Tribe, 1966; Deacon, 1976; Foley & Deacon, 1986a; Laing & Deacon, 1990; McQuilken *et al.*, 1992a). A potential problem with some of these reports is that nitrate was supplied as sole nitrogen source, and the mycoparasitic *Pythium* spp. with the exception of *P. nunn* (Laing & Deacon, 1990) cannot use nitrate-nitrogen. The potential cellulolytic activities of *P. acanthophoron* (CBS 337.29 and IMI 330 382), *P. oligandrum* (P14.1.2 and F9.2.3), *P. mycoparasiticum* (AR7A and AR5A), *P. periplocum* (P9.8.4), *P. ultimum* (G39) and *Fusarium culmorum* (CD9) were now tested on cellulose film overlying L-asparagine-mineral salts agar. As explained in Section 2.4.4, the cellulose strips were inoculated at one end with the fungi, six strips for each fungal isolate. Once the fungus had grown across the cellulose strip the degree of degradation of the cellulose film was assessed using a needle penetrometer. Uninoculated film was punctured by a weight of 70g at each tested point, so any reduction from this was taken to represent the degree of cellulose breakdown.

As shown in Table 3.18, after 7 days *F. culmorum* had caused complete loss of strength of the film along its length, such that the weight of the penetrometer alone (5.1g) was usually sufficient to puncture the film. Compared to this, *P. ultimum* caused slight loss of strength of the film, indicating possible weak cellulolytic activity. Both isolates of *P. acanthophoron* also caused very slight strength-loss and thus appeared to be weakly cellulolytic. The other mycoparasites, especially *P. oligandrum* and *P. mycoparasiticum*, caused very little strength loss and appear to be non-cellulolytic.

3.7 The effect of chloride on the growth of Pythium species

Martin & Hancock (1986) reported that *P. oligandrum* was more tolerant of elevated chloride concentrations than was *P. ultimum in vitro*. This was used to explain why soils with elevated chloride concentrations in irrigated Californian cotton fields were suppressive to disease caused by *P. ultimum*, because *P. oligandrum* could compete with the pathogen, and exclude it, when cotton crop residues were available for colonisation by both fungi after harvest. This report prompted an investigation of the tolerance of several mycoparasitic *Pythium* spp. to chloride concentrations *in*

Table 3.18 Comparison of the weight (g) supported by cellulose film overlying L-asparagine-mineral agar medium after inoculation with *Pythium* spp. and *Fusarium culmorum*; means with 95% confidence limits in parentheses for assessments with a penetrometer on each of six replicate pieces of film.

Fungal isolate	weight supported by cellulose film (g)*
Pythium oligandrum (F9.2.3)	67.8 (± 3.1)
Pythium oligandrum (P14.1.2)	65.7 (± 4.6)
Pythium mycoparasiticum (AR5A)	67.0 (± 1.5)
Pythium mycoparasiticum (AR7A)	65.0 (± 3.9)
Pythium periplocum (P9.8.4)	62.8 (± 4.6)
Pythium acanthophoron (IMI 330 382)	57.7 (± 4.9)
Pythium acanthophoron (CBS 337.29)	55.3 (± 4.4)
Pythium ultimum (G39)	49.8 (± 4.1)
Fusarium culmorum (CD9)	0.5 (± 0.5)

* Uninoculated cellulose film was punctured by 70g applied weight.

vitro, as a possible basis for promoting the activities of mycoparasites relative to plant-pathogenic *Pythium* spp. in soil.

P. oligandrum (P14.1.2), *P. acanthophoron* (IMI 330 382), *P. mycoparasiticum* (AR7A), *P. ultimum* (G39), *P. aphanidermatum* (CBS 634.70) and *P. graminicola* (IMI 91329) were inoculated into medical flats containing 12 ml of basal medium (Section 2.1) supplemented with glucose (20 gl^{-1}), L-asparagine (1.51 gl^{-1}) and thiamine hydrochloride (100 μgl^{-1}). The medium was supplemented with NaCl at 0, 100, 300 or 500mM concentration. Five flats of each treatment were incubated for 14 days at 25°C and the mycelial dry weights were assessed (Table 3.19).

All three plant-pathogenic *Pythium* species grew better than the mycoparasitic *Pythium* species in the salt-free medium, and their growth was progressively reduced with increase in NaCl concentrations. The growth of the mycoparasites was also progressively reduced as the NaCl content was raised. As a percentage of growth in the NaCl-free controls, the three mycoparasites were clearly less tolerant of NaCl than were the phytopathogens (Table 3.19).

In order to exclude the possible involvement of sodium ions as a cause of poor growth in the experiment above, both *P. oligandrum* (P14.1.2) and *P. ultimum* (G39) were inoculated into glucose-asparagine-mineral salts medium supplemented with Na_2SO_4, KCl or NaCl so that the chloride-containing media had Cl^- at 100 meq, 300 meq and 500 meq. Likewise the concentrations of NaCl and Na_2SO_4 were calculated to provide the same sodium concentrations ranging from 100 to 500meq. Growth, expressed as percentage of that in unsupplemented controls is shown in Figures 3.2 and 3.3, with one way analysis of variance carried out on arcsine-transformed data.

Both *P. oligandrum* and *P. ultimum* responded to KCl supplements as to NaCl supplements, with progressive reduction of growth; *P. ultimum* was the more tolerant of the increasing chloride concentrations (Figure 3.2). In a comparison of NaCl and Na_2SO_4 supplements (Figure 3.3), both fungi were more tolerant of the sulphate than chloride salt, but *P. oligandrum* was less tolerant of either salt than was *P. ultimum*.

The mycoparasitic species grow relatively poorly compared with *P. ultimum* in glucose-asparagine-mineral medium (Section 3.6.1), and this might have contributed to the increased sensitivity of the mycoparasites to sodium chloride. So the effect of sodium chloride concentration on *P. oligandrum* and *P. ultimum* was tested in both 3% molasses medium and in glucose-asparagine-mineral medium.

Table 3.19 Comparison of the mycelial dry weights (mg) of *Pythium* spp. in liquid culture medium with different NaCl concentrations (means ± SEM for 5 replicates; percent of growth in NaCl-free controls is shown in parentheses).

Fungal isolate	NaCl concentration (mM)			
	0	100	300	500
P. ultimum (G39)	72 ± 2.0	60 ± 1.4 (83.6)	48 ± 2.7 (66.7)	26 ± 1.2 (35.5)
P. aphanidermatum (CBS 634.70)	70 ± 2.5	44 ± 0.9 (61.9)	32 ± 2.5 (44.6)	11 ± 5.3 (15.0)
P. graminicola (IMI 91329)	69 ± 2.4	57 ± 5.3 (82.1)	15 ± 4.2 (21.9)	1 ± 0.7 (1.4)
P. acanthophoron (330 381)	15 ± 0.6	5 ± 0.9 (30.3)	1 ± 0.4 (7.9)	0.8 ± 0 6 (5.3)
P. oligandrum (P14.1.2)	15 ± 0.7	10 ± 1.6 (66.7)	0.8 ± 0.4 (5.4)	0.6 ± 0.5 (4.0)
P.mycoparasiticum (AR7A)	16 ± 1.0	8 ± 0.7 (48.8)	0.6 ± 1.3 (3.7)	0.2 ± 0.6 (1.2)

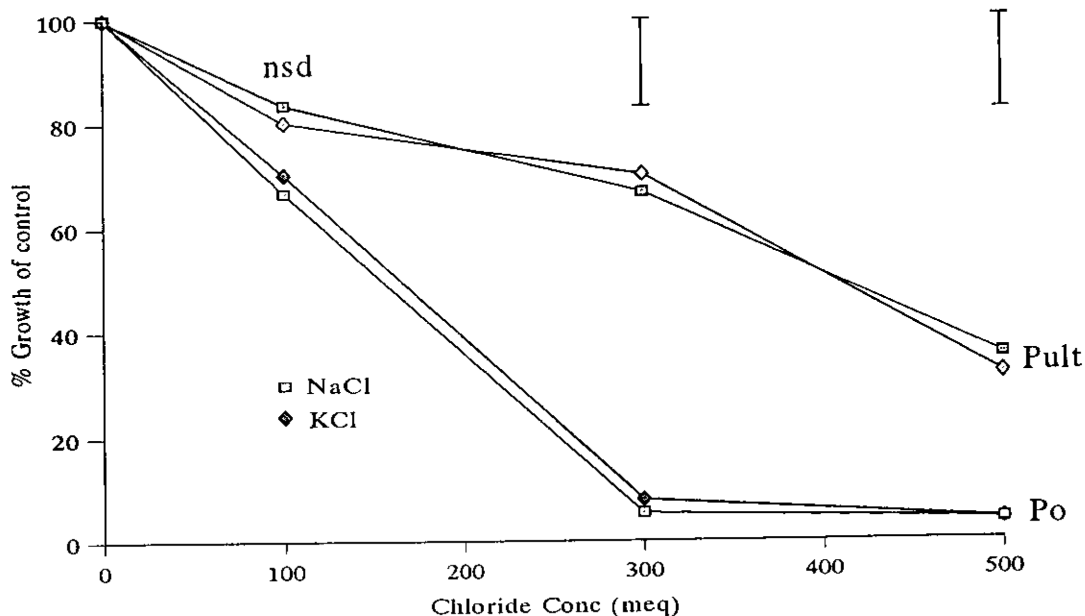


Figure 3.2 Effect of chloride concentration (meq) supplied as either NaCl or KCl on the growth of *P. ultimum* (Pult) and *P. oligandrum* (Po) in glucose-asparagine-thiamine-mineral liquid culture, as a percentage of growth in the salt-free control (means of 5 replicates with the bars representing L. S. D. at $P < 0.05$, nsd = no significant difference).

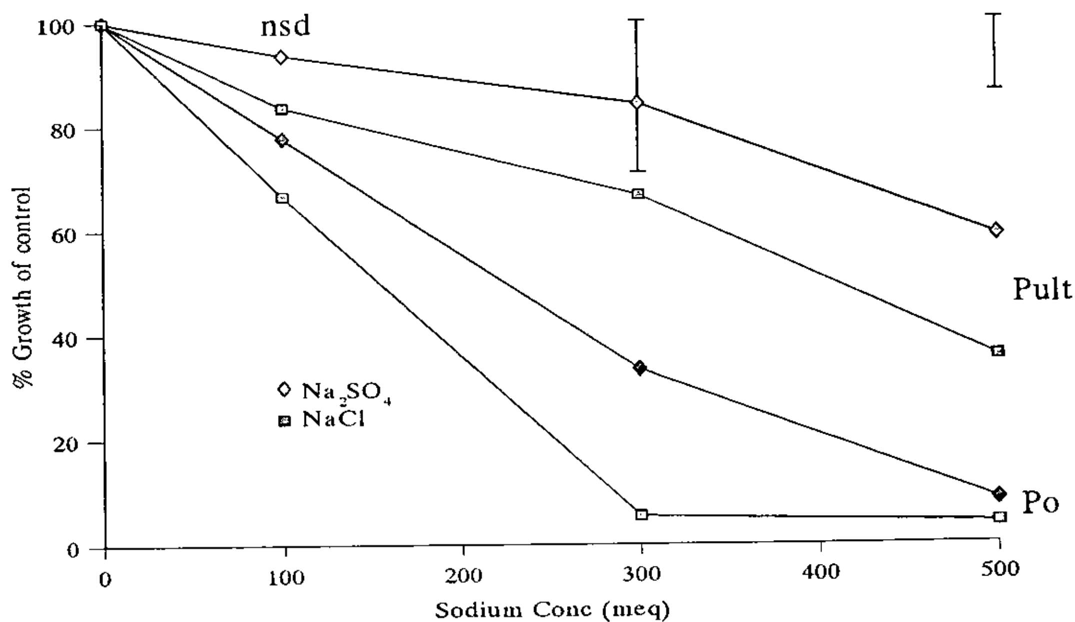


Figure 3.3 Effect of sodium concentration (meq) supplied as either NaCl or Na_2SO_4 on the growth of *P. ultimum* (Pult) and *P. oligandrum* (Po) in glucose-asparagine-thiamine-mineral liquid culture as a percentage of growth in the salt-free control (means of 5 replicates with the bars representing L. S. D. at $P < 0.05$, nsd = no significant difference).

As shown in Figure 3.4, *P. oligandrum* was, indeed, more tolerant of increasing NaCl concentrations in molasses medium than in the glucose-asparagine-mineral medium. Using one way analysis of variance on arcsine-transformed values of the percentage growth reductions, as shown in Figure 3.4, there was no significant effect of the two types of medium on *P. oligandrum* at 100mM NaCl, but significantly ($P < 0.01$) less growth reduction of *P. oligandrum* in molasses than in glucose-asparagine-mineral medium for both 300mM and 500mM NaCl. In contrast, the type of medium had no effect on the response of *P. ultimum* (Figure 3.4) to increased concentration of NaCl.

The effect of NaCl on oospore production by *P. oligandrum* in molasses medium (Table 3.20) was less than on total biomass production, especially for the lower NaCl concentrations. Even at 300mM NaCl the number of oospores had fallen proportionally less than had total biomass yield, but at 500mM NaCl concentration the production of oospores was markedly reduced.

3.8 Plant pathogenicity tests

P. oligandrum, *P. mycoparasiticum*, *P. periplocum* and *P. acanthicum* are reported to be non-pathogenic to seedling plants (Deacon & Henry, 1978; Pratt & Janke, 1980; Laing, 1989; Deacon *et al.*, 1991). The pathogenicity of *P. acanthophoron*, however, has not been studied in detail. Sideris (1932) found it to be an extremely weak parasite of the roots of *Ananas sativus* (pineapple); whilst Lodha & Webster (1990) isolated it from the soil around a healthy ginger rhizome but did not test pathogenicity. The pathogenicity of *P. oligandrum* (P14.1.2 and F9.2.3), *P. mycoparasiticum* (AR7A and AR5A), *P. periplocum* (P9.8.4) and *P. acanthophoron* (IMI 330 382 and CBS 337.29) was now tested on tomato (*Lycopersicon esculentum*), cucumber (*Cucumis sativus*) and pepper (*Capsicum annum*) seedlings, in comparison with the plant pathogens *P. ultimum* (G39 and G99) and *P. aphanidermatum* (CBS 634.70). The method (Section 2.3) involved placing seeds on agar discs in pots filled with perlite; control pots contained uninoculated agar discs. Seedling emergence was assessed every 5 days up to 25 days (Table 3.21).

Both *P. ultimum* and *P. aphanidermatum* killed all the seedlings before emergence. When the seeds were retrieved they were found to have rotted. None of the mycoparasitic isolates tested caused appreciable decrease in emergence of any of the plants compared to the control.

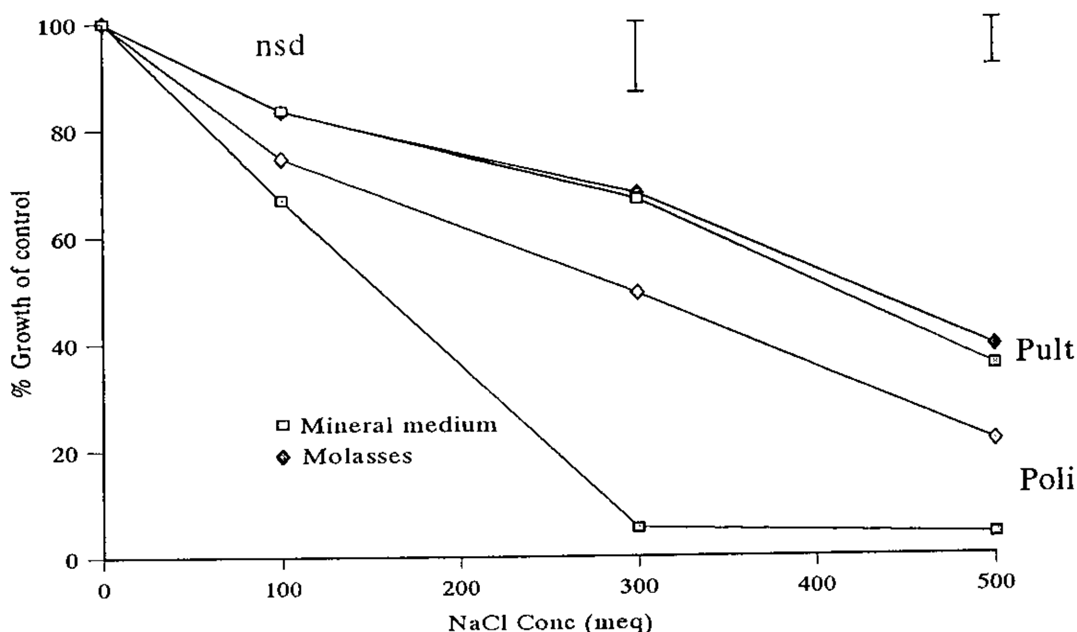


Figure 3.4 Effect of sodium chloride concentration (mM) on the growth of *P. oligandrum* (Po) and *P. ultimum* (Pult), as a percentage of the growth in the NaCl-free control, for glucose mineral medium and 3% molasses medium (means of 5 replicates).

Table 3.20 Comparison of the effect of sodium chloride concentration on total dry weight (mg) and oospore number (x10^5) of *Pythium oligandrum* grown in 3% molasses liquid culture (means of 5 replicates with SEM).

NaCl conc^n (mM)	Total dry weight (mg)	Oospore number (x10^5)	Oospores (x10^5) / mg total dry weight
0	25.2 ± 1.2	7.7 ± 0.5	0.31
100	18.8 ± 1.3	7.1 ± 0.5	0.38
300	12.4 ± 0.7	5.0 ± 0.7	0.40
500	5.4 ± 0.9	1.2 ± 0.3	0.22

Table 3.21 Pathogenicity of *Pythium* species to germinating seeds of tomato (cv Moneymaker), cucumber (cv Ridge Perfection) and pepper (cv California Wonder) after 25 days.

	Seedling emergence (maximum 12)		
Fungal isolate	Tomato	Pepper	Cucumber
Control	11	11	8
Pythium oligandrum (P14.1.2)	9	11	8
Pythium oligandrum (F9.2.3)	11	10	6
Pythium mycoparasiticum (AR7A)	12	12	9
Pythium mycoparasiticum (AR5A)	12	12	10
Pythium acanthophoron (IMI 330 382)	9	11	8
Pythium acanthophoron (CBS 337.29)	12	10	8
Pythium periplocum (P9.8.4)	12	11	8
Pythium ultimum (G39)	0	0	0
Pythium ultimum (G99)	0	0	0
Pythium aphanidermatum (CBS 634.70)	0	0	0

3.9 Discussion

The experiments in this chapter extended previous reports on the physiology and nutrition of mycoparasitic *Pythium* spp. in comparison with some representative phytopathogenic *Pythium* spp. Although there were earlier reports on the nutritional characteristics of many *Pythium* spp. (eg. Leonian & Lilly, 1938; Ridings *et al.*, 1969), Foley & Deacon (1986a) first drew attention to characteristic nutritional differences between mycoparasitic and plant-pathogenic *Pythium* spp., and that work was extended to further mycoparasitic *Pythium* spp. by Laing & Deacon (1990).

Several further isolates have been used in comparative studies, including both the original isolate of *P. acanthophoron* and a more recent isolate of this fungus. The significance of this work on *P. acanthophoron,* in particular, lies in the fact that Sideris's (1932) original description of this fungus is inadequate for full identification and the original culture is now sterile so further isolates are difficult to ascribe to this species (Lodha & Webster, 1990). Nutritional and other physiological features, as well as studies on mycoparasitic interactions (Chapter 6), can help in this respect.

Table 3.22 Summary of the nutritional requirements of the mycoparasitic *Pythium* species.

Species	Organic nitrogen requirement	Thiamine requirement
P. oligandrum	Yes	Yes
P. mycoparasiticum	Yes	Yes
P. acanthicum	Yes	Yes
P. periplocum	Yes	Yes
P. acanthophoron	Yes	No
P. nunn	No	No

Foley & Deacon, (1986a) found that an organic nitrogen and an exogenous thiamine source were required by the mycoparasites *P. oligandrum, P. acanthicum, P. periplocum* and *P. mycoparasiticum*, unlike the plant pathogens tested, which could utilise inorganic nitrogen and were self-sufficient for thiamine. This vitamin, as thiamine pyrophosphate, is required as a coenzyme for several enzymes involved in metabolism (Garraway & Evans, 1984). *P. acanthophoron* (IMI 330 382) has now been found to require an organic nitrogen source, such as asparagine, agreeing with the nutritional requirement of the mycoparasites in this respect. However it was found to be self-sufficient for thiamine like many plant pathogenic *Pythium* spp. This agrees with the findings of Ridings *et al.* (1969) for *P. acanthophoron*. The original isolate of Sideris, *P. acanthophoron* CBS 337.29, was found here to require organic nitrogen but be self-sufficient for thiamine, like isolate IMI 330 382 from Britain. It will be

shown later (Chapter 6) that both these isolates act as mycoparasites, while Table 3.21 shows that neither was pathogenic to seedlings of tomato, cucumber or pepper. Only *P. nunn*, of all the mycoparasitic *Pythium* species, has nutritional requirements more characteristic of the plant pathogenic species than the mycoparasitic species. Laing & Deacon (1990) found that it could utilise inorganic nitrogen sources and was partly self-sufficient for thiamine although the addition of thiamine promoted its growth slightly (Laing, 1989). On this basis, the current evidence for nitrogen and vitamin requirements of the mycoparasitic *Pythium* spp. is summarised in Table 3.22.

For some fungi, amino acids can serve as both a carbon and nitrogen source (Garraway & Evans, 1984), but none of the *Pythium* species tested here was found to grow appreciably on asparagine as sole carbon and nitrogen source. Yeast extract similarly supported poor growth in the absence of another carbon source. With regard to other carbon sources, mannitol can be utilised by some fungi (Jennings, 1974; Lewis & Smith, 1967) but not by many oomycetes. However mycoparasitic *Pythium* spp. have been reported to utilise mannitol (Foley & Deacon, 1986a), unlike some plant-pathogenic species. This would be compatible with the fact that mannitol is a common carbohydrate in the mycelia of non-oomycetes, including the known fungal hosts of mycoparasitic *Pythium* spp., but mannitol is relatively uncommon in green plants- the hosts of plant pathogenic *Pythium* spp. (Lewis & Smith, 1967). In this study, however, only *P. mycoparasiticum* and one isolate of *P. oligandrum* (F9.2.3) were found to utilise mannitol to any significant degree. The addition of cholesterol, calcium or both increased the growth on mannitol by most of the mycoparasitic *Pythium* isolates that were tested, so that growth in some cases approached that on glucose as the carbon source. In contrast, the plant pathogen *P. ultimum* made little growth using mannitol, compared with glucose, although again the addition of cholesterol and calcium was stimulatory. For only one mycoparasitic isolate (*P. oligandrum*, P14.1.2) did a cholesterol supplement alone significantly increase the growth on mannitol, whereas McQuilken *et al.* (1992a) and Child *et al.* (1969a) had found that *P. oligandrum* and *P. acanthicum* respectively utilised mannitol in the presence of cholesterol. In my work calcium increased the growth of mycoparasitic isolates on mannitol more often than cholesterol, and the addition of cholesterol plus calcium had no extra beneficial effect (Table 3.7). The sole exception was with *P. oligandrum* isolate P14.1.2, which, like *P. ultimum*, was not stimulated to grow on mannitol by the addition of calcium, but was stimulated by cholesterol. From even these limited data it is clear that the growth responses on mannitol vary not only between species but also between isolates.

Different sterols were also found to affect mannitol utilisation in different ways (Table 3.8). β-sitosterol was found to stimulate growth by *P. mycoparasiticum* (AR7A) on mannitol, but not by *P. oligandrum* (P14.1.2) or *P. acanthophoron* (IMI 330 382). In contrast, ergosterol was the only significantly stimulatory sterol for *P. oligandrum* (P14.1.2) but had no effect on *P. acanthophoron* (IMI 330 382) and significantly decreased the growth of *P. mycoparasiticum* (AR7A). This latter effect, however, could have been due to the use of ethanol as a solvent. *P. mycoparasiticum* was more sensitive to ethanol than the other mycoparasites, although the inhibitory effect on *P. mycoparasiticum* AR7A at least was overcome by ergosterol. Hendrix (1964) also observed that different sterols have different effects on the growth of *P. periplocum* on agar medium containing glucose. Whilst cholesterol increased the growth rate, ergosterol had no effect, and β-sitosterol decreased the growth rate. Sterols and calcium may either increase the uptake of mannitol by the mycoparasites or increase the metabolic conversion of mannitol. These possible mechanisms will be discussed later.

Lenny & Klemmer (1966) reported that addition of cholesterol and calcium, alone or in combination, increased the growth of *P. graminicola* on glucose as a carbon source. But glucose utilisation by the *Pythium* species in the present study was unaffected by these supplements. Any stimulatory effects of cholesterol and calcium on glucose utilisation by *Pythium* may be species - or isolate-dependent. Thus, Hendrix (1965) found that growth of *P. periplocum* on glucose-agar media was stimulated by the addition of cholesterol, whereas *P. proliferum* de Bary and *P. spinosum* failed to respond to cholesterol. Calcium was also observed by Erwin (1968) to increase the mycelial growth of *Phytophthora megasperma*, and the effect of calcium could be replaced by strontium. Here the early rate of growth of *P. oligandrum* in liquid medium was found to be significantly stimulated by calcium and, to a lesser degree, by cholesterol, but the final yield of biomass was the same irrespective of the supplements (Figure 3.1). Similar findings were reported by Child *et al.* (1969a, 1969b) for *P. acanthicum* and cholesterol. Brushaber, Child & Haskins (1972), however, found that cholesterol increased the initial growth of *P. acanthicum* on glucose, but the final biomass yield was less in the presence of cholesterol than in the absence of cholesterol.

The mechanism of the sterol effect on growth is unclear, but it has been suggested by Hendrix (1970) to be an effect on either membrane permeability or respiration. Sietsma & Haskins (1968) suggested that cholesterol was taken up by *P.*

acanthicum and incorporated into its plasma membrane, so that permeability of the membrane was changed, resulting in a reduction of leakage of intracellular constituents. They and Child *et al.* (1969b) noted that polyene antibiotics increased the leakage of ions from mycelium grown in the presence of cholesterol, but had hardly any effect when the mycelium was grown without cholesterol. Polyene antibiotics are thought to bind with sterols in the membrane creating polar pores through which ions leak (Gale *et al.*, 1981). The sensitivity of Oomycetes to polyene antibiotics when these fungi are supplied with sterols, which they cannot synthesise *de novo* from precursors (Hendrix, 1970), is evidence that exogenous sterols or their products are incorporated in the plasma membrane. But there is no simple way in which this could enhance the uptake of mannitol because this requires the presence of specific membrane-uptake proteins (Jennings, 1974). A possibility, however, is that sterols in some way facilitate mannitol uptake through a glucose permease with low affinity for mannitol (Jennings, 1974). Sterols have also been implicated in respiration processess; when grown with cholesterol, *P. ultimum* is reported to not only to take up glucose faster but also to catabolise it faster and produce more carbon dioxide per unit glucose than without cholesterol (Schlosser & Gottlieb, 1966, cited in Hendrix, 1970). In contrast, however, Sietsma & Haskins (1968) found that cholesterol had no effect on the rate of glucose uptake or increase of dry weight by *P. acanthicum*. Cholesterol has also been reported to increase activity of a number of enzymes involved in energy production (Sietsma, 1971, cited in Hendrix, 1974). However another explanation of the effect of cholesterol could be that cholesterol itself is used as a carbon source by some of the isolates. This could explain the growth observed with mannitol, in that the growth was attributable to utilisation of cholesterol and not mannitol. However, if the only effect of cholesterol was to act as a supplementary carbon source, an increase in the initial growth rate of *P. oligandrum* on glucose would not be expected; rather if no other nutrients were limiting, an increase in final yield would be expected. Calcium has also been suggested to stimulate growth by affecting membrane permeability or respiration (Hendrix, 1970). Again the mechanisms are poorly understood, but the work here (Tables 3.7 and 3.8) suggests that the effects of both calcium and various sterols on growth of *Pythium* spp. are species - or even strain-dependent. This lack of uniformity of response seems to preclude simple interpretations.

The mycoparasitic *Pythium* species have been reported to be unable to utilise cellulose as a carbon source (Deacon, 1979; Foley, 1983; Foley & Deacon, 1986a; Laing & Deacon, 1990). This was confirmed here for *P. oligandrum*, *P. periplocum*

and *P. mycoparasiticum*. However, two isolates of *P. acanthophoron* caused some weakening of cellulose film- in one case equivalent to that caused by *P. ultimum*, which has been shown weakly to degrade filter paper (Deacon, 1979). The significance of this apparent cellulolytic activity of *P. acanthophoron* merits further study, although it was low compared with the reported cellulolytic activities of some other *Pythium* spp. such as *P. irregulare, P. mamillatum* and *P. graminicola* (Deacon, 1979).

It has often been found that mycoparasitic *Pythium* spp. grow more poorly on relatively simple, chemically defined media than on more complex "natural" media (Foley & Deacon, 1986a; Laing, 1989). This was confirmed here for both *P. oligandrum* and *P. mycoparasiticum*. It indicates that the absolute nutritional requirements of most mycoparasitic *Pythium* spp. (eg. organic nitrogen and thiamine in many cases) do not truly reflect the requirements for optimal vegetative growth. Of the undefined media tested, molasses supported the greatest oospore production. Both sunflower seed extract and carrot extract also supported oospore production of both *P. oligandrum* and *P. mycoparasiticum*. All three of these media contain sterols which are required by *Pythium* spp. for sexual reproduction (Hendrix, 1970; Kerwin & Duddles, 1989). Potato-dextrose, whilst supporting the highest biomass production for both mycoparasites, did not support oospore production. Even when potato-dextrose was supplemented with either a natural sterol source (sunflower seed extract) or β-sitosterol it did not support oospore production. This could be because potato-dextrose is inhibitory to oospore production, or because it lacks one or more factors, apart from sterols, which are required to initiate sexual reproduction. Sunflower seed extract alone supplied these factors for good oospore production (Table 3.10), yet even a mixture of potato-dextrose and sunflower seed extract did not enable either *P. oligandrum* or *P. mycoparasiticum* to produce many oospores (Table 3.10). In contrast, mixture of molasses and potato-extract (without dextrose) led to production of many oospores (Table 3.11). From this and other evidence in Tables 3.10 and 3.11, it seems that potato dextrose, rather than potato extract was inhibitory to oospore production, and that this inhibition persisted for at least 28 days of culture, even though no further growth occurred between 14 and 28 days.

P. oligandrum is known to produce oospores rapidly in appropriate culture media after a short phase of vegetative growth (McQuilken *et al.* 1990a), presumably when nutrients are exhausted; this could be associated with autolysis of the mycelium and conversion of its resources into oospore reserves, as was reported for

development of sclerotia of several fungi by Christias & Lockwood (1973). However, a link between autolysis and oospore production does not seem to be supported by the data in Table 3.11, because autolysis (as evidenced by a substantial loss of biomass) was evident on prolonged incubation of *P. oligandrum* in all the media that did not support oospore production, whereas no significant loss of total biomass occurred in the media that supported oospore production.

In other studies on *P. oligandrum*, McQuilken *et al.* (1992a) suggested that the trigger for oospore production might not be nutrient depletion but rather a specific nutrient level or ratio. Both they and Child *et al.* (1969a) demonstrated that oospore production (by *P. oligandrum* and *P. acanthicum* respectively) increased at lower carbon to nitrogen ratios of the growth medium. Klemmer & Lenney (1965) showed that increased sexual reproduction of *P. vexans*, *P. graminicola* and *P. arrhenomanes* was not associated with increased growth rate. Yang (1964, cited in Child *et al.*, 1969a) reported that there was a negative correlation between growth and sexuality in three *Pythium* species- *P. debaryanum*, *P. ultimum* and *P. irregulare*. Therefore a medium that promotes good vegetative growth might not necessarily support good oospore production and evidence for this can also be seen in Table 3.11.

In molasses medium three different mycoparasitic *Pythium* spp. showed differences in their ability to grow and produce oospores, and substantial variation was also found between isolates of *P. mycoparasiticum*. This factor could be important in relation to production of biocontrol inocula, although it is equally important that the oospores are viable and germinable (Chapter 4). Evidently, also, the ability to produce oospores can be lost on prolonged storage or repeated sub-culturing of isolates, which is reflected in the fact that Sideris's original isolate of *P. acanthophoron* (CBS 337.19) did not produce oospores even though it grew well in molasses. This confirms the sterility of this isolate reported by Lodha & Webster (1990).

For production of commercial biocontrol inocula, shake cultures are prefered to static cultures (Papavizas *et al.*, 1984), and for *P. oligandrum* they were seen to yield more oospores per unit biomass (Table 3.14). This could be due to better aeration of the culture medium (although different volumes were used in the two systems to maximise the aeration of the static cultures) or to improved nutrient utilisation. McQuilken *et al.* (1990a) have also reported the efficient production of oospore of *P. oligandrum* in shaken liquid culture.

Experiments on radial growth of colonies on agar (Table 3.15) showed marked differences in the extension rates of the four tested mycoparasites, and also different responses of the different species on the different media tested. Both isolates of *P. mycoparasiticum* grew fastest on SSA, and slower on full-strength PDA than on most other media. In contrast the single isolate of *P. periplocum* grew fastest across molasses agar, while both isolates of *P. acanthophoron* and both of *P. oligandrum* grew fastest across potato-dextrose agar. This last finding is at variance with that of Foley & Deacon (1986a), who found that *P. mycoparasiticum*, *P. oligandrum*, *P. periplocum* and *P. acanthicum* were partly inhibited by a component of commercial potato-extract. However, all tested isolates *P. mycoparasiticum* (Table 3.16) grew somewhat better on half-strength than fuller-strength PDA, partly supporting the findings of Foley & Deacon (1986a). Linear extension on agar is not necessarily a good reflection of growth rate *per se*, but it could be relevant to the ecology of the different fungi- for example, their abilities to extend as fast as the hyphae of their fungal hosts (Chapter 5). Comparative biomass yields of the mycoparasites were studied only for *P. mycoparasiticum* and *P. oligandrum*. In carrot extract, molasses and sunflower seed extract liquid media the yields of *P. mycoparasiticum* were very close to those of *P. oligandrum* after 14 days, but in potato-dextrose broth *P. mycoparasiticum* did not give an equivalent yield to that of *P. oligandrum* (Table 3.9). This tends to support the findings for extension on the different agar media, in that potato-dextrose was least satisfactory for *P. mycoparasiticum*.

On vermiculite moistened with molasses- a particulate substratum- *P. mycoparasiticum* was found to colonise the vermiculite slower than *P. oligandrum* or *P. ultimum*, consistent with its slower extension rate. Also, unlike the other fungi, *P. mycoparasiticum* could not be detected in all vermiculite particles up to 70 days, even though it had colonised them by 14 days. Microscopic examination of the vermiculite particles at 14 days and thereafter revealed a large number of oospores of both *P. oligandrum* and *P. mycoparasiticum* on them. It is not known whether these fungi grew from vermiculite particles onto agar plates from residual mycelium or from germinating oospores. However, the failure of *P. mycoparaiticum* to grow from the particles at the later sampling times would be consistent with the finding (see Chapter 4) that oospores of *P. mycoparasiticum*, unlike those of *P. oligandrum*, consistently failed to germinate on agar.

The experiments on tolerance of NaCl and other salts, reported here, were prompted by the report of Martin & Hancock (1986) that soils suppressive to disease

caused by *P. ultimum* in irrigated Californian cotton fields were associated with high soil chloride contents and that these high chloride contents favour growth of *P. oligandrum* relative to *P. ultimum*. Martin & Hancock (1986) showed *in vitro* that growth of *P. oligandrum* was less sensitive to elevated chloride levels than was growth of *P. ultimum*, and that the interaction between these fungi in colonisation of cotton crop residues in soil was affected, in favour of *P. oligandrum*, by elevated chloride levels. However, in my experiments (Tables 3.19) the phytopathogens *P. ultimum* and *P. aphanidermatum* were seen to be the most tolerant of high NaCl concentrations when grown in glucose-asparagine-mineral liquid culture. The three mycoparasitic pythia- *P. oligandrum*, *P. acanthophoron* and *P. mycoparasiticum*- and, to a lesser extent, the plant pathogen *P. graminicola*, were less tolerant of NaCl. In further investigations of this, the toxic effect seemed to be due mainly to the chloride and not to the sodium ion, as seen by substituting KCl or Na_2SO_4 for NaCl. In all experiments however, *P. oligandrum* was more sensitive than was *P. ultimum* to increased concentrations of the salts. This could have been partly an osmotic effect rather than salt toxicity *per se*. But in any case it provides no reason to expect that antagonism of plant-pathogenic *Pythium* spp. by the mycoparasites might be favoured by the addition of salts to growth media. McQuilken *et al.* (1992b) also observed a decrease in mycelial extention rates for *P. oligandrum* when soil extract agar was supplemented with various concentrations of NaCl. They observed growth of *P. oligandrum* to cease between -2.5 amd -3.0 MPa water potential, but in my study, growth of *P. oligandrum* was almost completely inhibited at -1.27MPa osmotic potential (*c.*300 mM NaCl). Such differences might be explicable in terms of the type of media and culture system used, because *P. oligandrum* was found to be more tolerant of increasing NaCl concentrations in molasses medium than in glucose-asparagine-mineral medium. No such significant difference was seen for *P. ultimum*, which grew well in both the defined medium and in the molasses medium, whereas *P. oligandrum* was found to have a distinct preference for the molasses medium. Blomberg & Adler (1993) reported that the type of carbon source supplied to *Saccharomyces cerevisiae* Meyer ex Hansen influenced its tolerance of high NaCl concentrations. Using acetate, growth was supported up to only 0.45M NaCl, whereas on glucose medium the yeast grew at 1.5M NaCl concentration. In high salt conditions, NaCl enters the fungal hyphae, causing osmotic or salt stress. To compensate for this, fungi produce compatible solutes- often polyols such as glycerol. This compatible solute, glycerol, is derived from intermediates of the glycolytic sequence. In contrast to this, the yeast would not so readily be able to produce glycerol from acetate, because this would require the operation of gluconeogenic

pathways. A similar type of effect could explain the effect of nutrient compounds of the medium on salt-tolerance by *P. oligandrum*. The oomycetes produce few polyols; instead proline seems to be more important as a compatible solute in these fungi (Luard, 1982; Blomberg & Adler, 1993). Presumably, the poor growth of *P. oligandrum* in defined media than in rich and nutritionally diverse media could influence its ability to produce compatible solutes involved in water-stress tolerance. This still leaves unexplained the fact that *P. oligandrum* and the other mycoparasites studied here were less tolerant of elevated salt levels than were the phytopathogens, in contrast to the report by Martin & Hancock (1986). It is possible that the elevated chloride levels in irrigated cotton crops of the San Joaquin Valley of California have selected for strains of *P. oligandurm* with enhanced salt-tolerance. This possibility merits investigation by testing of strains from different field sites. Such postulated inter-strain variation in salt-tolerance would be compatible with several examples of inter-strain differences in the mycoparasitic *Pythium* spp. found in the present study.

CHAPTER 4

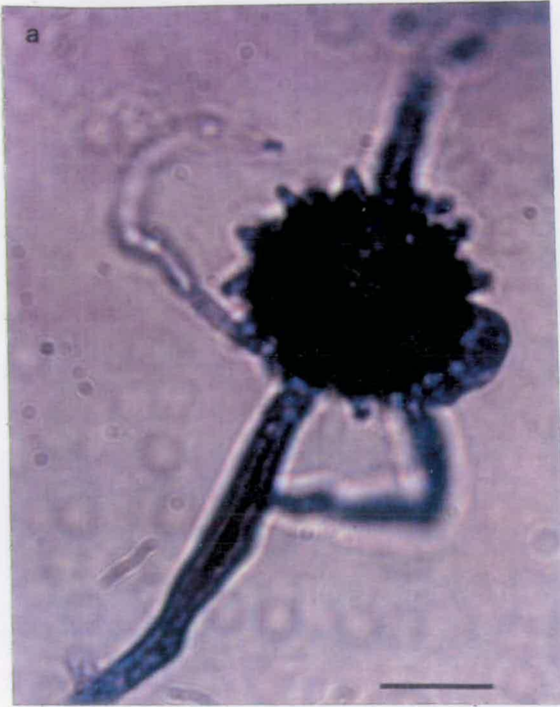
GERMINATION OF OOSPORES OF MYCOPARASITIC PYTHIUM SPECIES

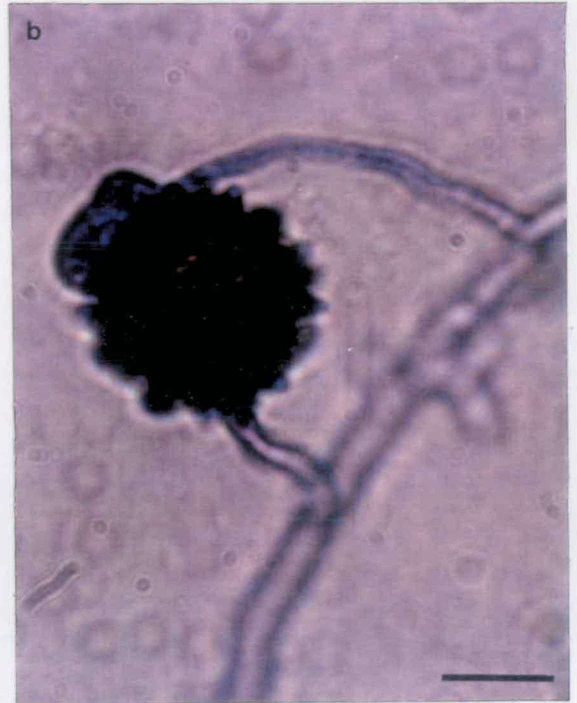
Oospores of mycoparasitic *Pythium* spp. have potential for use as biological control inocula. However, only oospores of *P. oligandrum* have been used to any extent experimentally for biocontrol (Vesleý, 1978a; Al-Hamdani *et al.*, 1983; Martin & Hancock, 1984, 1987; Lutchmeah & Cooke, 1985; Walther & Gindrat, 1987; McQuilken *et al.*, 1990b), and attempts to enhance their germination have been only partly successful (Al-Hamdani *et al.*, 1983; Walther & Gindrat, 1987a; McQuilken *et al.*, 1990a, 1992c). In this chapter, the germination of oospores of three mycoparasitic *Pythium* species (*P. oligandrum, P. acanthophoron* and *P. mycoparasiticum*) is investigated, with an aim to maximise germination by using different treatments.

4.1 Differences in sexual morphology in P. acanthophoron and P. oligandrum

The sexual morphology of *P. oligandrum* and *P. acanthophoron* has not been directly compared. In this section cultures of both *P. oligandrum* (P14.1.2) and *P. acanthophoron* (IMI 330 382) were grown on SSA at 25°C for 48 h. The colonies were then stained using 0.1% trypan blue in glacial acetic acid and observed microscopically.

The oogonia of both *P. acanthophoron* and *P. oligandrum* are echinulate. However, the spines of the oogonia of *P. acanthophoron* were shorter and more rounded at the ends (Figures 4.1a and b), compared to the longer sharp spines of the oogonia of *P. oligandrum* (Figure 4.1c). Antheridia were mostly lacking in *P. oligandrum*. In *P. acanthophoron* one or occasionally two antheridia were present per oogonium, and were monoclinous or diclinous in origin. The antheridial sac was observed to be bag - or 'bubble'-like with a slight constriction in the middle and making broad contact with the oogonium (Figures 4.1a and b). Often empty antheridial sacs were observed attached to mature oogonia.





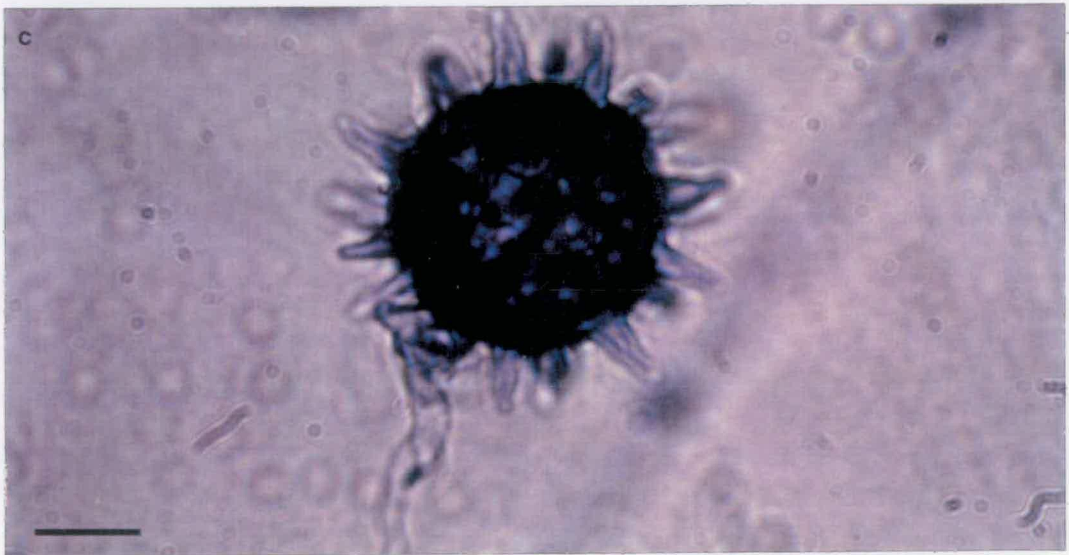


Figure 4.1a-c. Oogonia of *P. acanthophoron* (a,b) and *P. oligandrum* (c) on plates of sunflower seed extract agar; stained with trypan blue in glacial acetic acid. Bar represents 10μm. Note the short round-ended spines and antheridia of *P. acanthophoron*, compared with sharp spines and absence of antheridia in *P. oligandrum*.

4.2 Effect of incubation method on germination

4.2.1 Effect of incubation time

The method in Section 2.5 was used to produce oospores for this and subsequent experiments. It involved inoculating medical flats containing 12 ml of 3% molasses medium with a disc of a mycoparasite grown on PDA. The flats were incubated at 25°C in darkness for 35 days (R. P. Bradshaw-Smith, pers. comm.) unless otherwise stated. Five replicate flats were then harvested, to retrieve the biomass from the medium, the agar inoculum discs were removed and the biomass dried overnight (16 h) in a stream of sterile air on a laminar flow bench. The dried biomass was then resuspended in 1ml of sterile distilled water (SDW) and homogenised using an Ultraturrax T-25 blender at 20,500 rpm for 60 seconds. The resulting oospore and mycelial suspension was then centrifuged, washed with sterile distilled water, centrifuged again and the pellet was resuspended in SDW to achieve an oospore concentration of 10^5 oospores per ml. On each of 4 replicate PDA plates containing 200 μg vancomycin and 100 μg penicillin G per litre (McQuilken *et al.*, 1990b) 0.5 ml aliquots of the oospore suspension were spread. The plates were placed in a sealed plastic bag and incubated at 25°C in the dark. The germination of 500 oospores on each plate was assessed microscopically at 2 hourly intervals until some germination was observed and then every hour until maximum percentage germination was seen. Oospores were considered to have germinated if the germtube length was greater than the spore diameter. The time course of oospore germination for *P. oligandrum* (P14.1.2) and *P. acanthophoron* (IMI 330 382) is shown in Figure 4.2. Even after 28 h no germination of oospores of *P. mycoparasiticum* AR7A was observed.

For both *P. oligandrum* and *P. acanthophoron* no germination was seen before 14 h or 15 h. By 19 or 20 h, however, the fungi showed 25-26% germination. After this time, further assessment of germination was not possible because the extensive mycelial growth from germinated spores obscured observations. However, most of the remaining oospores were seen to be ungerminated. So, further experiments employed a standard assessment time of 18 h for *P. oligandrum* and 19 h for *P. acanthophoron*.

A distinct ooplast was visible in mature oospores of both *P. oligandrum* and *P. acanthophoron* harvested from 3% molasses medium. During incubation of these spores on PDA, the ooplast progressively disappeared from the germinating spores, but not from those that failed to germinate. Germination typically occurred by a single germ tube, which then often enlarged into a swelling near its point of

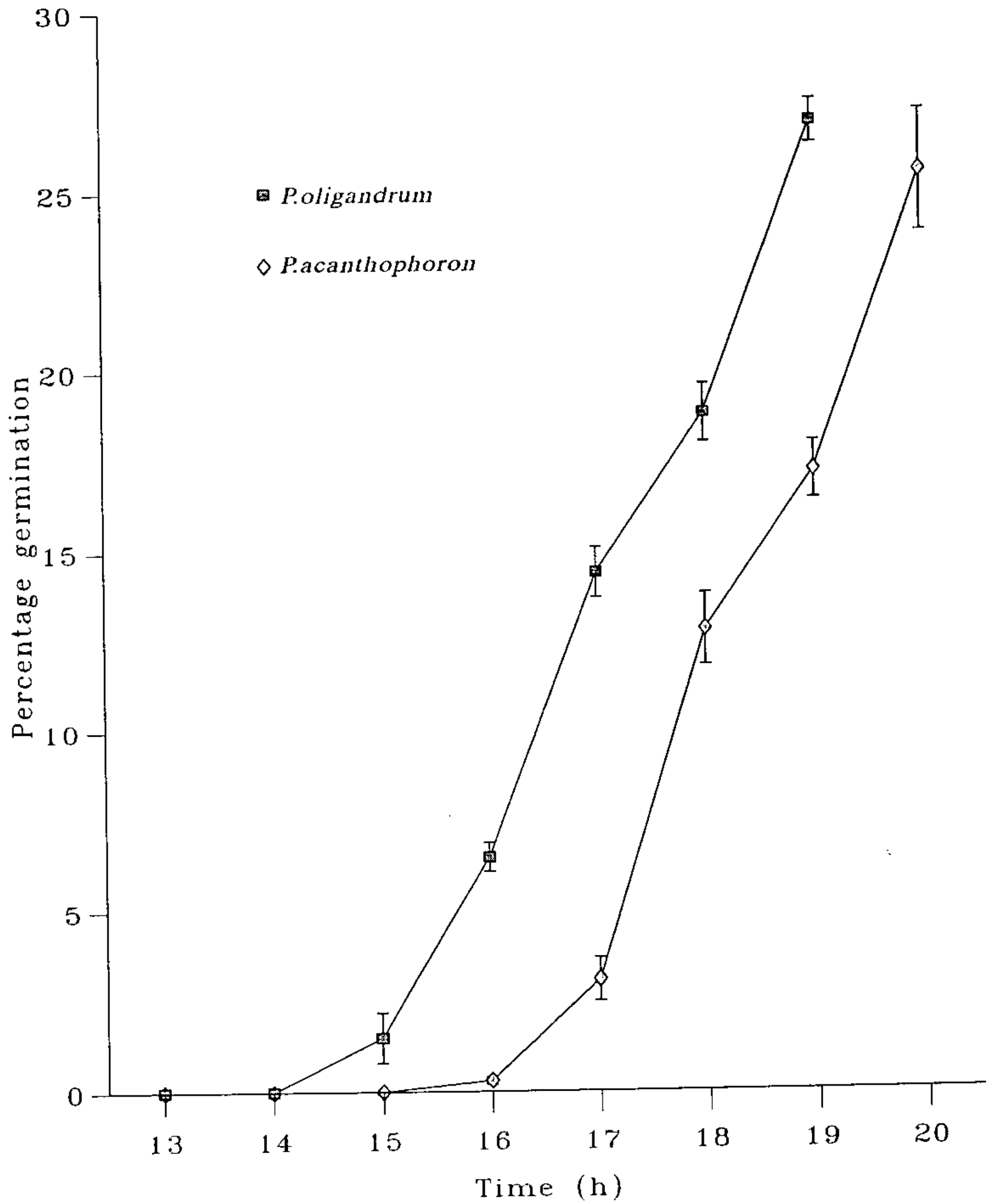


Figure 4.2 Percentage germination of oospores of *P. oligandrum* and *P. acanthophoron* harvested from 3% molasses and placed on potato-dextrose agar plates; mean of 4 replicates, error bars representing S.E.M.

emergence (Figures 4.3a-f, 4.4 and 4.5) and many branches were often seen to originate from this swelling (Figures 4.3a-f, 4.4 and 4.5). However, in many cases, observations suggested that the oospores of *P.oligandrum* and *P. acanthophoron* themselves did not germinate, but a new hypha developed from regrowth of an antheridial-like structure on the surface of the oospores. When this was observed depletion of the oospore contents or movement of oospore protoplasm into the developing hypha was not seen.

4.2.2 Effect of culture incubation time on the germination of oospores of P. oligandrum

Oospores of *P. oligandrum* (P14.1.2) were prepared as before but the 3% molasses cultures were harvested after 1, 3 and 7 days and then at 7 day intervals thereafter, up to 70 days. Germination was assessed after 18 h incubation on PDA plates at 25°C in the dark. The oospore numbers and mycelial dry weights of the cultures were also determined at each harvest time, using the method in Section 2.4.1. For oospore counts mycelium was homogenised in 1ml distilled water and counts were made with a haemocytometer.

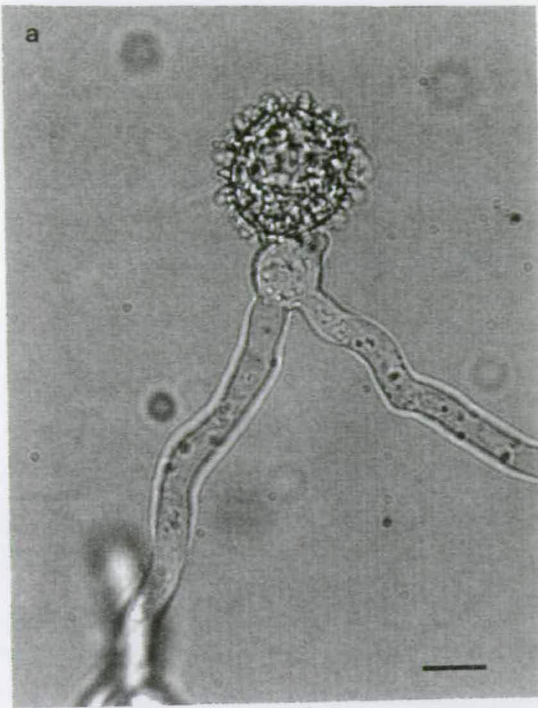
As shown in Figure 4.6, maximum biomass was reached after only 3 days incubation in 3% molasses and it remained relatively constant throughout the 70 day period. The oospore number reached a maximum at about 14 days, and also remained relatively constant thereafter. No difference in the structure of the oospores was seen at the various time intervals after this period.

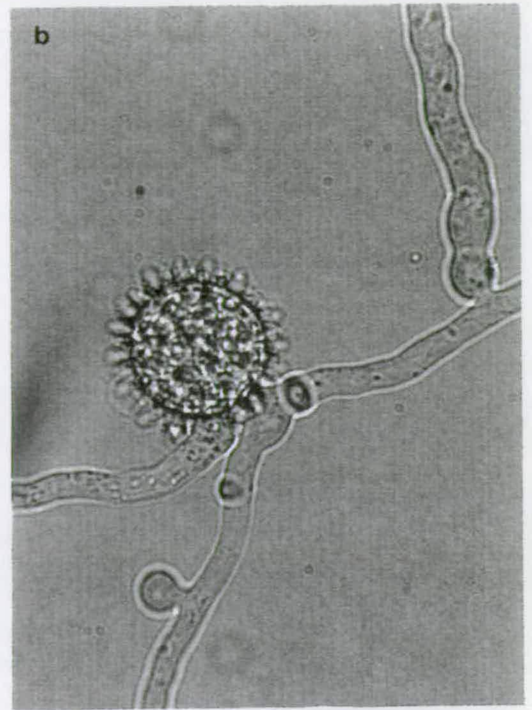
No germination of oospores was seen from cultures harvested at 7 days or less, and little germination was seen even from the 14 and 21 day cultures. With longer culture incubation time, the percentage germination progressively increased, up to a maximum of *c.*26% for oospores harvested after 49 days incubation.

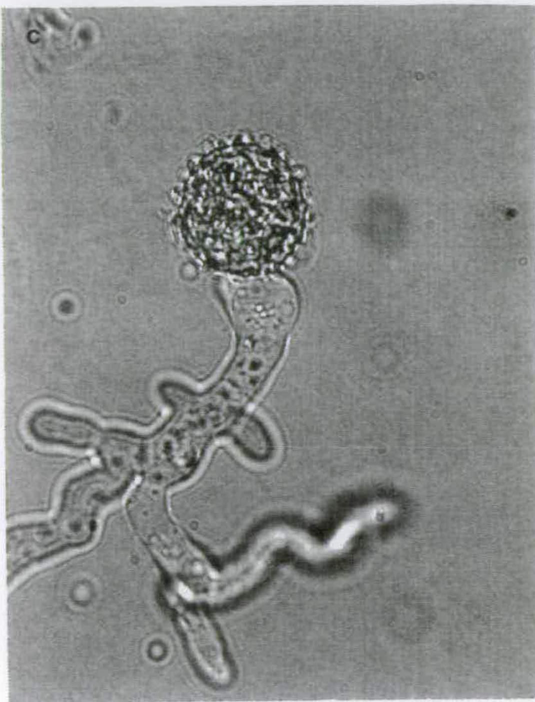
From the results, it seems that oospores are produced relatively early, after an initial phase of vegetative growth. But the oospores, once formed, appear to need a maturation period before they can germinate.

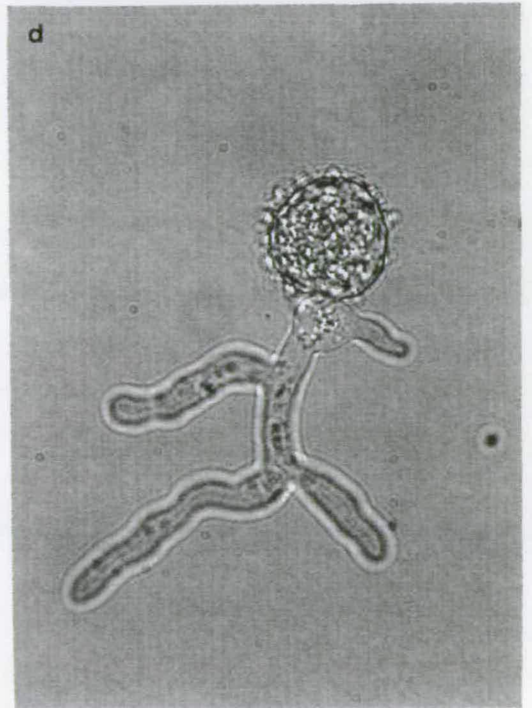
4.2.3 Effect of culture incubation temperature on percentage germination of oospores of P. oligandrum

P. oligandrum was grown as before in 3% molasses at either 25°C or 18°C and harvested after 35 days. Percentage germination of the harvested oospores was also









Figures 4.3a-d. Video copy prints of germinating oospores of *P. acanthophoron* (IMI 330 382) on PDA. Bar represents 10μm. Note the swelling of the germ tube near its point of emergence, and branches originating from the swelling.

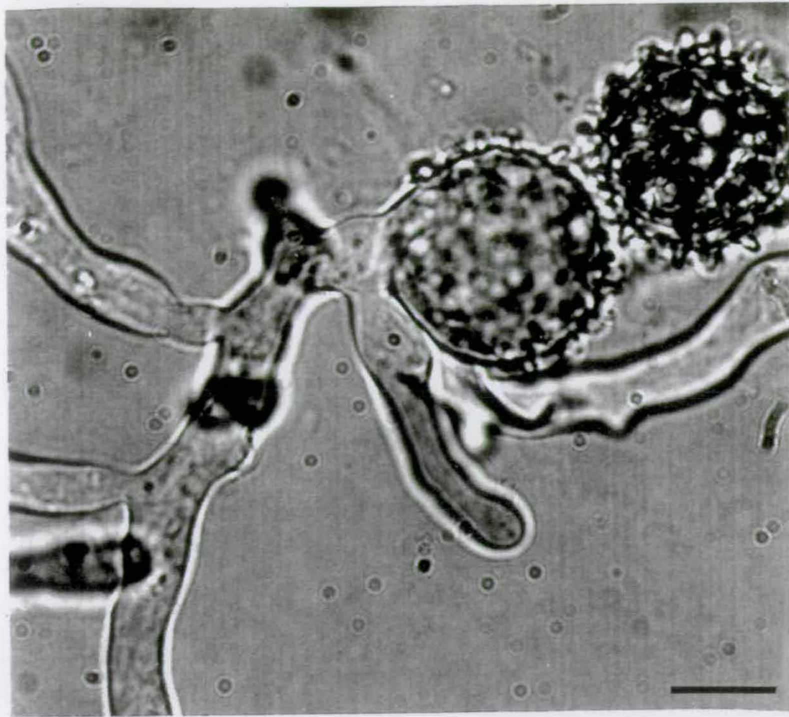


Figure 4.4 Germinating oospore of *P. acanthophoron* (IMI 330 382) on PDA. Bar represents 10μm. Note that the cytoplasm of the germ tube is continuous with that of the oospore.

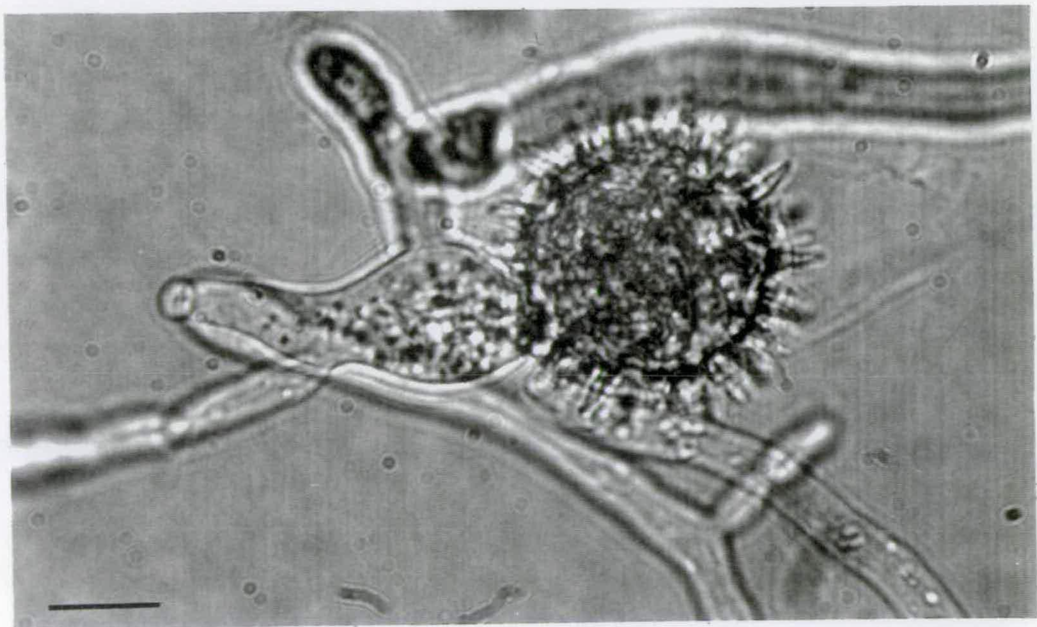


Figure 4.5 Germinating oospore of *P. oligandrum* (P14.1.2) on PDA plate. Bar represents 10μm. Note the swelling of the germ tube near its point of emergence, and numerous branches originating from the swelling.

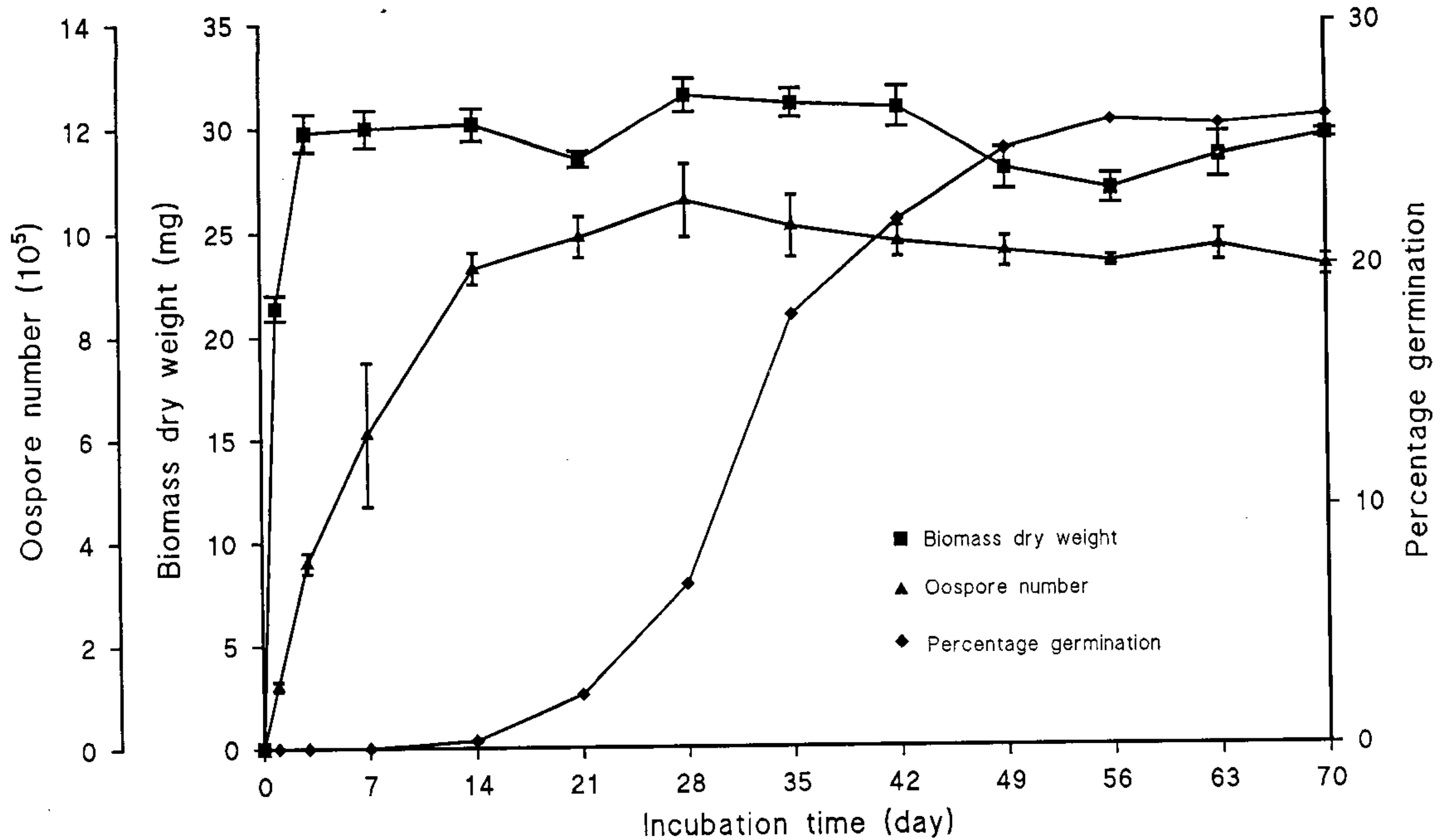


Figure 4.6 The effect of culture incubation time (day) on oospore number (x10^5), biomass dry weight (mg) and germinability of oospores of *P. oligandrum* grown in 3% molasses liquid medium. Mean of 5 replicates ± S.E.M for biomass and oospore counts, but 4 replicates for oospore germination.

assessed on agar plates incubated at 18°C and 25°C. Biomass and oospore counts were determined and compared between temperatures by t-test. Percentages of germination were transformed to arcsines and analysed by two-way analysis of variance.

There was no significant difference in final biomass yield or oospore counts between the two temperatures (Table 4.1) but oospores produced in the cultures at 25°C germinated significantly better than those produced at 18°C. Also, germination of the oospores from both types of culture was significantly higher after 18 h when the spores were incubated at 25°C than at 18°C. Oospores germinating at 18°C had much shorter germtubes than those germinating at 25°C, but this did not influence the assessment of germination because the 'non-germinated' spores had not even begun to produce germtubes.

4.2.4 Germination of oospores of P. oligandrum *produced in shake cultures compared to static cultures*

In earlier experiments (Section 3.3.4) *P. oligandrum* (P14.1.2) produced more oospores per mg of biomass in conical flasks incubated on an orbital shaker than in static cultures in medical flats. The percentage germination of the oospores produced by these two culture techniques was now compared.

Medical flats were used as above and incubated for 35 days, at 25°C in the dark. Shake cultures were prepared as explained in Section 3.3.4, with 250ml conical flasks containing 100ml of 3% molasses medium and incubated on an orbital shaker (110 rpm) for 35 days at 25°C in the dark. There were 5 replicates in both cases. Oospore germination was assessed after harvesting as before, on PDA plates incubated at 25°C in the dark for 18 h.

The germination of oospores produced in static culture (16.8%) was not significantly different from that of oospores produced in shake culture (18.4%).

4.3 Viability of oospores assessed with tetrazolium bromide

The maximum germination observed for *P. oligandrum* (P14.1.2) in the previous experiments was *c.*26%, so it was of interest to determine whether the remaining oospores were viable but non-germinable, or non-viable. Sutherland & Cohen (1983) used tetrazolium bromide as a stain for viability of oospores of several Oomycetes. The basis of this staining is that the colourless tetrazolium salt is reduced

Table 4.1 Effect of culture incubation temperature (25°C or 18°C) on the biomass yield, oospore count and germinability of oospores of *P. oligandrum* harvested from 3% molasses after 35 days in the dark. Means ± S.E.M. of 5 replicate flats for biomass and oospore counts (4 replicates for oospore germination).

		Culture incubation at 25°C	Culture incubation at 18°C
Biomass (mg)*2		27.0 ± 1.5	27.2 ± 1.5
Oospore count (10^5)*2		8.3 ± 0.7	7.3 ± 0.6
Germination *1	at 25°C	17.4 ± 0.4[c]	9.0 ± 1.0[b]
	at 18°C	3.3 ± 0.1[a]	1.1 ± 0.2[a]

*1 Values not followed by the same letter differ significantly ($P < 0.01$) by analysis of variance of arcsine-transformed data.

*2 No significant difference ($P < 0.05$) in means by t-test.

by the cellular dehydrogenases to its coloured formazan product if cells are viable. Differences in the colour of oospores were considered to represent different states of the oospores: a rose colour indicated dormancy, a blue colour indicated activation or germination, and black or clear oospores were considered to be ungerminable (Sutherland & Cohen, 1983).

This staining technique was now applied to oospores of *P. acanthophoron* (IMI 330 382), *P. oligandrum* (P14.1.2) and *P. mycoparasiticum* (AR7A). Oospore suspensions of approximately 2 x 10^5 oospores per ml were prepared as described earlier. A 0.1% solution of 3-(4-5-dimethylthiazol-2-yl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT; Aldrich Chemical Company) in sterile distilled water was used. One ml of this solution was added to 1ml oospore suspension in sterile test tubes. These were then incubated at 37 $\pm$ 1°C for 72 h. Samples were withdrawn after 2, 4, 8, 24, 32, 48, 56 and 72 h. Two replicates were set up per *Pythium* species, and assessments were made on a total 600 oospores for each fungus at each time.

As shown in Figure 4.7, more than 90% of the oospores of *P. acanthophoron*, and about 70% of those of *P. oligandrum* stained after 48 h incubation with MTT. Further incubation in the stain did not increase the number of stainable spores. *P. mycoparasiticum* (AR7A) only showed a maximum 58% of oospores that stained. Of the 92% of oospores of *P. acanthophoron* that were stained after 48 h, 91% were rose coloured (dormant) (Figure 4.8a) and only 1% were black (presumed non-viable). Of the 72% of oospores of *P. oligandrum* that stained after 48 h, 58% were rose coloured, 13% black and 1% were blue (indicative of activation). Many of the black oospores were grossly distorted (Figure 4.8b). All 58% of the stained oospores of *P. mycoparasiticum* were black (presumably non-viable).

Autoclaved oospores of *P. oligandrum* were incubated in MTT for 48 h. Over 60% of these oospores were observed to have stained, but all were black indicating that the oospores were non-viable.

The results indicate that the low percentage germination of oospores of both *P. oligandrum* P14.1.2 and *P. acanthophoron* IMI 330 382 was due to the oospores being dormant but viable, rather than being non-viable. However, the lack of germination of oospores of *P. mycoparasiticum* AR7A seems to have been due to a lack of viability by the criterion of Sutherland & Cohen (1983).

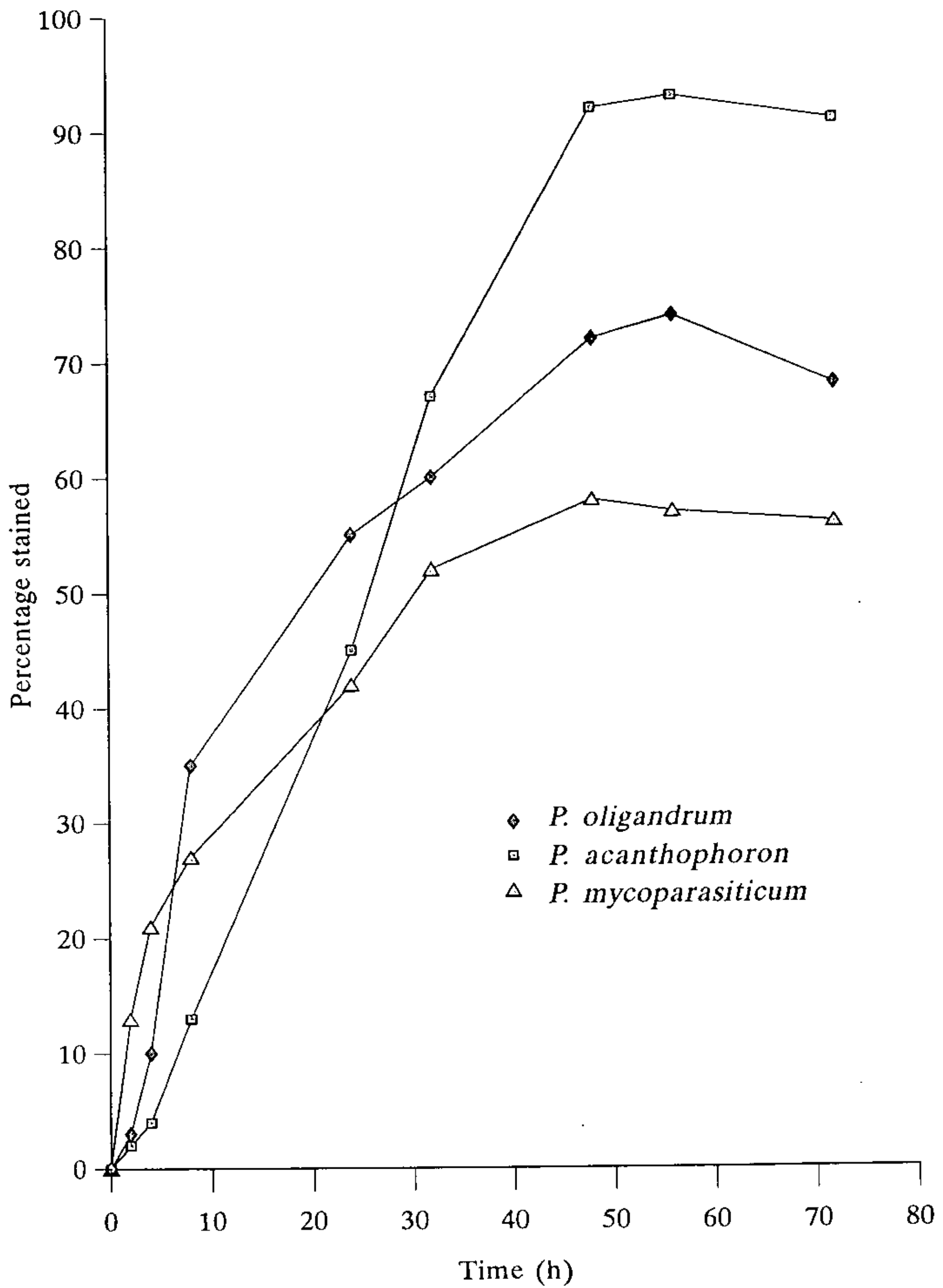


Figure 4.7 Effect of incubation time on percentage of oospores of *P. acanthophoron*, *P. oligandrum* and *P. mycoparasiticum* stained with tetrazolium dye (MTT); data points are means of 6 replicate assessments (total 600 oospores).

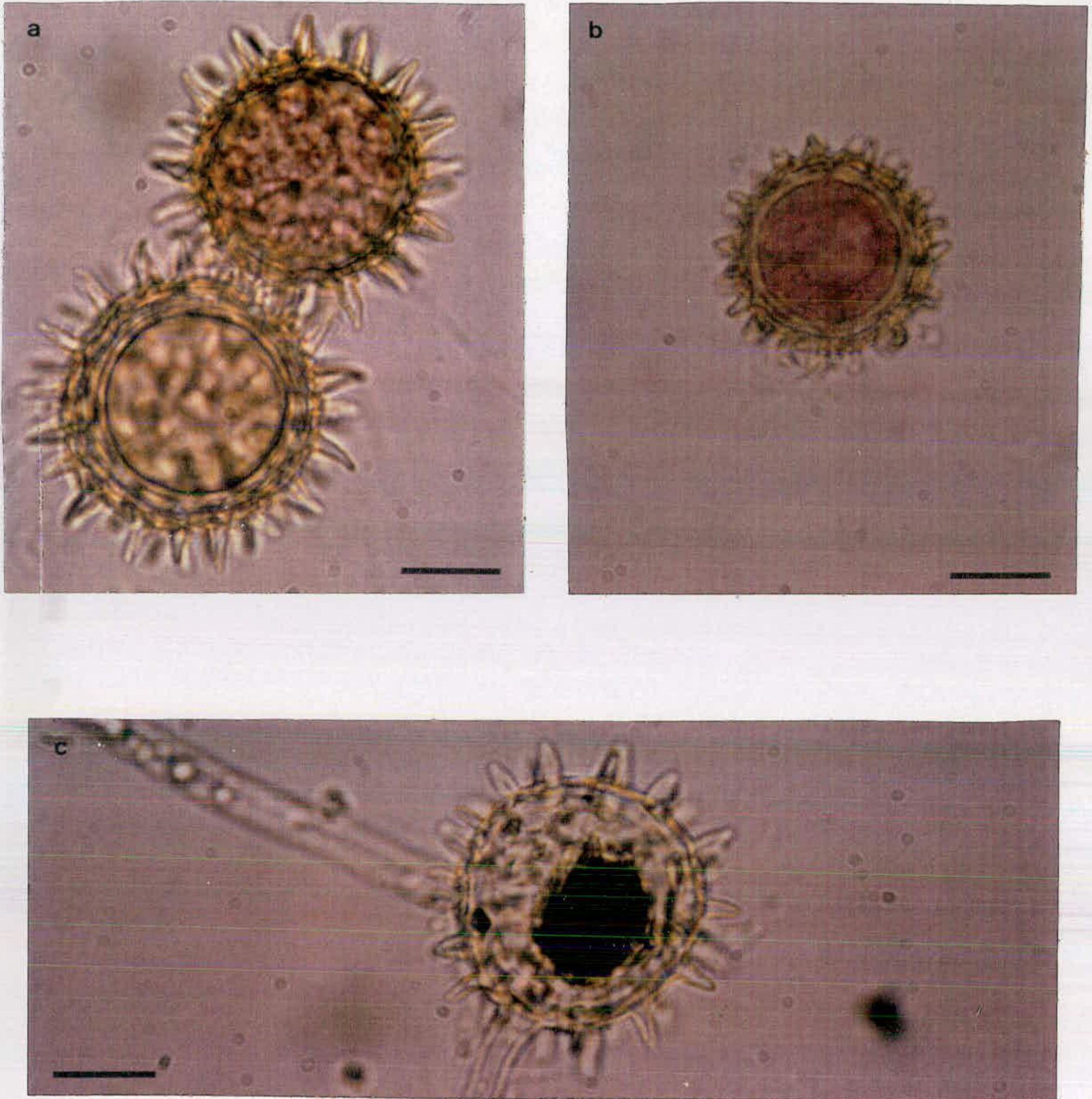


Figure 4.8 Oospores of *P. oligandrum* (a,c) and *P. acanthophoron* (b) stained with 3-(4-5-dimethylthiazol-2-yl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) stain. Bar represents 10μm. Viable but dormant oospores stain rose coloured; non-viable oospores stain black (c) and clear (a) .

4.4 Effect of nutrients on germination of P. oligandrum oospores

4.4.1 Effects of different agar media

The germination of oospores of *P. oligandrum* on four different agar media was tested. The media were potato-dextrose agar (PDA), malt extract agar (MEA), half-strength PDA (HS PDA) and sunflower seed extract agar (SSA). Oospore suspensions (35 day old) of *P. oligandrum* (P14.1.2) were prepared from 3% molasses medium as described earlier; the media for germination were supplemented with 200 μg vancomycin and 100 μg penicillin G per litre, and germination was assessed after 18 h at 25°C in darkness. Percentage data from 4 replicate agar plates were arcsine-transformed and compared by one-way analysis of variance.

The largest percentage germination was recorded on PDA (Table 4.2), with somewhat less (but not significantly so) on half-strength PDA, significantly less on MEA and very little germination on SSA.

4.4.2 Effects of peptone and malt extract

Oospores of *P. oligandrum* (P14.1.2), prepared as previously, were suspended in concentrations of bacteriological peptone or malt extract, ranging from 0.5% to 2%, and placed on cavity slides. The final oospore concentration was $1x10^5$ oospores per ml, and germination was assessed after incubation at 25°C in a moist chamber for 18 h.

As shown in Table 4.3, bacteriological peptone at both 2% and 1% supported a significantly higher percentage germination compared to malt extract at equivalent concentrations. In all cases, however, germination was strongly nutrient dependent, because no germination was recorded in distilled water, little was seen at 0.5% nutrient concentrations, and even 1% nutrient source gave significantly less germination than did 2%. Notable also is the finding that both a mixture of amino acids (bacteriological peptone) and a predominently sugar source (malt extract) triggered the germination of oospores.

Table 4.2 Percentage germination of oospores of *P. oligandrum* on different agar media after 18 h incubation at 25°C; means ± S.E.M. for 4 replicates.

Agar medium	Percentage germination *
PDA	18.7 ± 0.3[c]
HS PDA	16.5 ± 1.0[c]
MEA	13.0 ± 1.0[b]
SSA	1.3 ± 0.6[a]

* Values not followed by the same letter differ significantly ($P < 0.01$) by one-way analysis of variance of arcsine-transformed percentages.

Table 4.3 Effect of different nutrients on percentage germination of oospores of *P. oligandrum* after 18 hours incubation in the dark; means ± S.E.M. for 4 replicates.

Medium	Percentage germination *
Bacteriological peptone 2%	22.6 ± 0.4[e]
Bacteriological peptone 1%	10.0 ± 0.5[c]
Bacteriological peptone 0.5%	2.4 ± 1.5[a]
Malt extract 2%	14.5 ± 0.9[d]
Malt extract 1%	6.1 ± 1.2[b]
Malt extract 0.5%	2.5 ± 1.0[a]
Distilled water	0[a]

* Values not followed by the same letter differ significantly ($P < 0.01$) by one-way analysis of variance of arcsine-transformed data.

4.5 Effect of incubation of freshly harvested oospores of P. oligandrum in sterile distilled water on subsequent germination

Walther & Gindrat (1987a) reported that incubation of freshly harvested mycelial mats of *P. oligandrum* from carrot extract medium in sterile distilled water for 10 days increased the germination of 3 out of 4 isolates compared with equivalent prolonged incubation in the carrot extract medium. To investigate this effect further, cultures of *P. oligandrum* (P14.1.2) were grown in 3% molasses medium at 25°C in the dark, and 15 medical flats were harvested at each 7 day interval up to 49 days. Of the 15 flats, the biomass of 5 flats was air-dried on a laminar-flow bench overnight (16 h). The air-dried mycelia were pooled, resuspended in SDW and homogenised for 60 seconds at 20,500 rpm using an Ultraturrax T-25 blender. The resulting oospore suspension was washed by centrifugation and the pellet was resuspended in SDW to achieve a final concentration of $1x10^5$ oospores per ml. Of this suspension, 0.5ml aliquots were plated on each of 4 replicate PDA plates and incubated at 25°C for 18 hours in the dark. The biomass of another 5 flats was treated in a similar manner, but the contents of each flat were treated separately. The resulting oospore suspension from each flat was washed by centrifugation, resuspended in 12ml SDW and reincubated in a medical flat at 25°C in the dark for a further 7 days. Then the contents of these 5 flats were washed by centrifugation, and the pellet resuspended in SDW to get a final concentration of $1x10^5$ oospores per ml distilled water. The oospore germination was assessed as before. For the remaining 5 flats the mycelial mats were rinsed in SDW but not homogenised, then reincubated in 12ml SDW in medical flats for a further 7 days in darkness. After this, the mats were harvested, homogenised and assessed for germination as before.

As shown in Table 4.4, oospores harvested directly from molasses medium showed low germinability until the cultures were at least 28 days old, but near-maximum germination that was achievable in the conditions of these experiments once the cultures were 35 days old. When mycelial mats from molasses medium were washed and resuspended in distilled water for 7 days then used to prepare oospore suspensions, these oospores showed at least as much germination as the oospores left for a further 7 days in molasses culture (eg. in Table 4.4 compare the 21 day treatment "mycelial mat, harvested after 7 days in SDW" with the 28 day treatment "direct harvesting"). This indicates that the oospores required ageing or maturation period in order to germinate but this period was nutrient-independent. In contrast to these findings, oospores prepared as a suspension,

Table 4.4 Effect of reincubation of *P. oligandrum* in distilled water as both oospore suspension and as whole mycelial mats, on subsequent germination compared with that of oospores harvested direct from molasses culture: means of 4 replicates with arcsine-transformed mean ± S.E.M. in parentheses.

Culture age (days) in molasses	Percentage germination		
	Direct harvesting	Harvest after 7 days in SDW	
		Mycelial mat	Oospore suspension
7	0	7.6 (4.3 ± 0.8)	0
14	2.2 (1.3 ± 0.3)	4.5 (2.5 ± 0.2)	0
21	3.3 (1.9 ± 0.2)	8.2 (4.7 ± 0.4)	0.2(0.1 ± 0.03)
28	7.3 (4.2 ± 0.3)	19.9 (11.5 ± 0.6)	0.8(0.4 ± 0.1)
35	19.2 (11.1 ± 0.4)	21.3 (12.3 ± 0.2)	0.5(0.3 ± 0.1)
42	22.0 (12.7 ± 0.3)	25.7 (14.9 ± 0.3)	0.2(0.1 ± 0.04)
49	24.6 (14.2 ± 0.7)	NT*	NT

* NT, Not tested.

free from mycelia, immediately after harvest from molasses medium, then stored for 7 days in water showed consistently low germination. This might indicate that the maturation period needed for germinability must occur while the oospores are attached to parent mycelium. But there are alternative hypotheses, discussed in Section 4.11.

4.6 Effect of pH on oospore germination

For maximum biomass production of *P. oligandrum* in liquid culture, the optimum pH is between 6.0 and 7.0, though *P. oligandrum* can grow over the range of pH 5.0 to 9.0 (McQuilken *et al.*, 1992a) but not at pH 4.5 or pH 9.5. The germination of oospores at different pH values has not been investigated for *P. oligandrum*, and for *P. acanthophoron* there is no information on the effect of pH on either biomass production or oospore germination.

Oospores of both *P. oligandrum* and *P. acanthophoron* were prepared as previously from 35 day old cultures in 3% molasses medium in the dark at 25°C. Then the oospores were suspended in 1% bacteriological peptone with either citrate-phosphate buffer for the pH range 3.6 to 7.0, or phosphate-phosphate buffer for pH 6.0 to 8.0. The final oospore concentration was 1×10^5 per ml, in 0.2M buffer. The overlap of one pH unit for the two buffers enabled the effects of buffer components to be separated, to some extent, from effects of pH *per se*. For each pH value, 0.5 ml of oospore suspension was added to each of four cavity slides which were then incubated in a moist sealed chamber in the dark at 25°C for 18 h (*P. oligandrum*) or 19 h (*P.acanthophoron*). An unbuffered control (pH 6.7) comprised 1% bacteriological peptone alone.

Both *P. oligandrum* and *P. acanthophoron* showed a similar germination response to pH (Figures 4.9 and 4.10). The optimum pH was from 5.6 to 7.0 (of the pH values tested). Significantly less germination occurred at pH 5.0 ($P < 0.01$ by one-way analysis of variance of arcsine-transformed percentages) and little germination occurred at pH 4.6 or 7.6. Unbuffered controls showed 9% germination for *P. oligandrum* and 9.2% for *P. acanthophoron*.

4.7 Effect of oospore concentration on germination

In order to test the possibility that oospores of *P. oligandrum* exhibit self-inhibition at the concentrations used in previous experiments, a series of oospore suspensions ranging from 10^3 to 10^6 oospores ml^{-1} distilled water was spread on PDA

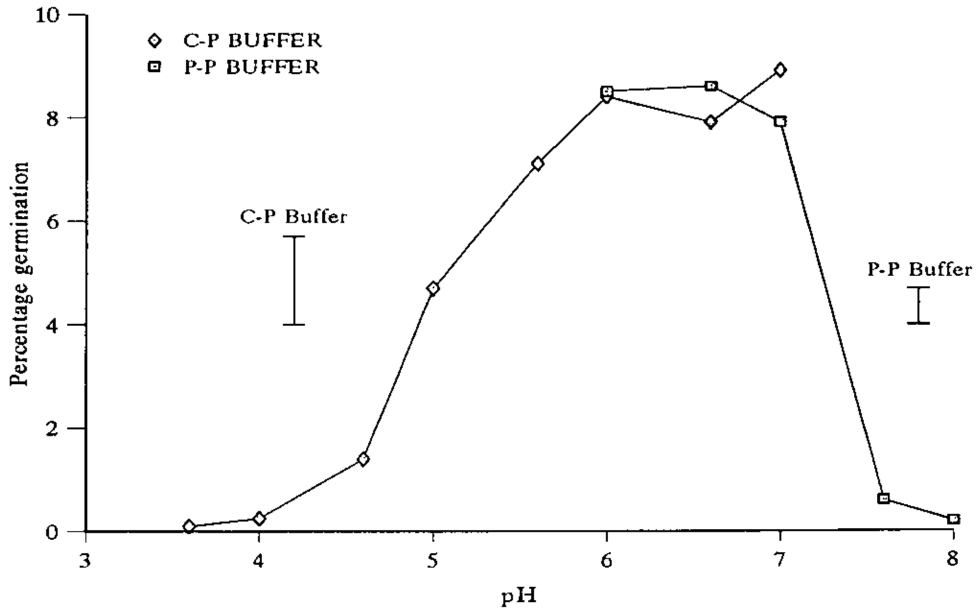


Figure 4.9 Effect of pH on the germination of oospores of *P. acanthophoron*, in 1% bacteriological peptone, using citrate-phosphate (C-P) and phosphate-phosphate (P-P) buffers; means of 4 replicates with the bars representing L.S.D. at P < 0.05.

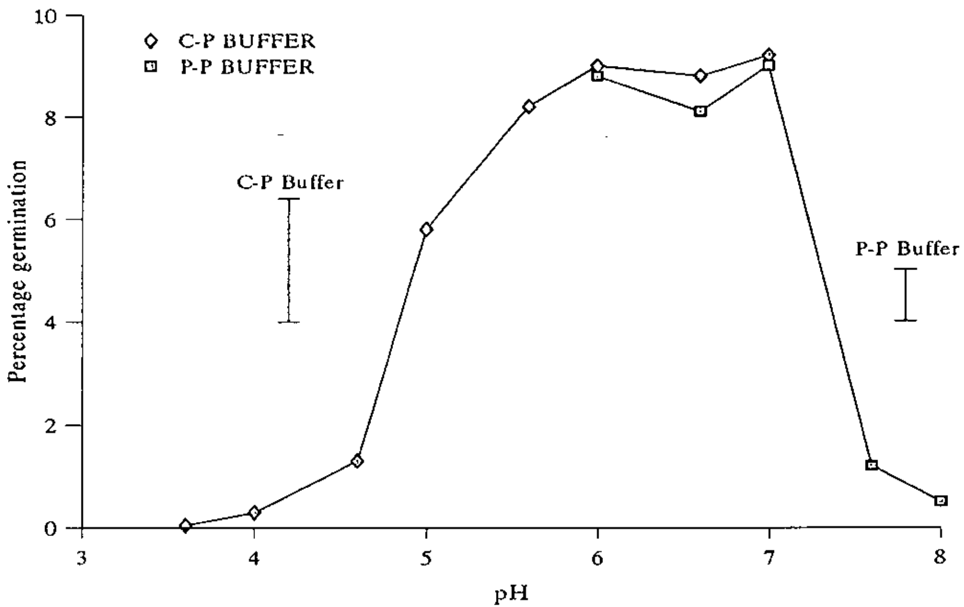


Figure 4.10 Effect of pH on the germination of oospores of *P. oligandrum* in 1% bacteriological peptone using citrate-phosphate (C-P) and phosphate-phosphate (P-P) buffers; means of 4 replicate with the bars representing L.S.D. at P < 0.05.

plates containing antibiotics, but in all cases the percentage germination lay between 19.2 and 20.5, with no significant difference.

4.8 Effect of potassium permanganate on oospore germination

Ruben *et al.* (1980) reported that germination of oospores of *P. aphanidermatum* was increased by treating desiccated oospores with 0.1% potassium permanganate solution following a short incubation at high temperature. Similar treatment of oospores of *Phytophthora megasperma* f. sp. *medicaginis* was also reported to increase their germination (El-Hamalawi & Erwin, 1986). The effect of potassium permanganate on the germination of oospores of *P. oligandrum* was thus investigated.

Oospores, prepared as usually, were suspended in 10ml of freshly prepared potassium permanganate solution at 0.1%, 0.01% or 0.001% concentration to give a final oospore concentration of 1 x 10^4 ml^{-1}, then vigorously agitated with a vortex mixure for 10 min and washed three times, with centrifugation at 3600 rpm (2333*g*) for 2 min. The pellets from the final wash were resuspended in sterile distilled water to achieve an oospore concentration of 1 x 10^4 ml^{-1}. A control involved oospores treated in the same way but using sterile distilled water instead of potassium permanganate solution. Germination was assessed after incubation on PDA supplemented with antibiotics for 18 h at 25°C in darkness.

As shown in Table 4.5 pretreatment of oospores with 0.1% potassium permanganate significantly decreased the percentage germination compared to the control. Both 0.01% and 0.001% potassium permanganate, however, significantly increased the percentage germination compared to the control, with the 0.001% concentration being the most effective.

4.9 Germination of oospores of different *P. oligandrum* isolates

The percentage germination of nine *P. oligandrum* isolates was compared to that of *P. oligandrum* isolate P14.1.2, using the standard conditions for culturing of the fungi and preparation of oospores, then incubating the oospores on PDA with antibiotics, for 18 h at 25°C in darkness. There were four replicate plates throughout, and 500 oospores were assessed on each plate.

Table 4.5 The effect of potassium permanganate on oospore germination of *P. oligandrum*; means ± S.E.M. for 4 replicates.

Potassium permanganate concentration	Percentage germination *
0	16.8 ± 0.2^{b}
0.1	5.1 ± 0.3^{a}
0.01	27.8 ± 0.6^{c}
0.001	30.8 ± 0.3^{d}

* Values not followed by the same letter differ significantly ($P < 0.01$) by one-way analysis of variance of arcsine-transformed data.

Table 4.6 Germination of oospores of different *P. oligandrum* isolates after 18 hours incubation on PDA plates in the dark; means of 4 replicates with arcsine-transformed means ± S.E.M. in parentheses.

P. oligandrum isolate	Percentage germination
P14.1.2	21.5 (12.4 ± 0.3)
Fc9.2.3	N.C.*
FcB.2.4	19.2 (11.0 ± 0.4)
BcB.8.3	8.5 (4.9 ± 0.3)
FcB.4.1	12.7 (7.3 ± 0.4)
Bc3.1	6.2 (3.6 ± 0.9)
Bp9.2.4	N.C.
P7.4	1.9 (1.1 ± 0.2)
F13.9	3.0 (1.7 ± 0.3)
P14.4.8	18.8 (10.8 ± 0.5)

*N.C. = Not counted, due to extensive germling growth.

As shown in Table 4.6, the percentage germination varied considerably for different isolates. For two isolates (F 9.2.3 and Bp9.2.4) the germination could not be quantified due to extensive germling growth indicating that these two isolates germinate sooner than the others. Two other isolates showed similar percentage germination to that of P14.1.2 and two isolates showed very low germination.

In a similar experiment, 10 isolates of *P. mycoparasiticum* were compared. But none showed germination after 28 h or 72 h at 25°C on PDA.

4.10 Effect of storage of dried culture biomass or oospore suspensions on subsequent oospore germination by P. oligandrum

Cultures of *P. oligandrum* were grown in 3% molasses medium at 25°C, harvested and dried on a laminar air bench overnight as previously described. Half of the dried biomass, as before, was used to produce an oospore suspension of $1x10^5$ oospores ml^{-1} distilled water. The oospore germination of a sample was determined immediately, as in previous experiments. Then samples of this oospore suspension were stored in the dark at room temperature (*c.*22°C) or at 5°C. The rest of the dried biomass was split between two Petri dishes and these were sealed with parafilm and stored in desiccators, over silica gel, at either 5°C or room temperature. In all cases, samples of oospores were tested for germination on PDA plates after 1, 3, 7, 14, 21, 28, 42, 56, 70, 84, 112 and 168 days storage. In the case of stored culture biomass, representative samples were taken at these times and used to prepare oospore suspensions. A one-way analysis of variance of arcsine-transformed percentage germination was carried out on the results after 21 and 42 days storage and a t-test on the results from the dried biomass after 84 days storage.

As shown in Figure 4.11, storage in any condition led to an initial rapid reduction in percentage germination of the oospores, from an initial 16.5% germination. But oospores in dried biomass retained at least some germinability when stored at both temperatures.

In this experiment oospore suspensions were tested for viability using MTT stain as in Section 4.2. Initially, 63% of oospores stained rose and 1% stained blue (both indicative of viability) whereas 15% stained black (non-viable) and the remainder did not stain. After storage of the dried biomass at room temperature for 6 months, 55% of the oospores stained rose and 23% stained black. Of those stored at 5°C, 60%

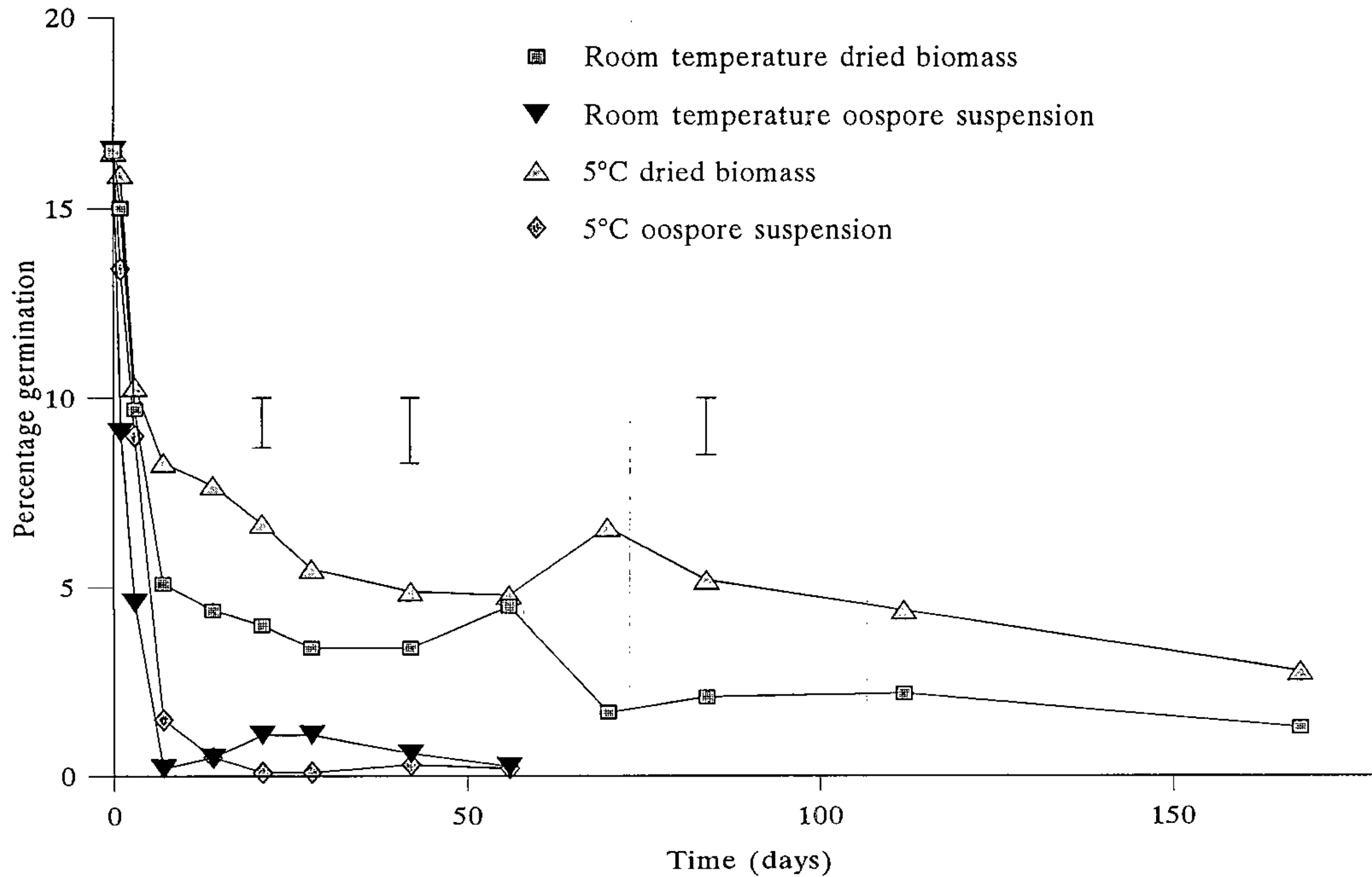


Figure 4.11 Effect of storage as oospore suspension or as dried biomass at room temperature or at 5°C on percentage oospore germination; means of 4 replicates with the bars representing L.S.D. at $P < 0.05$ at 21, 42 and 84 days.

stained rose coloured, 2% blue and 17% black. It therefore seems that the oospores retained their viability, but only a low percentage germinated.

4.11 Discussion

Oogonia of *P. acanthophoron* were seen to be morphologically distinct from those of *P. oligandrum*, having shorter spines with a rounded apex, compared to the longer sharp spines of *P. oligandrum*. Whilst antheridia were only rarely seen on oogonia of *P. oligandrum*, 1 or occasionally 2 bubble-like antheridia were seen on those of *P. acanthophoron*.

The germination process of both *P. oligandrum* and *P. acanthophoron* was very similar in that the ooplast progressively disappeared from the germinating spores, which normally germinated by a single germ tube. In most cases, this germ tube was then seen to enlarge near its point of emergence, with many branches developing from this swelling. In some cases, however, it seemed that the oospores *per se* did not germinate but a new hypha originated from regrowth of an antheridial-like structure on the surface of the oospore. When this occurred there was no evidence of depletion of the oospore contents or movement of oospore protoplasm into the developing hypha.

The culture method described here gave a very high yield of oospores of *P. oligandrum* relative to mycelium. Rapid vegetative growth occurred in the first 3 days of culture, then there was a shift to sexual reproduction, with the oospore number reaching a maximum after about 14 days incubation. Percentage germination increased with time up to a maximum of *c.*26% for oospores harvested after 49 days. However, no appreciable germination was seen of oospores from cultures harvested before 28 days, indicating that a period of maturation was required by the oospores before they are germinable. This has also been reported for *P. ultimum*, where oospore germination increased from 0% to more than 90% over a 6 week period in non-sterile soil extract (Ayers & Lumsden, 1975) suggesting that a period of maturation is required before the oospores are germinable.

Walther & Gindrat (1987a) reported that oospore germinability of 3 out of 4 tested isolates of *P. oligandrum* increased if the mycelia were washed and incubated in distilled water. This was, however, not the case with the isolate of *P. oligandrum* (P14.1.2) tested here. Instead, the increase in germination that occurred during storage

in water seemed to be due merely to the increased incubation time required for maturation of the oospores: similar germination occurred if the mycelia were harvested and subsequently incubated for a further 7 days in SDW, or if they were incubated in 3% molasses for the additional 7 days. This shows, however, that the maturation period is nutrient-independent. No such increase in germination occurred if the mycelia were macerated and the resulting oospores stored in water. This might indicate that the enhanced germinability is due to accumulation of nutrients or autolytic products from the starved mycelia into the oospores. Alternatively, the maceration procedure for producing mycelium-free oospores might have damaged these such that they were still germinable when initially tested but not after prolonged incubation. Laing (1989) also observed that the oospore maturation period required intact mycelia, but the percentage oospore germination that he recorded was not as low as here. The main difference between my experiment and that of Laing (1989) was that the oospores were dried here before rehydrating and incubating in SDW. Desiccation has been suggested by Ruben *et al.* (1980) to increase the permeability of the oospores by weakening the cell wall due to contraction of the wall polymers during desiccation and then expansion during rehydration. This might then result in leakage of nutrients from the oospore causing a decrease in viability during storage.

The low maximum percentage germination of the oospores observed could have been due to two factors: low viability of the oospores or dormancy controlled by endogenous or exogenous factors. Exogenously dormant spores fail to germinate due to unfavourable environmental conditions, whilst in constitutively or endogenously dormant spores the development is delayed due to an innate property of the spore itself (Sussman & Halvorson, 1966). Oospores of many *Pythium* spp. have been reported to have a dormancy period (Ayers & Lumsden, 1975; Lumsden & Ayers, 1975). This has also been suggested for *P. oligandrum* (Walther & Gindrat, 1987a).

The viability of oospores of *P. oligandrum* was tested using tetrazoliun bromide (MTT) as described by Sutherland & Cohen (1983). The basis of the test is that the cellular dehydrogenases in viable cells reduce the colourless tetrazolium salt to its coloured formazan product. Fifty-nine percent of the oospores were found to be viable, with the majority being in the dormant category as evidenced by this staining method. Similarly, the low germinability of oospores of *P. acanthophoron* was not due to lack of viability since over 91% were 'viable, but dormant' when stained with MTT. No germination of oospores of *P. mycoparasiticum* AR7A was observed, and all those that stained with MTT showed black colouration which is reported to indicate non-viability. Whether the lack of oospore germination for the other *P.*

mycoparasiticum isolates tested was also due to non-viable oospores is not known. It is possible that the wrong culture conditions were used for production of viable oospores of *P. mycoparasiticum*. Kent (1946, cited in Sussman & Halvorson, 1966) reported that spores (conidia) of *Diplodia zeae* (*Diplodia maydis* (Berk.) Sacc.) from colonies grown on oatmeal agar germinated much more effectively than those from any other medium. Similarly, temperature of spore production can influence viability and germinability. Van Arsdel *et al.* (1956, cited in Sussman & Halvorson, 1966) reported that teliospores of the rust *Cronartium ribicola* A. Fisch. formed at 16°C germinated very well, but those formed at 20°C germinated erratically.

Germination of oospores of *P. oligandrum* was seen to be strongly influenced by nutrients, with bacteriological peptone (a mixture of amino acids and peptides) and malt extract (mainly sugars) supporting germination. At each concentration, bacteriological peptone was superior to malt extract, indicating that germination is enhanced by a mixture of amino acids more than by a mixture of sugars. On the agar media, the nutrients supplied by PDA stimulated more germination than those provided by MEA. Sunflower seed extract, however, supported very low oospore germination, indicating that either sterols are not required for oospore germination or that SSA does not contain enough or not the right nutrients to initiate oospore germination. Halving the concentration of PDA gave no significant reduction of the percentage germination compared to full strength PDA, suggesting that even at this concentration, it contains sufficient nutrients to initiate germination of the activated oospores. None of the different nutrient sources tested appeared to increase germination above the *c.* 19% achieved on full-strength PDA. Other specific nutrients, however, might have increased germination further. For example, lecithin was observed by Ruben *et al.* (1980) to significantly increase germination of oospores of *P. aphanidermatum*, whilst Flowers & Littrell (1972) reported increased germination of oospores of *P. aphanidermatum* by addition of casein and gallic acid. Calcium has also been reported to be required for germination of oospores of *P. aphanidermatum* (Stanghellini & Russell, 1973).

Culture incubation temperature also plays an important role in germinability of oospores. There was no difference in the biomass dry weight or oospore number after 35 days for *P. oligandrum* grown at 25°C compared with 18°C. However oospores produced at 25°C had a higher percentage germination than those produced at 18°C; the amount of germination of oospores produced during 35 days at 18°C (Table 4.1) was close to that of oospores produced during 28 days incubation at 25°C in other experiments (Table 4.4). This could be due the oospores maturing faster at the higher

temperature, rendering them more germinable. Oospores produced at either temperature also germinated at a higher percentage, or more rapidly, at 25°C than at 18°C. This is of interest because 25°C is abnormally high for most natural soil environments where *P. oligandrum* is known to occur, and slow or low germination at 18°C could disadvantage *P. oligandrum* relative to several plant-pathogenic *Pythium* spp. during competition for seed exudates or other sources of nutrients.

Endogenous dormancy has been postulated to be caused by one of three factors: lack of permeability of the wall or membrane, presence of self-inhibitors or other types of metabolic impairment (Sussman & Halvorson, 1966; Garraway & Evans, 1984). Spores of certain fungi possess compounds that inhibit their germination. Many rust uredospores exhibit self-inhibition by producing compounds such as cinnamic acid derivatives. These inhibit germination by preventing hydrolysis of wall plug material to produce a pore through which the germtube emerges (Garraway & Evans, 1984). In *P. oligandrum*, however, maximum germination seemingly was not governed by self-inhibitors, since the percentage germination of high concentrations of oospores were not significantly different from that of low concentrations of oospores.

Ruben *et al.* (1980) reported that pretreatment of desiccated oospores of *P. aphanidermatum* with 0.1% potassium permanganate substantially increased their germination. Here treatment of desiccated oospores of *P. oligandrum* with 0.1% potassium permanganate was found to decrease germination but 0.01% and 0.001% permanganate significantly increased germination compared to that in controls. Potassium permanganate at 0.1% was reported also to increase the germination of oospores of *Phytophthora megasperma* f. sp. *medicaginis*, but 0.5% permanganate killed most of the oospores (El-Hamalawi & Erwin, 1986). Ruben *et al.* (1980) suggested that the activating effect of potassium permanganate treatment probably involved the chemical oxidation of wall components, in particular oxidation of the lipid components of the oospore wall, possibly altering the permeability of this. Beakes, El-Hamalawi & Erwin (1986) however suggested that the permanganate penetrates the oospore wall of *Phytophthora megasperma* and could affect the cell membrane. Prolonged treatment or use of higher potassium permanganate concentrations ultimately reduced oospore viability which suggests that this compound is toxic if allowed sufficient time to penetrate into the cytoplasm. *P. oligandrum* therefore seems to be more sensitive to potassium permanganate than are *P. aphanidermatum* and *Phytophthora megasperma*.

The last mechanism of endogenous dormancy, i.e. metabolic impairment, involves the presence of a metabolic block which affects key enzymes associated with respiration or other pathways, or inhibition of RNA and protein synthesis. However this mechanism might also involve self-inhibition or permeability effects (Sussman & Halvorson, 1966; Garraway & Evans, 1984).

Treatment with 0.001% potassium permanganate was the only treatment that caused any significant increase in percentage germination above controls, but even then the maximum germination was below 35%. A combination of treatments might be required to activate the oospores of mycoparasites. On the other hand, the presence of the host, in this case another fungus, might stimulate oospore germination. Barton (1957) reported that germination of oospores of *Pythium mamillatum*, a plant pathogen, was increased in response to exudates from living seedlings, and similar treatments might be tested for mycoparasites with their fungal hosts.

Storage of oospores as both dried biomass and oospore suspensions decreased their germinability. Those stored as dried biomass however, retained some germination, unlike those stored as oospore suspensions, where no appreciable germination was observed after 42 days storage. As suggested previously, the desiccation of the oospores and then rehydration to produce the oospore suspension has been indicated to increase the permeability of oospore walls (Ruben *et al.*, 1980). This could then result in loss of nutrients from the oospores, reducing their viability. The results with the dried biomass were similar to those reported by McQuilken *et al.* (1990a), i.e. reduction of germination was less pronounced when the oospores were stored at 5°C compared to 20°C or 25°C. Walther & Gindrat (1987a) however reported that treatment of oospores with myo-inositol increased germination during storage, possibly because myo-inositol protects spores from desiccation by substituting hydroxyl groups for water molecules in the microbial cell wall (Webb *et al.*, 1964). The decrease of germination in my study was not observed to be due to a loss of viability. Storage, however, might increase the time required for the oospore to germinate, or make the breaking of dormancy more difficult.

The germination of oospores of *P. acanthophoron* (IMI 330 382) was similar to that of *P. oligandrum*, with *c.*17% germination recorded after 19 h incubation for 35 day old cultures on PDA. The percentage germination of *P. acanthophoron* at the different pH values was again similar to that for *P. oligandrum* (P14.1.2), with the optimum being between pH 5.6 and 7.0. For both fungi, pH below 4.6 or above 7.6 was inhibitory to oospore germination. The pH range for oospore germination for *P.*

oligandrum is more narrow than that reported for vegetative growth (McQuilken *et al.*, 1992a).

For mass production of biocontrol inocula, shake or stirred cultures would be more efficient in terms of space and production capabilities. In the previous chapter, shake cultures were seen to yield more oospores per unit of biomass, and here it was shown that the percentage germination of oospores was the same as for those produced in static cultures. However for use as biological control inocula, such as for seed treatment, the generally low oospore germination of mycoparasitic *Pythium* may still be the limiting factor. Different *P. oligandrum* isolates were seen to have different inherent germination rates under identical conditions, confirming the findings of Walther & Gindrat (1987a). The use of isolates with a naturally high oospore germinability might therefore be advantageous for biocontrol. In this respect *P. acanthophoron* does not seem to offer advantages over *P. oligandrum*, although only one isolate of *P. acanthophoron* has been studied for oospore germination. If *P. mycoparasiticum* is to be used as an inoculant biocontrol agent then the lack of oospore germinability and viability must be overcome. In this study many attempts were made to induce germination by *P. mycoparasiticum* but they were unsuccessful and so have not been detailed here.

CHAPTER 5

ECOLOGY AND DETECTION OF MYCOPARASITIC PYTHIUM SPECIES FROM SOIL

Of the mycoparasitic *Pythium* species, *P. oligandrum* has been most frequently isolated from soil, followed by *P. acanthicum* (Schmitthenner, 1962; Vaartaja & Bumberis, 1964; Vaartaja, 1968; Hendrix & Campbell, 1970; Van der Plaats-Niterink, 1975; Deacon & Henry, 1978; Foley & Deacon, 1985; Dick & Ali-Shtayeh, 1986; Ribeiro & Butler, 1992; Mulligan & Deacon, 1992). Both *P. periplocum* (Hendrix & Campbell, 1970; Van der Plaats-Niterink, 1975; Ribeiro & Butler, 1992) and *P. acanthophoron* (Sideris, 1932; Quimio & Abilay, 1977; Lodha & Webster, 1990) have been isolated less frequently. *P. nunn* has, so far, only been reported from soil in Colorado, U.S.A (Lifshitz *et al.*, 1984a). *P. mycoparasiticum* has been detected only in Britain, and always by means of the precolonised plate method (Deacon & Henry, 1978; Foley & Deacon, 1985). Using this technique, however, it was found to be common in arable and other disturbed soils. Due to the scarcity of information about the occurrence of *P. mycoparasiticum* in soil, the experiments in this chapter were concerned with investigating the isolation of *P. mycoparasiticum* in comparison with other mycoparasitic *Pythium* species from soil.

5.1 Isolation of P. mycoparasiticum from soil

5.1.1 Precolonised agar plates

Both Deacon & Henry (1978) and Foley & Deacon (1985) isolated *P. mycoparasiticum* (= *Pythium* 'SWO') from soil less frequently than *P. oligandrum*, using agar plates precolonised by a *Phialophora* sp. However dilution of the soils using sand increased the detection of *P. mycoparasiticum*. Mulligan & Deacon (1992) reported that detection of different mycoparasites using the precolonised agar plate method was strongly influenced by the host fungus used. They, however, did not detect *P. mycoparasiticum*.

Here the method described in Section 2.6.2.1 was used, whereby plates of PDA were precolonised with one of five host fungi, *Phialophora* sp. (IMI 187786), *Fusarium culmorum*, *Fusarium oxysporum*, *Botrytis cinerea* or *Botryotrichum*

piluliferum. Each plate was cut into 6 equal sectors, and each of these was placed into a clean nonsterile plastic Petri dish. Samples of air-dried sieved soil were serially diluted 2-fold, using sterile, washed, sieved sand. Aliquots (0.4ml) of soil/sand mixture were placed on the youngest parts of 5 replicate host sectors. These were then incubated at room temperature, and microscopically examined after 7, 10 and 14 days for the characteristic oogonia of the mycoparasitic *Pythium* species. To obtain pure cultures of the mycoparasites, small squares of agar were cut from these sectors and placed on fresh PDA plates. For identification, the mycoparasites were subcultured onto SSA (Section 2.1), to enable production of their characteristic oogonia.

P. oligandrum was identified by its spiny-walled oogonia on the host plates. *P. periplocum* was also identified by its spiny wall oogonia, but distinguished from those of *P. oligandrum* by the antheridial arrangement (Waterhouse, 1968; Van der Plaats-Niterink, 1981). *P. mycoparasiticum* was identified by its characterisitic smooth-walled oogonia which had numerous antheridia attached (Deacon *et al.* 1991). The other three mycoparasitic *Pythium* spp. were not isolated in this study.

Selection of soils was based on the observation of Foley (1983) and Foley & Deacon (1985) that *P. mycoparasiticum* was most frequently isolated from arable soils, especially cereal and potato soils. Thus three cereal soils and one potato soil were used. They are labelled A - D (see Table 2.2).

Initially with one soil (soil A) 5 host fungi were used on 'detector plates' for the mycoparasitic *Pythium* spp. and for each host there were five replicate sectors for each of six soil dilution levels (1, 1/2, 1/4, 1/8, 1/16, 1/32). As summarised in Table 5.1, *P. mycoparasiticum*, *P. oligandrum* and *P. periplocum* were detected in this soil, with a predominance of *P. oligandrum*. The most efficient hosts on the detector plates seemed to be *F. culmorum*, *B. cinerea* and *Phialophora* sp. So these host fungi were used in equivalent tests on the other 3 soils. The results for all 4 soils are shown in Table 5.2.

P. periplocum was not detected in any of the other 3 soils, so its only occurrence throughout this study was on a single plate of *Phialophora* sp. when soil A was diluted 8-fold.

P. mycoparasiticum was detected in each of the four soils, and on each of the three host fungi for each soil. Comparison of the total number of host sectors bearing

Table 5.1 Summary of the number of detected occurrences of mycoparasitic *Pythium* spp. when various dilutions of soil A were added to sectors of agar precolonised by different host fungi (30 sectors for each host).

Precolonised host fungus	Total number of detected occurrences of the mycoparasitic *Pythium* spp.		
	P. mycoparasiticum	*P. oligandrum*	*P. periplocum*
Phialophora sp.	3	11	1
Botrytis cinerea	5	12	0
Fusarium culmorum	2	11	0
Botryotrichum piluliferum	0	9	0
Fusarium oxysporum	0	0	0

Table 5.2 The number of detected occurrences of mycoparasitic *Pythium* species when soil dilutions were placed on sectors of agar precolonised by different host fungi (5 replicate sectors for each host fungus for each dilution).

Mycoparasite detected on each host fungus	Number of detected occurrences of the mycoparasitic *Pythium* sp. at each dilution						
	1*	1/2	1/4	1/8	1/16	1/32	Total
SOIL A							
HOST: PHIALOPHORA SP.							
P. mycoparasiticum	0	1	1	1	0	0	3
P. oligandrum	5	3	2	0	1	0	11
P. periplocum	0	0	0	1	0	0	1
HOST: FUSARIUM CULMORUM							
P. mycoparasiticum	0	0	2	0	0	0	2
P. oligandrum	4	2	1	3	1	0	11
HOST: BOTRYTIS CINEREA							
P. mycoparasiticum	0	2	0	1	2	0	5
P. oligandrum	5	2	4	1	0	0	12
SOIL B							
HOST: PHIALOPHORA SP.							
P. mycoparasiticum	4	2	3	3	1	0	13
P. oligandrum	0	0	0	0	0	0	0
HOST: FUSARIUM CULMORUM							
P. mycoparasiticum	4	4	2	3	2	0	15
P. oligandrum	0	0	0	0	0	0	0
HOST: BOTRYTIS CINEREA							
P. mycoparasiticum	4	4	5	1	1	3	18
P. oligandrum	1	0	0	0	0	0	1

* no dilution

Table 5.2 continued.

Mycoparasite detected on each host fungus	Number of detected occurrences of the mycoparasitic *Pythium* sp. at each dilution						
	1	1/2	1/4	1/8	1/16	1/32	Total
SOIL C							
HOST: PHIALOPHORA SP.							
P. mycoparasiticum	5	4	4	2	0	1	16
P. oligandrum	0	0	0	0	0	0	0
HOST: FUSARIUM CULMORUM							
P. mycoparasiticum	5	5	3	4	1	1	19
P. oligandrum	0	0	0	0	0	0	0
HOST: BOTRYTIS CINEREA							
P. mycoparasiticum	2	3	3	0	0	0	8
P. oligandrum	1	0	0	0	0	0	1
SOIL D							
HOST: PHIALOPHORA SP.							
P. mycoparasiticum	1	3	4	5	2	0	15
P. oligandrum	4	2	0	1	0	1	8
HOST: FUSARIUM CULMORUM							
P. mycoparasiticum	1	1	1	0	0	1	4
P. oligandrum	4	4	2	1	1	0	12
HOST: BOTRYTIS CINEREA							
P. mycoparasiticum	2	3	2	1	1	0	9
P. oligandrum	1	0	0	1	0	0	2
TOTAL FOR ALL SOILS							
HOST: PHIALOPHORA SP.							
P. mycoparasiticum	10	10	12	11	3	1	47
P. oligandrum	9	5	2	1	1	1	19
HOST: FUSARIUM CULMORUM							
P. mycoparasiticum	10	10	8	7	3	2	40
P. oligandrum	8	6	3	4	2	0	23
HOST: BOTRYTIS CINEREA							
P. mycoparasiticum	8	12	10	3	4	3	40
P. oligandrum	8	2	4	2	0	0	16
Total for all hosts for each dilution							
P. mycoparasiticum	28	32	30	21	10	6	127
P. oligandrum	25	13	9	7	3	1	58
P. periplocum	0	0	0	1	0	0	1

P. mycoparasiticum for the four soils showed that no significant difference (X^2) between the precolonised host fungi for detection of *P. mycoparasiticum*: there were 47 detections on *Phialophora* sp., compared with 40 on *F. culmorum* and 40 on *B. cinerea*. In contrast to *P. mycoparasiticum*, *P. oligandrum* was uncommon in two of the soils (B and C) with only one detectable occurrence in each case, but *P. oligandrum* was detected commonly in the other two soils. Again, the total numbers of detected occurrences of *P. oligandrum*, combined for the four soils, showed no significant difference in detection efficiency by the three host fungi (19, 23 and 16 occurrences, respectively).

Further analysis of the detection data in Table 5.2 shows that for the soils in which *P. oligandrum* was uncommon (soils B and C), successive dilution of the soil led to a decrease in the number of detections of *P. mycoparasiticum*. However, when *P. oligandrum* was common in a soil (soils A and D) *P. mycoparasiticum* was seldom detected by plating of undiluted soil but the frequency of detection increased in the early dilutions, coinciding with decrease in detection of *P. oligandrum*.

It had originally been intended that the dilution series could be used to obtain values for the most probable number of propagules of *P. oligandrum* and *P. mycoparasiticum* in soils, but the apparent interference of detection of *P. mycoparasiticum* by *P. oligandrum* precluded this. Instead, in an attempt to test for significance of interference, an approximation of a log dose : probit response curve, when untransformed, was used. The aim of this was to linearise the data for efficiency of detection against dilution of soil, which would be expected to fit a sigmoid curve. So the number of detections of *P. mycoparasiticum* was expressed as a percentage, then transformed to a probit and plotted against logarithm of soil dilution, the undiluted soil being assigned a log value of 1.5 corresponding to 32 units of soil, and the final dilution (1 in 32) representing 1 unit of soil (log = 0). This procedure was done first for the total number of detections of all mycoparasitic pythiums (combined for all hosts for all soils) at each dilution (Figure 5.1A). It gave a very highly significant fit to a straight line, confirming the original expectation that the untransformed data would fit a sigmoid curve. The near-perfect correlation also, incidentally, showed that efficiency of detection of total mycoparasitic *Pythium* populations was not influenced by other factors such as other microorganisms that could be diluted out in series. Next the procedure was repeated for detection of *P. oligandrum* only (Figure 5.1B). The result was almost identical, indicating that *P. oligandrum* was detected at the same efficiency, with regard to its lower population

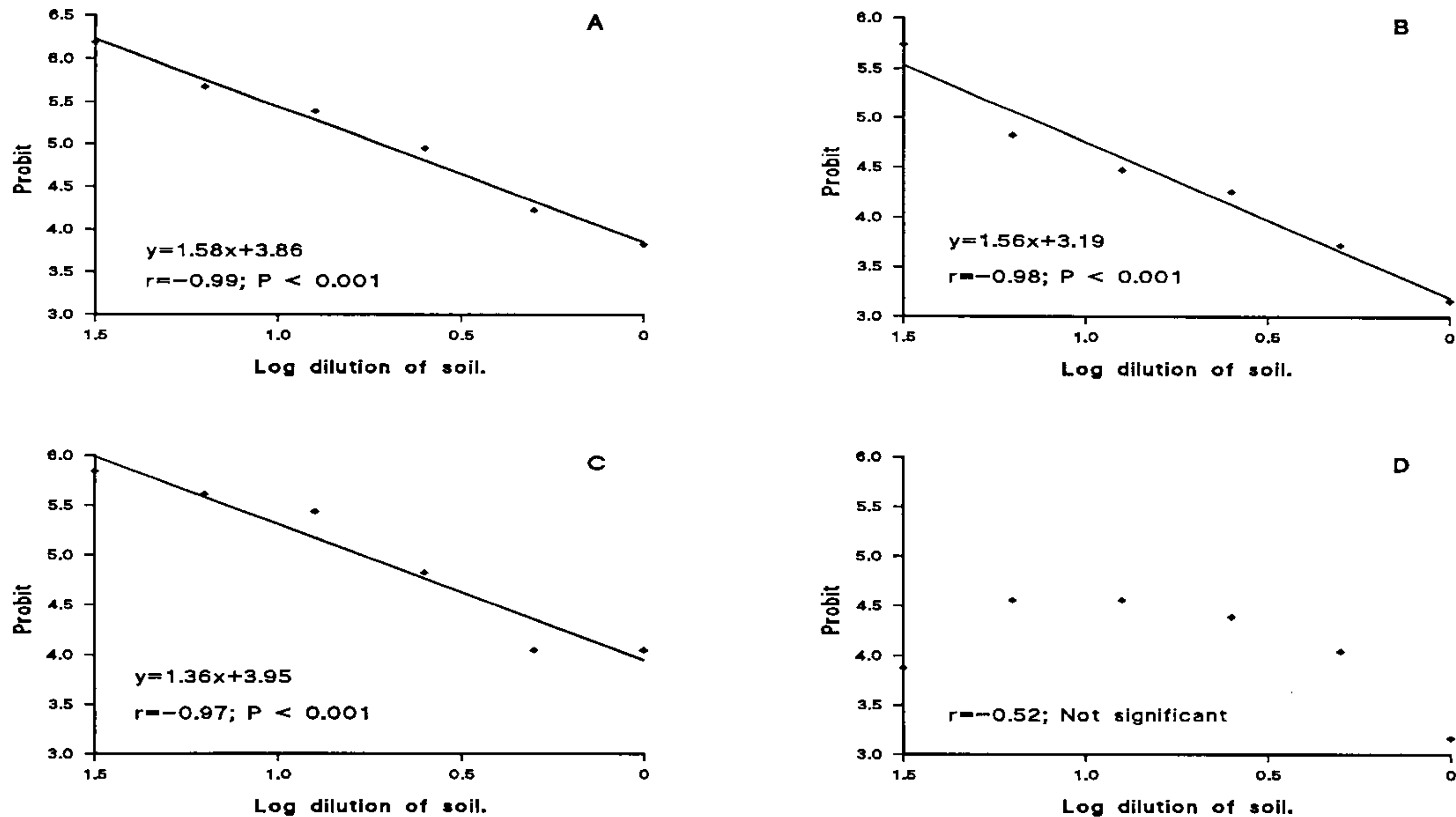


Figure 5.1 Relationship between probit-transformed percentage occurrence of mycoparasites and log of soil dilution for (A) total mycoparasitic *Pythium* spp. in 4 soils; (B) *P. oligandrum* in 4 soils; (C) *P. mycoparasiticum* in 2 soils with little/no *P. oligandrum* present; (D) *P. mycoparasiticum* in 2 soils with *P. oligandrum* present. In all cases the data were pooled for three types of host-precolonised sector (see Table 5.2). Undiluted soil was deemed to have 32 units of soil (log=1.5), and the highest dilution (1 in 32) to have 1 unit of soil (log=0).

level in dilution, across the dilution series. The procedure applied to *P. mycoparasiticum* in soils B and C (with virtually no *P. oligandrum* present) also gave a straight-line relationship (Figure 5.1C). However for *P. mycoparasiticum* in soils A and D (*P. oligandrum* present) there was no significant relationship between detection and dilution level of the soil (Figure 5.1D). This indicated that the efficiency of detection of *P. mycoparasiticum* in these two soils was subject to an interfering factor- most probably the presence of *P. oligandrum* which precluded detection of *P. mycoparasiticum* in the lower dilutions of the soil because *P. oligandrum* competed with *P. mycoparasiticum* on the host-precolonised agar.

The data from this experiment also revealed other features of interest. As shown in Table 5.3, a significantly higher number of *P. mycoparasiticum* detections were recorded after 10 days incubation of the sectors (X^2 test, $P < 0.01$) compared to 7 days, though there was no further increase during incubation for 14 days. In contrast, *P. oligandrum* was detected at near its maximum frequency after 7 days. Comparison of the data for the three hosts shows that most of the increased detection of *P. mycoparasiticum* after 10 days was accounted for by the slower development of this fungus on the sectors precolonised by *Phialophora* sp. (Table 5.3). During this experiment, also, *Papulaspora* sp. was detected on sectors of *B. cinerea*, but not on the other two hosts, and *Gliocladium* sp. and *Trichoderma* sp. similarly were detected on *B. cinerea* but not on the other hosts. The detections of these other mycoparasites were not quantified. It is unlikely that they interfered with the detection of *P. mycoparasiticum* because the frequency of detection of *P. mycoparasiticum* on *B. cinerea* was similar to that on the other hosts. But the other mycoparasites did interfere with the isolation of *P. oligandrum* or *P. mycoparasiticum* into pure culture from *B. cinerea* sectors; such isolation was best achieved from the *Phialophora* sp. detector plates.

5.1.2 Attempted isolation on glucose agar plates

White *et al.* (1993) found that *P. oligandrum* could readily be detected when soil was spotted onto 0.1% glucose agar plates, as described in Section 2.6.2.2. However when this method was used for soil A and various dilutions of this soil it never yielded *P. oligandrum* or *P. mycoparasiticum*. Instead, it yielded several other pythiaceous fungi which were not identified but which did not resemble the mycoparasites when transferred to plates of SSA to support reproductive development.

Table 5.3 The number of detected occurrences of *P. mycoparasiticum* (Pm) and *P. oligandrum* (Po) after different times of incubation, when soils or soil:sand dilutions were plated on sectors of agar precolonised by different host fungi (total 120 sectors for each host fungus scored after 7, 10 and 14 days incubation).*

	7 DAYS		10 DAYS		14 DAYS	
Host fungus	**Pm**	**Po**	**Pm**	**Po**	**Pm**	**Po**
Phialophora sp.	8	12	46	20	47	20
Fusarium culmorum	26	23	39	23	40	23
Botrytis cinerea	37	16	40	16	40	16
Total	71	51	125	59	127	59

* Data pooled for soils A-D; see Table 5.2

host-precolonised sector (see Table 5.2). Undiluted soil was deemed to have 32 units of soil (log=1.5), and the highest dilution (1 in 32) to have 1 unit of soil (log=0).

5.2 Isolation of P. mycoparasiticum from soil using precolonised cellulose strips

The mycoparasitic *Pythium* species are known to be non-cellulolytic (Tribe, 1966; Deacon, 1976; Foley & Deacon, 1986a; Laing & Deacon, 1990) and grow very poorly across transparent cellulose film when inoculated onto it alone. However, Tribe (1966) reported that *P. oligandrum* grew well on cellulose film when coinoculated with some cellulolytic host fungi, such as *F. culmorum* or *B. piluliferum*. This prompted an experiment in which strips of host-precolonised cellulose film were incubated in soil and subsequently placed on precolonised host sectors for attempted isolation of *P. oligandrum* and *P. mycoparasiticum*.

The method is described in Section 2.6.3. Autoclaved strips of cellulose film (2.5 x 2.0 cm) were precolonised with either *F. culmorum*, *B. cinerea* or *Phialophora* sp. and were then placed in square Petri dishes filled with soil or sand : soil dilutions, prepared as before. The soils were brought to 50% saturation with water and incubated at room temperature for 3 or 7 days. Then the cellulose strips (9 per soil dish) were excavated, cut into four equal sized portions and these were placed singly on PDA sectors precolonised by either *B. cinerea*, *F. culmorum* or *Phialophora* sp. Any soil particles attached to the cellophane film were removed carefully. There were 6 replicates of each of the three types of host precolonised cellulose strips on each of the three types of host precolonised sectors for each soil dilution at each sampling time. The sectors were incubated at room temperature and inspected microscopically after 7, 10 and 14 days. Soil D was used for this experiment, as it was known to contain both *P. oligandrum* and *P. mycoparasiticum*.

Table 5.4 summarizes the results of this experiment, pooled for the different host sectors and all soil dilutions. It shows that both *P. mycoparasiticum* and *P. oligandrum* could colonise the detector plates from cellulose film buried in the soil for 3 and 7 days, with no significant difference (X^2) between the times of burial. So the results for 3 and 7 days' burial were pooled for further analysis (Table 5.5). For these pooled data there was a significant difference (X^2, $P < 0.05$) in detection of *P. mycoparasiticum* on film precolonised by the three hosts. *F. culmorum* was the best host fungus on cellulose film (96 detections overall) and *Phialophora* sp. the poorest (65 detections overall). For subsequent detections of *P. mycoparasiticum* on host agar sectors there was also a significant difference (X^2, $P < 0.05$) in the ability of the three hosts, when used to precolonise agar plates, to detect *P. mycoparasiticum*. In this case *Phialophora* sp. was the best host (99 detections, compared with 73

Table 5.4 Number of detected occurrences (maximum 108 in each case) of *P. mycoparasiticum* (Pm) and *P. oligandrum* (Po) from cellulose film precolonised by *Fusarium culmorum*, *Botrytis cinerea* or *Phialophora* sp. and then buried in soil for 3 or 7 days before being placed on host-precolonised agar sectors*.

Host used to precolonise cellulose		3 days	7 days
F. culmorum	**Po**	57	65
	Pm	48	48
B. cinerea	**Po**	65	73
	Pm	39	36
Phialophora sp.	**Po**	57	64
	Pm	32	33
TOTAL	**Po**	179	202
	Pm	119	117

* Data pooled for burial of film in different dilutions of soil with sand and for detection on agar sectors precolonised by three fungi (see Table 5.5)

Table 5.5 Number of detected occurrences of *Pythium mycoparasiticum* (Pm) and *P. oligandrum* (Po) when cellulose strips precolonised with different host fungi were retrieved from soil serially diluted with sand then placed on agar sectors precolonised by different host fungi (12 replicate sectors per dilution per combination of host-precolonised cellulose and host-precolonised agar)*1

		Detected occurrences at dilution shown.						
		1	1/2	1/4	1/8	1/16	1/32	Total
F. CULMORUM PRECOLONISED CELLULOSE								
Fc sectors*2	**Po**	12	11	10	8	6	5	52
	Pm	1	1	2	4	6	4	18
Bc sectors	**Po**	12	8	6	6	5	4	41
	Pm	1	6	7	4	8	10	36
Ph sectors	**Po**	7	9	8	2	2	1	29
	Pm	5	3	6	10	9	9	42
TOTAL	**Po**	31	28	24	16	13	10	122
	Pm	7	10	15	18	23	23	96
PHIALOPHORA SP. PRECOLONISED CELLULOSE								
Fc sectors	**Po**	8	8	4	5	11	0	36
	Pm	3	4	7	4	1	2	21
Bc sectors	**Po**	12	11	9	9	10	5	56
	Pm	0	2	3	3	3	4	15
Ph sectors	**Po**	8	6	5	1	9	0	29
	Pm	4	6	7	7	3	2	29
TOTAL	**Po**	28	25	18	15	30	5	121
	Pm	7	13	17	14	7	8	65

Table 5.5 continued.

B. CINEREA PRECOLONISED CELLULOSE								
Fc sectors	**Po**	12	8	9	8	5	3	45
	Pm	0	4	3	4	5	9	25
Bc sectors	**Po**	11	12	10	6	9	3	51
	Pm	1	0	2	5	5	9	22
Ph sectors	**Po**	9	8	7	6	8	4	42
	Pm	3	4	5	6	5	5	28
TOTAL	**Po**	32	28	26	20	22	10	138
	Pm	4	8	10	15	15	23	75
OVERALL TOTAL	**Po**	91	81	68	51	65	25	381
	Pm	18	18	42	47	45	54	236

*1 Data pooled for retrieval of buried film at 3 and 7 days.

*2 *Fusarium culmorum* (Fc), *Botrytis cinerea* (Bc) and *Phialophora* sp. (Ph).

detections on *B. cinerea* and 64 on *F. culmorum* sectors). This trend was seen irrespective of the host used to precolonise the cellulose strips. For *P. oligandrum* there was no significant difference (X^2) in detection frequency for cellulose film precolonised by different fungi, but there was a significant difference (X^2, $P < 0.05$) in the detection efficiency on the different host precolonised agar sectors- *Phialophora* sp. was the least effective detector fungus (100 detections compared with 148 and 133 detections respectively for *B. cinerea* and *F. culmorum*), and this trend was seen irrespective of the host used to precolonise the cellulose strips. *P. oligandrum* and *P. mycoparasiticum* were occasionally detected on the same host-precolonised agar sector- in 25 cases out of a possible 592 'positive' sectors for these fungi. However when detected on the same sector, they were seen on different parts of the sector.

Of interest, there was no evidence that efficiency of detection from one type of burial treatment (eg. *F. culmorum*-colonised film) was enhanced by plating the retrieved cellulose on agar precolonised by the same fungus. From Table 5.5 the detection of *P. oligandrum* from such 'like' combinations was 132 instances compared with 249 instances in 'unlike' combinations, which is not significantly different from the expectation of a 1:2 ratio (X^2). The same is true of detection of *P. mycoparasiticum* (69 and 167 occurrences, respectively).

For both *P. oligandrum* and *P. mycoparasiticum*, the total number of detections from each soil dilution was calculated as a percentage, then probit-transformed and plotted against log dilution as described earlier. As shown in Figure 5.2, there was a very highly significant negative correlation between detection and progressive soil dilution for *P. oligandrum*. In contrast there was a significant increase in frequency of detection of *P. mycoparasiticum* with progressive dilution of the soil with sand. Reflecting this difference, the frequency of detection combined for the mycoparasitic *Pythium* spp. remained relatively constant over the dilutions (Table 5.5). The negative association between *P. oligandrum* and *P. mycoparasiticum* revealed by these data strongly indicates competition from the faster-growing *P. oligandrum*, such that it decreases the ability to detect *P. mycoparasiticum* from undiluted soil. Moreover, the data from the higher dilutions (eg. 1 in 32) indicate that the population level of *P. mycoparasiticum* in this soil was higher than that of *P. oligandrum* even though *P. oligandrum* was detected at the higher frequency overall. Using the total counts at the 1/32 dilution, the population of *P. mycoparasiticum* was significantly higher ($X^2 = 11.47$, $P < 0.01$) than that of *P. oligandrum*. This trend is seen even if only the data for *B. cinerea*-precolonised agar are used: *P. mycoparasiticum* was detected

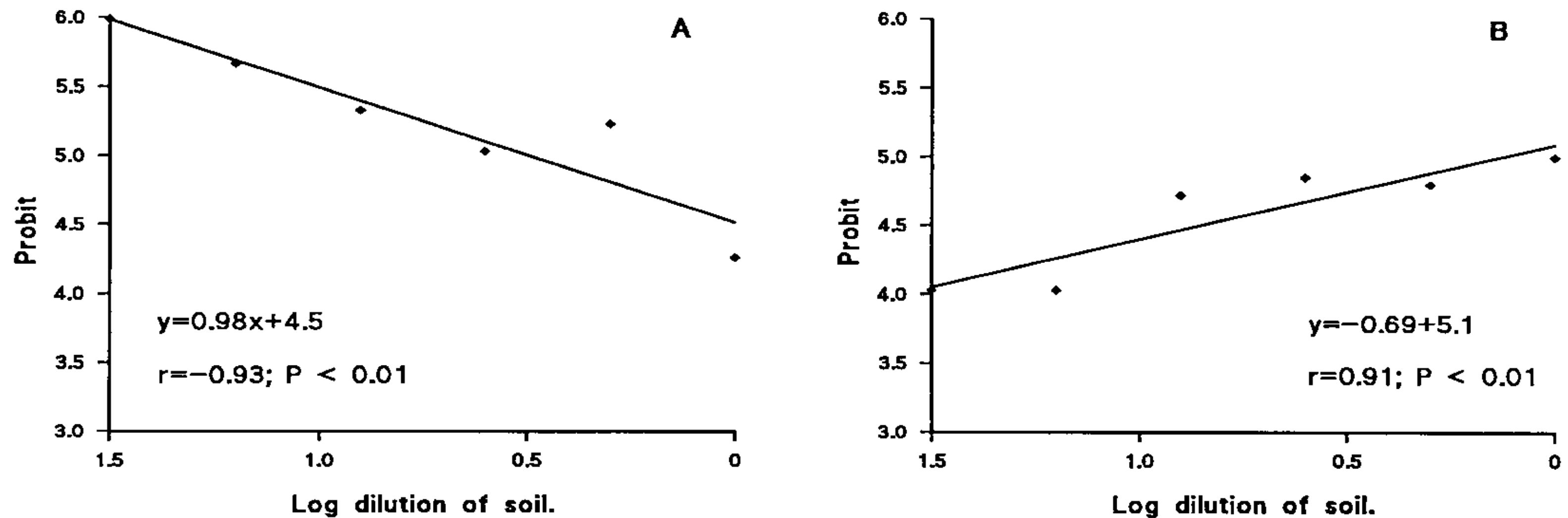


Figure 5.2 Probit-transformed percentage occurrence of (A) *P. oligandrum* and (B) *P. mycoparasiticum*, from soils diluted in series with sand, when cellulose film precolonised by different host fungi (pooled data) were retrieved from soil and placed on agar sectors precolonised by different host fungi (pooled data).

on 23 sectors at the 1/32 dilution, compared with 12 sectors for *P. oligandrum*, and yet *B. cinerea* was the best detector for *P. oligandrum* and not the best for *P. mycoparasiticum*.

Comparing the results with those for soil D in the previous experiment (Table 5.2), at the most dilute soil concentrations the frequency of detection of both *P. mycoparasiticum* and *P. oligandrum* was higher using this technique of soil baiting with host-precolonised cellulose strips than when soil/sand dilutions as such were placed onto host-precolonised agar sectors.

5.3 Isolation of mycoparasitic Pythium species from precolonised plant root segments

In the previous experiment both *P. oligandrum* and *P. mycoparasiticum* were isolated from host-precolonised cellulose strips. The effect of precolonising a more complex, natural substrate was now investigated.

The method was as described in Section 2.6.4. Briefly, seeds of broad bean and pea were germinated on damp tissue paper, the roots were excised when approximately 6 to 8cm long, cut into 2 cm lengths and placed on PDA colonies of *P. aphanidermatum* or *P. ultimum* for 24h at 25°C to allow colonisation of the segments by the pathogens. Control root pieces were placed on moist sterile filter paper in Petri dishes for 24h at 25°C. Six root pieces (2 controls, 2 with *P. aphanidermatum* and 2 with *P. ultimum*) were then buried between nylon mesh in each of 5 boxes of soil D at 50% saturation and incubated at room temperature for 5 days. After retrieval the root pieces were cut into two 1cm lengths one of which was placed on an agar sector precolonised by *F. culmorum*, and the other on a sector precolonised by *Phialophora* sp. These two hosts were chosen as being suitable for detection of both *P. oligandrum* and *P. mycoparasiticum*, but *Phialophora* sp. had been found to be somewhat better for *P. mycoparasiticum*, and *F. culmorum* somewhat better for *P. oligandrum* (Table 5.6). After the root pieces had been halved there were 10 replicate pieces of both pea and bean roots (for each treatment) for plating onto each type of host precolonised agar sector. The sectors were incubated at room temperature and examined microscopically after 7, 10 and 14 days.

As shown in Table 5.6, most root pieces yielded one or other of *P. mycoparasiticum* or *P. oligandrum*, although bean roots were somewhat better than pea roots (28 of 30 compared with 22 of 30 respectively yielded mycoparasites, as

Table 5.6 The number of detected occurrences of *P. mycoparasiticum* (Pm) and *P. oligandrum* (Po) from pea or bean root pieces that had been untreated or precolonised with either *P. aphanidermatum* or *P. ultimum*, then buried and retrieved from soil, cut in half and plated on agar precolonised by *Fusarium culmorum* or *Phialophora* sp. (10 replicates for each precolonised root type and each host-precolonised plate).

Root precolonised with host fungi		Number of detections (max. 10) of Pm or Po on host plates				No. of 'whole' 2cm root pieces with Po or Pm*2
		P. mycoparasiticum		*P. oligandrum*		
		on Fc*1	on Ph	on Fc	on Ph	
Bean roots	Control	2	0	7	6	10
	P. ultimum	0	1	9	8	9
	P. aphanidermatum	1	3	6	2	9
Pea roots	Control	1	1	5	4	7
	P. ultimum	0	0	9	8	9
	P. aphanidermatum	0	0	4	4	6
Total		4	5	40	32	

*1 *Fusarium culmorum* (Fc), *Phialophora* sp. (Ph) precolonised agar sectors.

*2 Data pooled for the mycoparasitic *Pythium* spp. and host detector fungi and for the two halves of each 2 cm root piece.

shown in the final column of Table 5.6). There was no significant difference between the detector fungi for detection of either *P. oligandrum* or *P. mycoparasiticum*. There was also little difference between the root-precolonisation treatments overall: the total detection of mycoparasitic *Pythium* spp. from controls, *P. ultimum* and *P. aphanidermatum* treatments was respectively (of 20 maximum 'whole' root pieces) 17, 18 and 15. However, when the data are analysed for frequency of detection on the 'root halves' there was a significant difference (X^2, $P < 0.05$) in the detection of *P. oligandrum* from root halves precolonised in different ways: control root pieces yielded *P. oligandrum* in 22 of the possible 40 platings, compared with 34 for roots precolonised by *P. ultimum* and only 16 for roots precolonised by *P. aphanidermatum*. Consistent with this, after retrieval, roots precolonised by *P. ultimum* were seen to contain many oogonia of *P. oligandrum* when crushed onto microscope slides, but only few oogonia of *P. oligandrum* were observed in crushed root pieces precolonised by *P. aphanidermatum*. These observations, however, were not quantified, and even the analysis of *P. oligandrum* frequency on root halves can be taken as only indicative because in many cases a detection would have been counted twice (on each half of an original 2 cm root length).

Of the mycoparasites, *P. oligandrum* was detected most frequently, on a total 72 of the possible 100 1 cm root 'halves' placed on precolonised agar sectors, and *P. mycoparasiticum* was detected on only 9 of the possible 100, presumably because of competition from *P. oligandrum* on the agar sectors. In this respect it is notable that some of the original root pieces, when halved, yielded *P. mycoparasiticum* from one half and *P. oligandrum* from the other.

5.4 Discussion

The use of host-precolonised agar plates was effective for detection and isolation of both *P. oligandrum* and *P. mycoparasiticum* from soil, but its efficiency was strongly influenced by the host fungus used. *F. culmorum*, *B. cinerea* and *Phialophora* sp. were suitable for detecting both *P. oligandrum* and *P. mycoparasiticum* from soil; but, in limited tests *B. piluliferum* was effective only for *P. oligandrum*, and *F. oxysporum* did not detect either mycoparasite. These findings support the conclusion by Mulligan & Deacon (1992), that different precolonising fungi on agar plates can strongly influence the detection of mycoparasites from soil. It is also confirmed that *B. cinerea*, *F. culmorum* and *Phialophora* sp. are suitable for detection of *P. oligandrum* (Mulligan & Deacon, 1992) and shown now that the same

three fungi can be used similarly for *P. mycoparasiticum*. However, there does not seem to be any simple way of predicting the host fungi that can be used successfully on precolonised plates. Both *F. oxysporum* and *B. piluliferum* are susceptible to parasitism by *P. oligandrum* and *P. mycoparasiticum* (Laing & Deacon, 1991) and yet at least *F. oxysporum* was ineffective as a detector fungus on precolonised plates.

Although this thesis does not report the frequency of isolation of mycoparasites into pure culture from precolonised plates, it was consistently difficult to obtain pure cultures of *P. oligandrum* or *P. mycoparasiticum* from colonies of *B. cinerea* or *F. culmorum*, but relatively easy to isolate mycoparasites into pure culture from *Phialophora* sp. This may be because *Phialophora* sp. is the more susceptible to mycoparasitism as shown by Laing & Deacon (1990, 1991). It could also be because *Phialophora* sp. is relatively slow-growing, thus enabling *P. oligandrum* and *P. mycoparasiticum* to outgrow it on PDA isolation plates. *P. mycoparasiticum* is much slower growing than *P. oligandrum* on PDA (4.9mm/day compared with 27.5mm/day; Section 3.4) and so is the more difficult to isolate in pure culture from host-precolonised sectors especially from a faster growing host fungus such as *F. culmorum*. In addition to these points, another factor influenced the efficiency of isolation of mycoparasites from detector plates of *B. cinerea*. Detector plates of this host supported growth of a wider range of mycoparasites from soil, including *Trichoderma* spp., *Gliocladium* spp. and a fungus classified as a presumptive mycoparasite by Mulligan & Deacon (1992), *Papulaspora* sp. These interfered with the isolation into pure culture of the mycoparasitic *Pythium* spp. Thus, although several host fungi were suitable for detection of *P. mycoparasiticum* and *P. oligandrum* from soil, *Phialophora* sp. was the best for subsequent isolation of these mycoparasites.

Despite the fact that there are six reportedly mycoparasitic *Pythium* spp., only three were detected in this study, and one of them- *P. periplocum*- was encountered only once. All these mycoparasites can be distinguished from one another on the basis of their sexual reproductive structures- the presence or absence of spines on the oogonia, shape and size of these spines (where present) and presence/absence or arrangement of antheridia. With practice, they can all be distinguished from one another on precolonised agar plates, without the need for isolation into pure culture. It must thus be asked why only *P. oligandrum* and *P. mycoparasiticum* have been detected frequently from British soils on precolonised plates. Even in a previous major study of 164 soils or natural samples, Foley & Deacon (1985) detected *P.*

acanthicum in only 2 samples, and never reported *P. periplocum, P. acanthophoron* or *P. nunn*. Mulligan & Deacon (1992), in a study of 28 soils, did not even detect *P. mycoparasiticum*, using up to seven precolonising host fungi on detector plates. A possible reason why *P. nunn* was not detected in these or my study is that *P. nunn* does not readily form oogonia (Lifshitz, *et al.* 1984a; Laing & Deacon, 1990) and thus would not have been seen on precolonised plates. An alternative possibility, of course, is that this fungus does not occur in Britain. *P. acanthophoron* is known to occur in Britain, although it seems to have been detected here only once (Lodha & Webster, 1990), from a sandy site in Devon. It also seems to be rare (or rarely isolated) elsewhere, having been reported by Sideris (1932) and Quimio & Abilay (1977). But *P. acanthicum* was reported by Apinis (1964) to be relatively common in Britain and it was also reported by Dick & Ali-Shtayeh (1986) from farm and park land.

Mulligan & Deacon (1992) suggested that one of the possible reasons why a wider range of mycoparasites were not detected in their study with precolonised plates was that inappropriate host fungi were used. An alternative has now been demonstrated in my work on *P. mycoparasiticum*, namely competition from the more common or faster-growing mycoparasites can preclude detection of other mycoparasites. The isolation of *P. mycoparasiticum* from soil was seen to be strongly influenced by the presence of *P. oligandrum*. In soils where *P. oligandrum* was uncommon (soils B and C), dilution of the soil decreased the detection of *P. mycoparasiticum*, whereas in soils where *P. oligandrum* was common (soils A and D) *P. mycoparasiticum* was infrequently isolated from the undiluted soil, but was found more often in soils diluted with sand. In order to analyse this effect of soil dilution it was necessary to convert the detection frequencies to a form in which they might fit a theoretical curve. Log of dose against probit of infection has been used successfully to analyse inoculum dosage effects of plant pathogens (eg Wastie, 1962; Garrett, 1970). The resulting plot should be a straight line, and conformity to this can be tested by regression analysis. A highly significant negative correlation was observed for the detection of the 'total' mycoparasitic *Pythium* spp. in all four soils, for the detection of *P. oligandrum* in all four soils, and the detection of *P. mycoparasiticum* in soils where *P. oligandrum* was uncommon (soils B and C). In other words, progressive dilution of soil in all these cases resulted in the expected progressive reduction of detection of the mycoparasites on host-precolonised agar. However there was no significant relationship for *P. mycoparasiticum* in soils where *P. oligandrum* was common (soils A and D): dilution did not cause a progressive reduction in detection

of *P. mycoparasiticum*. On the contrary, *P. mycoparasiticum* was detected more often in the early stages of the dilution series than in undiluted soil, while detection of *P. oligandrum* declined in these dilutions. The likely reason is that *P. oligandrum* outcompeted the slower growing *P. mycoparasiticum* on the host precolonised agar sectors. Supporting this is the observation that whilst the greatest detection of *P. oligandrum* on host precolonised agar sectors was after 7 days incubation, the highest frequency of detection of *P. mycoparasiticum* was after 10 days. However most of the increased detections of *P. mycoparasiticum* after 10 days were observed on *Phialophora* sp. host sectors, not on the two other hosts *B. cinerea* and *F. culmorum*. On the whole, *P. mycoparasiticum* seems to develop slower on the host precolonised sectors therefore allowing *P. oligandrum* to establish itself first. *P. mycoparasiticum* was also reported by Laing & Deacon (1990), who studied the abilities of the mycoparasitic *Pythium* spp. to grow across host precolonised agar, to be less aggressive towards host fungi than was *P. oligandrum*. In a limited study, dilution of soil using sand was also observed by Foley & Deacon (1985) to increase the detection of *P. mycoparasiticum* on *Phialophora*-precolonised agar sectors.

In contrast to the use of precolonised agar plates, White (1992) and White *et al.* (1993) used 0.1% glucose agar plates to isolate *P. oligandrum* from field soils, and found that *P. oligandrum* could readily be detected on these plates by its characteristic colony morphology. However, attempts to use 0.1% glucose agar in my work were unsuccesful; the plates supported growth of several unidentified pythiaceous fungi but *P. oligandrum* and *P. mycoparasiticum* were never detected.

In view of the finding that *P. oligandrum* seemed to outcompete *P. mycoparasiticum* on precolonised plates (from undiluted soil) it was of interest to investigate this effect in conditions closer to those likely to occur in nature. Tribe (1966) reported that *P. oligandrum* was able to colonise cellulose film in soil, apparently by growing in association with cellulolytic fungi that colonised the cellulose film and degraded it. So here, strips of cellulose film were buried in soil that had been diluted to various degrees with sand and, after 3 or 7 days, the film was placed on precolonised agar sectors to detect the mycoparasites. However instead of using uncolonised agar films, the strips of film were precolonised by *B. cinerea*, *Phialophora* sp. or *F. culmorum*. These 'baits' seemed more efficient in detecting mycoparasites from soil than was direct plating of soil onto host-precolonised agar sectors. Even at the highest soil dilution (1/32) nearly all the pieces of cellulose film placed on the detection plates were found to yield either *P. oligandrum* or *P.*

mycoparasiticum. None of the other 4 mycoparasitic *Pythium* spp. was detected. As, before, *P. mycoparasiticum* was detected with increasing frequency with progressive dilution of the soil with sand, while the frequency of detection of *P. oligandrum* declined with soil dilution. This was clearly seen from the log dose : probit response curve (Figure 5.2). Whereas for *P. oligandrum* there was a very highly significant negative correlation between detection frequency and soil dilution, a highly significant positive correlation was seen for *P. mycoparasiticum*. This again suggests that the faster growing *P. oligandrum* outcompetes *P. mycoparasiticum*, but in this instance the competition would have occurred for the buried cellulose film rather than for host precolonised agar sectors. In only 25 out of a total of 592 'positive' agar sectors were *P. oligandrum* and *P. mycoparasiticum* seen on the same detector plate. Even then they were seen on different parts of the sector. The population level of *P. mycoparasiticum*, as indicated by the difference in the frequency of detection of the two fungi from the highest soil dilution (1/32), appears to be higher than that of *P. oligandrum* in the soil.

No difference was seen in the frequency of detection of *P. oligandrum* and *P. mycoparasiticum* when host-precolonised cellulose film was retrieved from soil after 3 days compared with after 7 days. For *P. mycoparasiticum* detections, *F. culmorum* was found to be the best fungus on cellulose film, whilst there was no difference between the host fungi for *P. oligandrum* detection. On the other hand, irrespective of the host used to precolonise cellulose strips the best host for precolonising agar sectors to detect *P. mycoparasiticum* was *Phialophora* sp., whilst the same host was the worst for detecting *P. oligandrum*.

Another baiting technique was used by Ribeiro & Butler (1992) whereby air-dried sclerotia of *Sclerotinia sclerotiorum* were incubated in soil, then retrieved and placed on agar plates to isolate mycoparasitic *Pythium* species from them. *P. periplocum* and *P. acanthicum* as well as *P. oligandrum* were isolated from soil using this technique. *P. mycoparasiticum*, *P. acanthophoron* and *P. nunn* were not detected. There was no evidence of penetration or internal growth on the baited sclerotia of *S. sclerotiorum* by these mycoparasitic species. However it was suggested that the sclerotial walls of *S. sclerotiorum* contain substances such as amino acids, amino sugars and glucose. These were suggested to attract swimming zoospores or hyphae of the mycoparasitic *Pythium* spp. at or near the sclerotial surface.

In my work *P. oligandrum* and *P. mycoparasiticum* were able to colonise and be isolated from diseased plant material placed in soil and subsequently transferred to

host-precolonised agar plates. *P. oligandrum* was isolated at a higher frequency from both pea and bean root segments than was *P. mycoparasiticum*; this again was probably due to *P. oligandrum* outcompeting *P. mycoparasiticum* either during colonisation of the baits or during colonisation of the host precolonised agar plates. The mycoparasitic *Pythium* species were found to be isolated from both bean root pieces and pea root pieces, and irrespective of whether these had been colonised by phytopathogenic *Pythium* spp. before burial. However, this does not necessarily mean that the mycoparasites can invade living plant tissue, because the roots could have lost viability rapidly when severed and buried in soil. Overall, *P. oligandrum* was isolated less frequently from root pieces precolonised by *P. aphanidermatum* than from root pieces that had been precolonised by *P. ultimum* or not precolonised. Microscopical observation of crushed root pieces revealed many oogonia of *P. oligandrum* in roots precolonised by *P. ultimum*, but very few were seen in roots precolonised by *P. aphanidermatum*. Berry *et al.* (1993) reported that these phytopathogenic *Pythium* spp. differ in susceptibility to antagonism by *P. oligandrum*. *P. ultimum* was relatively susceptible whereas *P. aphanidermatum* was seen to antagonise *P. oligandrum*. Further evidence of this is reported in Chapter 6. Precolonisation of root pieces by *P. aphanidermatum* may thus have reduced or prevented secondary colonisation by the mycoparasitic *Pythium* species. Berry *et al.* (1993) also showed that whilst oospore production by *P. oligandrum* was unaffected by coinoculation with *P. ultimum* on agar films *in vitro*, *P. aphanidermatum* significantly decreased the oospore production by *P. oligandrum*. Drechsler (1943a) observed that *P. oligandrum* was often isolated from diseased pea roots along with three well known phytopathogens- *P. ultimum*, *P. debaryanum* and *P. irregulare*. He proposed that *P. oligandrum* was not a primary invader but a secondary coloniser of diseased plant tissue. *P. oligandrum* was also reported by Martin & Hancock (1986) to compete with *P. ultimum* for saprophytic colonisation of substrates in soil. These workers also found that prior colonisation of organic matter with *P. oligandrum* precluded subsequent colonisation by *P. ultimum*. Paulitz & Baker (1988b) similarly reported that another mycoparasite, *P. nunn*, could invade bean leaf residues previously colonised by *P. ultimum* and was also able to displace *P. ultimum* from the substrate.

A general feature in all these studies and in the work reported here is that *P. oligandrum*, *P. mycoparasiticum* and other mycoparasitic *Pythium* spp. were able to colonise substrata such as agar plates, cellulose strips and plant tissue which other fungi have previously colonised. This contrasts with the behaviour of non-mycoparasitic *Pythium* spp. such as *P. mamillatum*, which Barton (1960, 1961)

found to be inhibited by the staling products of other fungi, therefore limiting it to colonisation of virgin substrates only. The mycoparasitic *Pythium* spp. evidently are tolerant of the metabolic products of at least some fungi, enabling them to be secondary colonisers.

The extent and significance of this secondary colonising ability in agricultural soils can only be assessed when methods are developed for detecting the presence and population levels of the mycoparasites in soil. The work in this chapter has shown that the frequency of *P. mycoparasiticum* compared to *P. oligandrum* would often be underestimated if soils were not diluted to reduce or preclude competition from *P. oligandrum*. The reasons why other mycoparasitic *Pythium* spp. were seldom or never detected in this study remains unknown. Only when standardised methods are applied to a wide range of soils, and from a wide range of geographical locations, will it be possible to assess whether the mycoparasitic *Pythium* spp. have different distributions; at present such reported differences in distributions could be artifacts of sampling and assessment methods.

CHAPTER 6

FUNGAL INTERACTIONS

The mode of mycoparasitic action of *P. oligandrum* has been intensively studied (Deacon, 1976; Lutchmeah & Cooke, 1984; Lewis *et al.* 1989; Laing & Deacon, 1991; Berry *et al.* 1993). Similar work has been carried out on *P. acanthicum* (Haskins, 1963; Hoch & Fuller, 1977; Deacon & Henry, 1978), *P. nunn* (Lifshitz *et al.*, 1984c) and *P. mycoparasiticum* (Laing & Deacon, 1991). Laing & Deacon (1990, 1991) compared the aggressiveness of *P. oligandrum*, *P. mycoparasiticum* (*Pythium* SWO) and *P. nunn*, and reported different aggressiveness in the mycoparasites towards host fungi, with the host fungi also differing in susceptibility. The aggressiveness and host range of *P. acanthophoron* compared to *P. oligandrum* is not known, and will be the focus of the work in this chapter. The studies involved both growth of mycoparasites on precolonised agar plates and videotaped interactions between individual hyphae of mycoparasites and their hosts.

During the course of this work, *P. aphanidermatum* was found to antagonise *P. oligandrum* in some interactions. A preliminary report on this has been published (Berry *et al.* 1993). It was investigated further in this chapter.

6.1 Growth on precolonised plates

Mycoparasitic *Pythium* spp., unlike most other soil fungi, have been reported to be able to grow across agar previously colonised by other fungi (Deacon, 1976; Deacon & Henry, 1978; Foley & Deacon, 1986b; Laing & Deacon, 1990). Laing & Deacon (1990) reported that the different mycoparasitic *Pythium* spp. differed in their 'host range' in such tests, with *P. oligandrum* being able to overgrow the most fungi, *P. mycoparasiticum* overgrowing fewer and *P. nunn* overgrowing fewer still. Here the potential host range of *P. acanthophoron* (IMI 330 382) was compared to those of *P. oligandrum* (P14.1.2) and *P. mycoparasiticum* (AR7A) in terms of growth on precolonised agar plates.

The method described in Section 2.7.1 was used, whereby the host fungi were inoculated on one side of PDA plates and incubated at 25°C until they reached the opposite side. A PDA disc of the mycoparasite was then placed on the colony margin and the plate reincubated at 25°C. For each host fungus and mycoparasite

combination, 5 plates were used. On the base of each plate, 3 adjacent strips of agar, 5mm diameter, were marked so that the centre strip contained both inoculum discs. After 7 days the centre strip was cut and removed, then cut traversely into successive 5mm squares and these were placed on large PDA discs precolonised with the susceptible host fungus *Phialophora* sp. These were then incubated for 7 days to enable the mycoparasite to grow and produce reproductive structures on the *Phialophora* disc if it had been present on the square of agar removed from the original host plate. The presence or absence of reproductive structures was then assessed microscopically to indicate the extent of growth of the fungus on the original agar strip. The second and third strips were treated similarly after 14 and 21 days respectively.

As shown in Table 6.1, *P. oligandrum* (P14.1.2) grew rapidly and completely across colonies of *Phialophora* sp., *Botrytis cinerea*, *Humicola grisea*, *Botryotrichum piluliferum*, *Zygorynchus moelleri*, *Fusarium culmorum* and *Fusarium oxysporum*, more slowly across *Trichoderma aureoviride*, *Pythium ultimum*, *Pythium vexans*, *Pythium sylvaticum* and *Phytophthora cinnamomi*, and little or not at all across *Pythium graminicola*, *Pythium dissotocum*, *Pythium aphanidermatum*, *Pythium catenulatum* and *Rhizoctonia solani*. In these last mentioned cases there was no evidence that the mycoparasite progressively grew across the host colonies after the initial 7 days.

In comparison with *P. oligandrum*, *P. acanthophoron* (IMI 330 382) grew rapidly and completely across fewer fungi (Table 6.2), namely *Phialophora* sp., *B. cinerea*, *H. grisea*, *Z. moelleri*, *F. culmorum* and *B. piluliferum*; it also grew completely across *P. sylvaticum*, but after an initial lag. But all other tested fungi supported either no growth or an initial amount of growth that did not progress at successive assessment times.

P. mycoparasiticum (AR7A) grew across even fewer fungi (Table 6.3), namely *Phialophora* sp., *B. cinerea* and *H. grisea* but made little or no growth across all the others.

For hosts with abundant aerial mycelia, such as *F. culmorum*, the mycoparasites were seen to cause collapse of this and the extent of growth of the mycoparasite could be gauged by the extent of collapse of the mycelium (Figure 6.1a).

Table 6.1 Growth (mm, maximum 75) of *P. oligandrum* (P14.1.2) after 7, 14, 21 days across plates of potato-dextrose agar previously colonized by host fungi (means of 5 replicates ± SEM, where appropriate).

	Time of assessment (days)		
Host fungus	7	14	21
Phialophora sp.	75	75	75
Botrytis cinerea	75	75	75
Humicola grisea	75	75	75
Botrytrichum piluliferum	75	75	75
Zygorynchus moelleri	75	75	75
Fusarium culmorum	75	75	75
Fusarium oxysporum	75	75	75
Trichoderma aureoviride	48 ± 2.0	65 ± 6.1	75
Pythium sylvaticum	3.0 ± 2.2	60 ± 1.6	75
Pythium vexans	38 ± 2.0	75	75
Pythium ultimum	33 ± 4.6	73 ± 1.2	75
Pythium graminicola	28 ± 3.0	28 ± 2.0	25
Pythium dissotocum	25 ±	35 ± 10.0	27 ± 7.3
Pythium catenulatum	1 ± 1.0	5 ± 5.0	0
Pythium aphanidermatum	0	0	0
Phytophthora cinnamomi	60 ± 10.0	66 ± 6.8	71 ± 3.0
Rhizoctonia solani	0	0	0

Table 6.2 Growth (mm, maximum 75) of *P. acanthophoron* (IMI 330 382) after 7, 14, 21 days across plates of potato-dextrose agar previously colonized by host fungi (means of 5 replicates ± SEM, where appropriate).

	Time of assessment (days)		
Host fungus	7	14	21
Phialophora sp.	75	75	75
Botrytis cinerea	75	75	75
Humicola grisea	75	75	75
Botrytrichum piluliferum	65 ± 5.2	75	75
Zygorynchus moelleri	75	75	75
Fusarium culmorum	57 ± 4.9	73 ± 1.2	75
Fusarium oxysporum	23 ± 8.0	23 ± 6.4	19 ± 4.8
Trichoderma aureoviride	0	0	0
Pythium sylvaticum	2.0 ± 1.2	19 ± 5.6	75
Pythium vexans	32 ± 7.2	34 ± 6.6	27 ± 2.5
Pythium ultimum	5 ± 2.2	10 ± 1.6	8 ± 4.6
Pythium graminicola	0	0	0
Pythium dissotocum	1 ± 1.0	0	0
Pythium catenulatum	1 ± 1.0	0	0
Pythium aphanidermatum	0	0	0
Phytophthora cinnamomi	3 ± 1.2	0	0
Rhizoctonia solani	0	0	0

Table 6.3 Growth (mm, maximum 75) of *P. mycoparasiticum* (AR7A) after 7, 14, 21 days across plates of potato-dextrose agar previously colonized by host fungi (means of 5 replicates ± SEM, where appropriate).

	Time of assessment (days)		
Host fungus	7	14	21
Phialophora sp.	67 ± 2.5	75	75
Botrytis cinerea	57 ± 1.2	75	75
Humicola grisea	28 ± 2.5	75	75
Botrytrichum piluliferum	0	0	0
Zygorynchus moelleri	8 ± 5.8	7 ± 4.4	7
Fusarium culmorum	25 ± 2.2	24 ± 2.9	0
Fusarium oxysporum	0	0	0
Trichoderma aureoviride	3 ± 2.0	1 ± 1.0	0
Pythium sylvaticum	0	1 ± 1.0	0
Pythium vexans	0	0	0
Pythium ultimum	3 ± 2.0	3 ± 2.0	0
Pythium graminicola	0	0	0
Pythium dissotocum	1 ± 1.0	0	0
Pythium catenulatum	0	0	0
Pythium aphanidermatum	0	0	0
Phytophthora cinnamomi	5 ± 1.6	0	0
Rhizoctonia solani	0	0	0

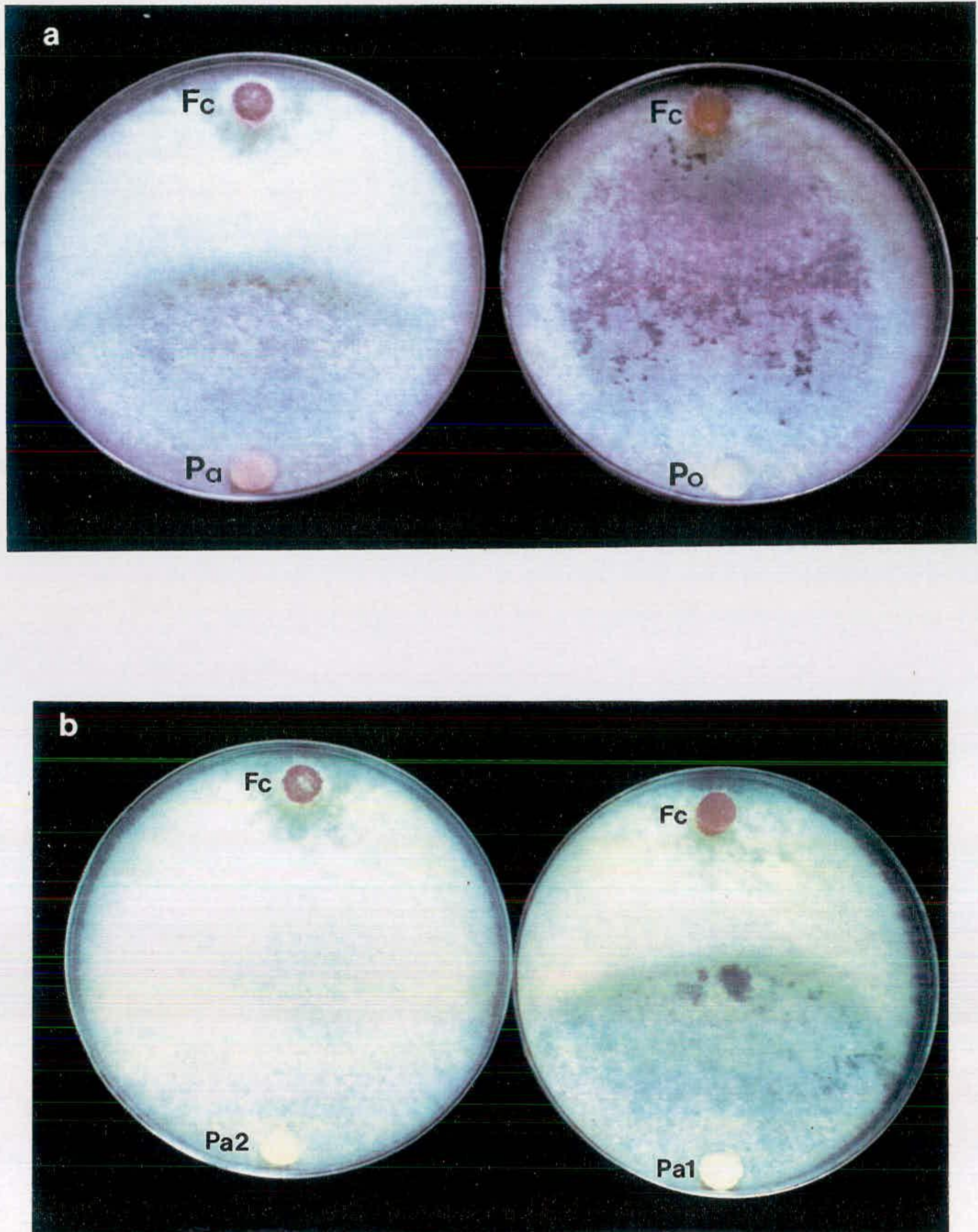


Figure 6.1 Agar plates precolonised by *F. culmorum* (Fc) and inoculated with (a) *P. oligandrum* (Po) or *P. acanthophoron* (IMI 330 382)(Pa) (b) *P. acanthophoron* (IMI 330 382)(Pa1) or *P. acanthophoron* (CBS 337.29)(Pa2). Note the extent of collapse of aerial mycelium of *F. culmorum*.

To determine whether the observations recorded for *P. acanthophoron* (IMI 330 382) were representative of this species, the original isolate of Sideris (1932) now preserved as CBS 337.29, was tested for ability to grow across *F. culmorum*. Five plates were used and treated as before. Since this isolate no longer produces reproductive bodies it could not be assessed by placing squares on discs of *Phialophora* sp. Instead the 5mm pieces were placed on PDA plates (5 per plate) and incubated at 25 °C to detect outgrowth of the mycoparasite.

No collapse of the aerial mycelia of *F. culmorum* was seen when colonies were inoculated with *P. acanthophoron* (CBS 337.29) (Figure 6.1b), and no *P. acanthophoron* colonies were seen to grow onto PDA from any of the 5mm pieces.

6.2 Videotaped hyphal interactions

For the experiments on videotaped hyphal interactions the method described in Section 2.7.2. was used. Glass coverslips bearing films of distilled water agar were inoculated with small agar discs of host and mycoparasite and videotaped observations were begun when hyphae at the colony margins were just about to contact one another. Precise tip-to-tip contacts were very rare. Instead, the interactions were usually betweeen a tip and the lateral wall (side) of another hypha close to its tip. So the interactions were categorised as of two types:

1) a mycoparasite tip contacting a host side (tip-to-host side),

2) a host tip contacting a mycoparasite side (side-to-host tip).

6.2.1 Comparison of mode of action of P. acanthophoron *and* P. oligandrum

Interactions between individual hyphae were recorded for both *P. acanthophoron* and *P. oligandrum* against the non-*Pythium* hosts, *Fusarium oxysporum* and *Trichoderma aureoviride*, and the *Pythium* hosts, *P. ultimum* and *P. aphanidermatum*. The mode of parasitism of, and host susceptibility to, *P. oligandrum* was reported to be different for these two different host groups (Laing & Deacon, 1991) and they will be considered separately in this section. *F. oxysporum* and *T. aureoviride* were selected since it had been found that *P. oligandrum* grew across agar plates precolonised by them, whereas *P. acanthophoron* did not grow across their colonies (compare Tables 6.1 and 6.2). *P. ultimum* was chosen because its colonies also supported growth by *P. oligandrum* but not by *P. acanthophoron*,

whereas *P. aphanidermatum* supported growth of neither mycoparasite on precolonised agar plates.

6.2.1.1 Interactions with non-*Pythium* hosts

The two mycoparasites were seen to have an identical mode of parasitism. They exhibited no notable pre-contact tropism, but soon after contact with host hyphae two main classes of behaviour were seen:

1) cytoplasmic coagulation or vacuolation of the host contents, which was usually followed by penetration by the mycoparasite;

2) lysis of the host at the point of contact, sometimes followed by penetration of the host hypha by the mycoparasite, or by proliferation of the mycoparasite in the host lysate.

These two categories are described in more detail below, using a representative example of each.

1) Coagulation of host cytoplasm.

In this case, a hyphal tip of *P. acanthophoron* (IMI 330 382) contacted the side of a *F. oxysporum* hypha (Figure 6.2a), was deflected and continued to grow alongside the host hypha at an unaltered growth rate (Figure 6.2b). A surge of the host protoplasm was seen 195 sec after contact and then stoppage of cytoplasmic streaming in the host. After a further 52 sec (4 min 7 sec after contact) the host protoplasm coagulated and penetration of the host by *P. acanthophoron* was seen (Figure 6.2c, arrow). The coagulation was restricted to one host compartment, which the mycoparasite first contacted. Penetration occurred from a branch of the mycoparasite that formed close to the first point of contact. This branch continued to grow inside the host hypha, reaching the first septum at 8 min 28 sec after initial contact and it then grew out through the lateral wall of the host hypha (Figure 6.2d). The *P. acanthophoron* branch continued to grow alongside a second *F. oxysporum* hypha (Figure 6.2e) and coagulation of this adjacent host hypha then occurred (Figure 6.2f). Meanwhile, the next hyphal compartment of the first-contacted host hypha showed coagulation at 12 min 29 sec (Figure 6.2e), and an internal hypha of *P. acanthophoron* was seen to have penetrated the septum and continued to grow through the second compartment (Figure 6.2f).

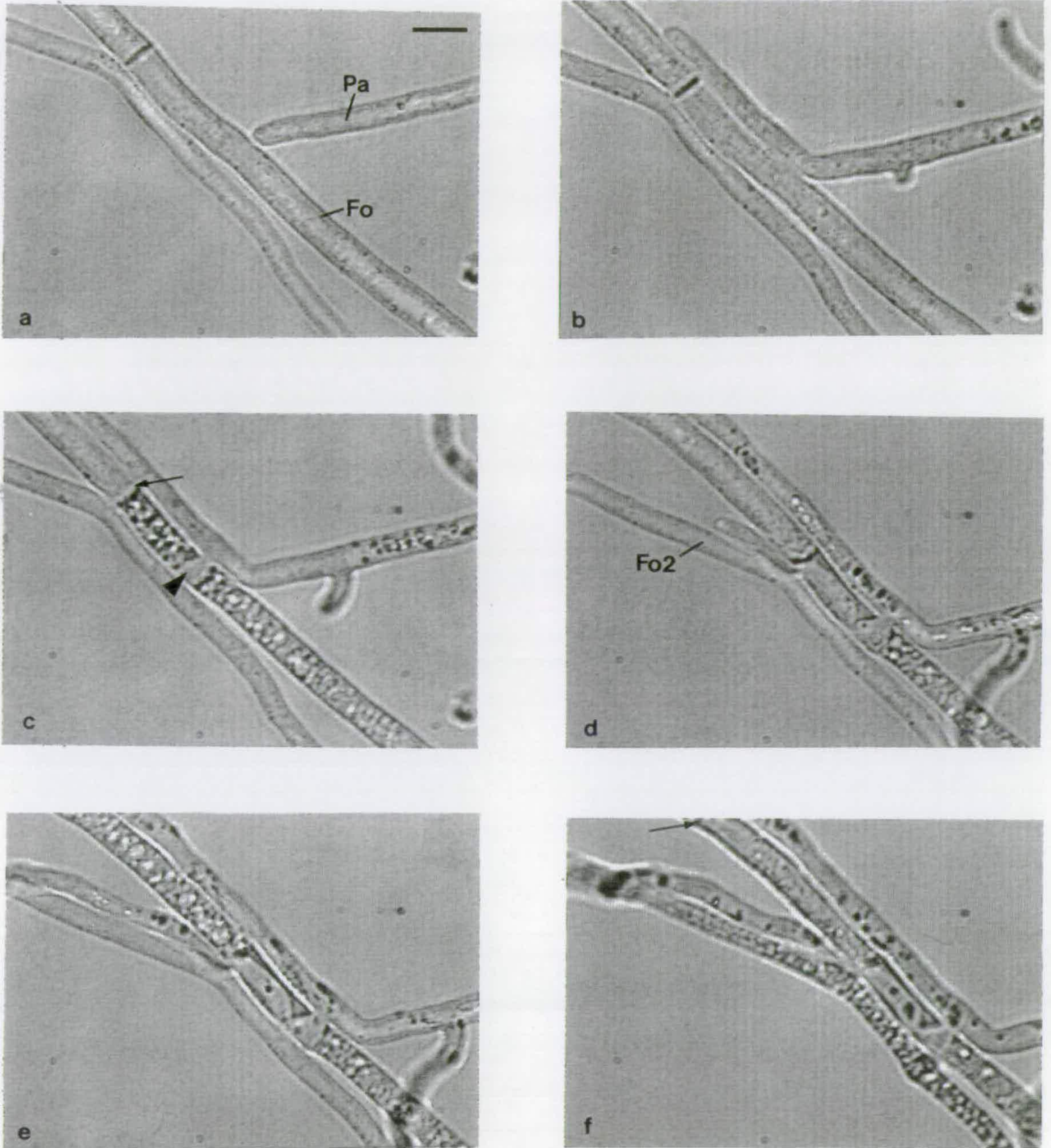


Figure 6.2a - f Stages in parasitism of *F. oxysporum* (Fo) by *P. acanthophoron* (Pa), videotaped through a film of agar. Bar=10μm. (a) Hypha of *P. acanthophoron* (Pa) making contact with side of *F. oxysporum* hypha (Fo). (b) 3 min 12 sec post-contact; the tip of *P. acanthophoron* continues to grow alongside the host hypha. (c) 4 min 7 sec post-contact; coagulation of *F. oxysporum* cytoplasm up to the first host septum (arrow) and early stage of growth of an internal hypha of Po (arrowhead). (d) 10 min 25 sec post-contact; the internal branch of Po has reached the host septum and left the host hypha through the lateral wall; it now grows alongside a second host hypha (Fo2). (e) 14 min 14 sec post-contact; coagulation of second compartment of the first host hypha. (f) 18 min 43 sec post-contact; penetration branch continues to grow inside compartment (tip arrowed) and the second host hypha has coagulated.

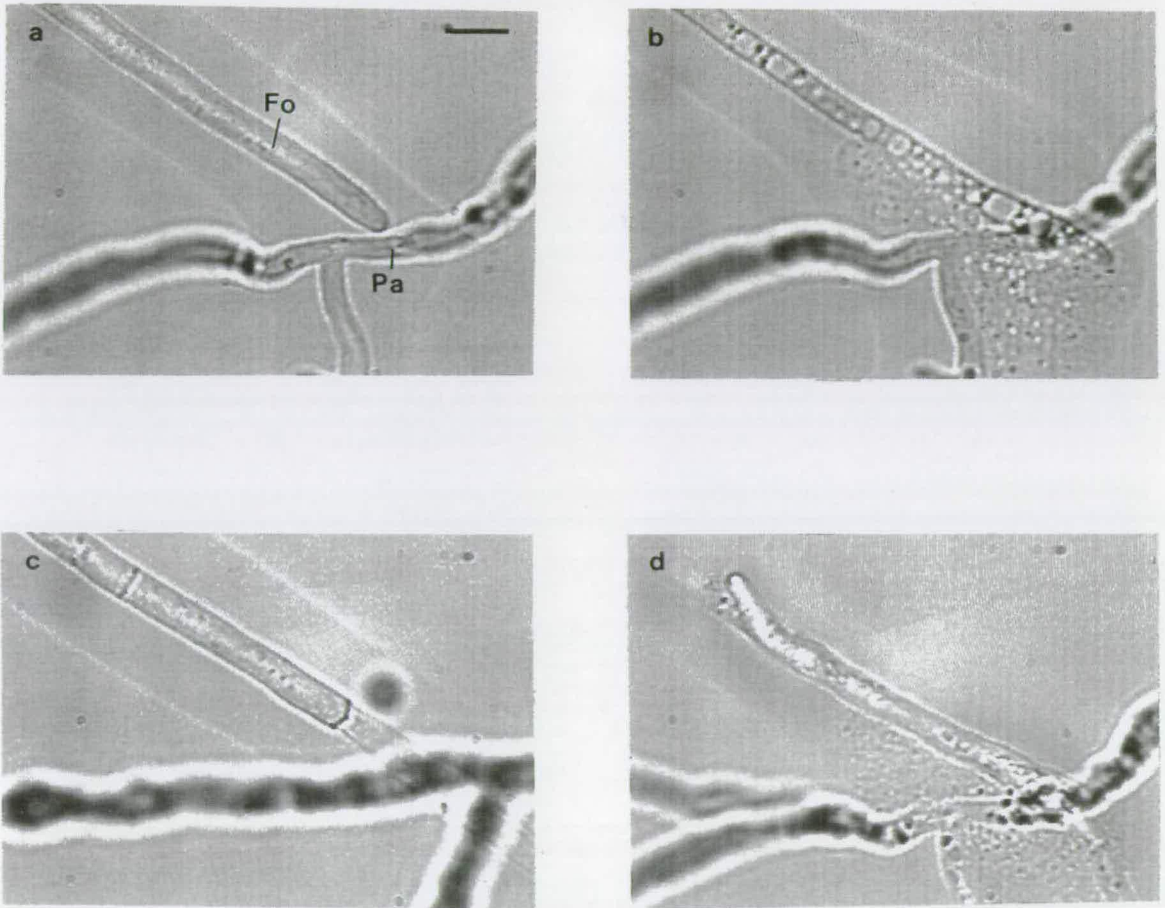


Figure 6.3a - d Stages in parasitism of *F. oxysporum* (Fo) by *P. acanthophoron* (Pa), videotaped through a film of agar. Bar=10μm. (a) *F. oxysporum* hyphal tip makes contact with side of a *P. acanthophoron* hypha. (b) 7 min 42 sec post-contact; cytoplasmic discharge of *F. oxysporum* hypha has occurred at the initial point of contact with Pa. (c) Host hypha further back from the contact point, showing the septum of the healthy sub-apical compartment bulging into the damaged apical compartment (d) 54 min 51 sec post-contact; penetration branch of *P. acanthophoron* has grown into the damaged host hyphal tip and then exits through the lateral wall.

2) Lysis of host hypha.

In this interaction the tip of a *F. oxysporum* hypha contacted the side of a *P. acanthophoron* (CBS 337.29) hypha (Figure 6.3a), and continued to grow with an unaltered growth rate after contact. A surge of *F. oxysporum* cytoplasm, towards the point of contact, was observed at 5 min 7 sec post-contact, and was followed by lysis of the *F. oxysporum* hypha a few seconds later (Figure 6.3b). At the same time as the surge was observed, the *F. oxysporum* tip stopped growing. Only the apical compartment, in contact with the *P. acanthophoron* hypha, was affected; the septum between the apical and sub-apical host compartments restricted the damage, and this septum was seen to bulge into the damaged apical compartment (Figure 6.3c), indicating that the sub-apical compartment was turgid. At 26 min 54 sec post-contact a *P. acanthophoron* branch was seen to have developed at the point of contact and it subsequently penetrated the affected *F. oxysporum* hypha and grew inside it. Finally, at 54 min 51 sec post-contact this internal branch grew out through a lateral wall of the host (Figure 6.3d).

Many other instances of lysis and coagulation were seen in contacts between either *P. acanthophoron* or *P. oligandrum* and *T. aureoviride* or *F. oxysporum*. The incidence and timings of these events were assessed as described below.

6.2.1.1.1 Interactions of *P. acanthophoron* and *P. oligandrum* with *Fusarium oxysporum*

With both *P.oligandrum* (P14.1.2) and *P. acanthophoron* (IMI 330 382) almost every interaction resulted in either lysis or coagulation (or both) of the *F. oxysporum* hyphae (Table 6.4). There were a total 21 recorded interactions for *P. oligandrum* (10 mycoparasite tip to host side and 11 mycoparasite side with host tips) and 21 for *P. acanthophoron* (11 and 10 cases of tip-side and side-tip respectively). In a few cases (2 for *P. acanthophoron*, and 5 for *P. oligandrum*) coagulation was seen to be followed by lysis. But in the majority of interactions the conspicuous mycoparasitic event involved either coagulation or lysis, not both. As summarized in Table 6.4, lysis was, overall, the most common event when *F. oxysporum* hyphal tips contacted the side of a mycoparasite hypha, whereas coagulation was common in interactions between mycoparasite tips and the sides of *F. oxysporum* hyphae.

Host lysis occurred in a total 13 cases out of an overall 21 interactions for *P. acanthophoron* (IMI 330 382) with times ranging from 83 sec to 35 min 49 sec

Table 6.4 Incidence (in parentheses) and timings (sec, means ± SEM) of different disruptive events after contact between hyphae of *F. oxysporum* and either *P. oligandrum* or *P. acanthophoron* (IMI 330 382).

	First response (of any type)*4	Coagulation	Lysis	Penetration
Tip to host side*				
P. oligandrum P14.1.2	270 ± 70 (10/10)	272 ± 122 (5/10)*3	250 ± 80 (6/10)	n.d.*5 (10/10)
P. acanthophoron IMI 330 382	316 ± 76 (11/11)	364 ± 85 (10/11)	335 ± 125 (4/11)	n.d. (11/11)
Side to host tip*2				
P. oligandrum P14.1.2	210 ± 32 (10/11)	260 ± 47 (5/11)	301 ± 56 (9/11)	n.d. (6/11)
P. acanthophoron IMI 330 382	187 ± 29 (10/10)	178 (1/10)	393 ± 220 (9/10)	n.d. (9/10)

* Interactions in which a mycoparasite hyphal tip contacted the side of a host hypha.

*2 Interactions in which a host hyphal tip contacted the side of a mycoparasite hypha.

*3 Signifies that coagulation occurred in 5 of 10 recorded interactions.

*4 First response of either mycoparasite or host hypha, e.g. surge of host or mycoparasite cytoplasm, lysis or cytoplasmic coagulation.

*5 n.d. = not determined.

post-contact (mean, 6 min 15 sec); there was no significant difference between 'host side to parasite tip' and 'host tip to parasite side' interactions in the mean time of lysis. For *P. oligandrum*, lysis was observed in 15 out of a total 21 interactions, with times ranging from 52 sec to 9 min 54 sec post-contact (mean, 4 min 41 sec), and again there was no difference in timing between 'host side to parasite tip' and 'host tip to parasite side' interactions. Almost invariably when host tips contacted the sides of hyphae of *P. oligandrum* or *P. acanthophoron* the first visible sign of an effect was surging of host protoplasm towards the contact point, coinciding with the stoppage of *F. oxysporum* tips. Coagulation or lysis then followed almost immediately.

Coagulation of *F. oxysporum* cytoplasmic contents occurred in 11 cases out of a total 21 interactions with *P. acanthophoron*, with times ranging from 72 sec to 14 min 41 sec (mean, 5 min 46 sec). For *P. oligandrum*, coagulation was observed in 10 out of a total 21 interactions, with times ranging from 52 sec to 12 min 27 sec (mean, 4 min 26 sec) and no obvious difference in timing between 'host side to parasite tip' and 'host tip to parasite side' interactions. For all interactions involving the host tip compartment, stoppage of the host tip coincided with coagulation of host cytoplasm.

In most interactions, both *P. oligandrum* and *P. acanthophoron* were seen to branch after disruption of the host, and then penetrated the host hypha. For *P. oligandrum*, in the 16 interactions where penetration was observed, it occurred by a branch that arose from the main hypha close to the initial point of contact. In 12 of the cases the branches were seen only after disruption of host cytoplasm, in 1 case at the same time as disruption and in 3 cases before disruption (mean 29 sec before disruption). With *P. acanthophoron* penetration occurred in all but one case, usually by a branch but in 2 cases by the main hypha involved in the initial contact event. The penetrating branches were most often seen (15 of the 19 cases) only after disruption of the host hypha. In a few cases where the host was not penetrated after lysis the mycoparasite branched prolifically in the spilled host contents.

Once the parasites penetrated a host hypha they often continued to grow internally, causing disruption of subsequent compartments, and were occasionally seen to produce branches which exited the host hypha. The internal hyphae of the mycoparasites grew slower than the normal external hyphae (Table 6.5). *P. acanthophoron* grew internally in *F. oxysporum* at 58% of the external growth rate,

Table 6.5 Growth rates (μm min^{-1}) of hyphae of *P. oligandrum* and *P. acanthophoron* inside the hyphae of *F. oxysporum* and *T. aureoviride*, compared with the normal external growth rates (means ± SEM for 5 replicates for the mycoparasites in each case). Also shown are equivalent external and internal growth rates of Sideris's original isolate of *P. acanthophoron* (CBS 337.29).

Parasite	Growth rate: External	Growth rate: Internal, *F. oxysporum*	Growth rate: Internal, *T. aureoviride*
P. oligandrum P14.1.2	10.4 ± 1.7	7.9 ± 2.0 (76%)*	8.2 ± 2.9 (79%)
P. acanthophoron IMI 330 382	12.5 ± 1.1	7.2 ± 1.6 (58%)	8.3 ± 1.8 (66%)
P. acanthophoron CBS 337.29	4.6 ± 1.2	4.1 ± 1.2 (89%)	n.d.*2

* Percentage of the rate of external hyphae.

*2 n.d. = not determined.

and *P. oligandrum* grew internally in *F. oxysporum* at 76% of the external growth rate. However there was no significant difference in these internal growth rates for *P. oligandrum* (P14.1.2) or *P. acanthophoron* (IMI 330 382). Regrowth of *F. oxysporum* was also occasionally observed from a healthy compartment into the disrupted empty compartment.

For comparison with the recently isolated strain of *P. acanthophoron*, described above, interactions were also recorded between *F. oxysporum* and Sideris's isolate of *P. acanthophoron* (CBS 337.29). The outcome of these interactions is shown in Table 6.6. The original isolate behaved in an essentially similar way to the newer strain, causing either lysis or coagulation of *F. oxysporum* in all recorded interactions, and in some respects faster than the newer strain. *P. acanthophoron* CBS 337.29 showed less tendency to penetrate *F. oxysporum* hyphae when the host tips contacted it, but it frequently branched and proliferated in the host lysate. As shown in Table 6.5, when *P. acanthophoron* (CBS 337.29) penetrated the host it grew internally at 89% of its external rate.

6.2.1.1.2 Interactions of *P. acanthophoron* and *P. oligandrum* with *Trichoderma aureoviride*

The same overall trend was seen in interactions with the host *T. aureoviride* (Table 6.7) as with *F. oxysporum* (Table 6.4). Every contact event with *P. acanthophoron* or *P. oligandrum* resulted in either lysis or coagulation of *T. aureoviride* hyphae (and coagulation followed by lysis in one case). All interactions involving 'host tip to mycoparasite side' with both *P. acanthophoron* and *P. oligandrum* resulted in lysis, as did most of those involving 'mycoparasite tip with *T. aureoviride* side'. On the whole, compared to interactions with *F. oxysporum* (Table 6.4), more interactions with *T. aureoviride* resulted in lysis of host hyphae (41 out of 45 cases). The damage to hyphae of *T. aureoviride* often was faster than with *F. oxysporum* in equivalent contact events (cf. Tables 6.7 and 6.4).

For *P. acanthophoron* there was no significant difference in the timings of the parasitic events between 'mycoparasite tip to *T. aureoviride* side' and '*T. aureoviride* tip to mycoparasite side' interactions. However for *P. oligandrum* the timings for interactions between 'mycoparasite tip to *T. aureoviride* side' were faster ($P < 0.05$) than for '*T. aureoviride* tip to mycoparasite side' interactions. In terms of both the incidence and timing of lysis there was no significant difference between *P. oligandrum* and *P. acanthophoron*. For *P. oligandrum*, host lysis occurred in a total

Table 6.6 Incidence (in parentheses) and timings (sec, means ± SEM) of different disruptive events after contact between hyphae of *F. oxysporum* and *P. acanthophoron* (CBS 337.29).

	First response (of any type)	Coagulation	Lysis	Penetration
Tip to host side*	135 ± 29 (13/13)	212 ± 106 (3/13)*3	158 ± 30 (13/13)	n.d.*4 (11/13)
Side to host tip*2	219 ± 26 (10/10)	476 (1/10)	229 ± 32 (10/10)	n.d. (3/10)

* Interactions in which a mycoparasite hyphal tip contacted the side of a host hypha.

*2 Interactions in which a host hyphal tip contacted the side of a mycoparasite hypha.

*3 Signifies that coagulation occurred in 3 of 13 recorded interactions.

*4 n.d. = not determined

Table 6.7 Incidence (in parentheses) and timings (sec, means ± SEM) of different disruption events after contact between hyphae of *T. aureoviride* and either *P. oligandrum* or *P. acanthophoron* (IMI 330 382).

	First response (of any type)*	Coagulation	Lysis	Penetration
Tip to host side*2				
P. oligandrum P14.1.2	84 ± 12 (11/11)	198 ± 24 (2/11)	88 ± 16 (10/11)	n.d.*4 (10/11)
P. acanthophoron IMI 330 382	102 ± 18 (12/12)	138 ± 12 (3/12)	123 ± 14 (9/12)	n.d. (12/12)
Side to host tip*3				
P. oligandrum P14.1.2	132 ± 12 (12/12)	n.d. (0/12)	182 ± 57 (12/12)	n.d. (10/12)
P. acanthophoron IMI 330 382	132 ± 24 (10/10)	n.d. (0/10)	138 ± 24 (10/10)	n.d. (9/10)

* First response of either mycoparasite or host hypha, e.g. surge of host or mycoparasite cytoplasm, lysis, or cytoplasmic coagulation.

*2 Interactions in which a mycoparasite hyphal tip contacted the side of a host hypha.

*3 Interactions in which a host hyphal tip contacted the side of a mycoparasite hypha.

*4 n.d. = not determined

22 interactions out of a possible 24, with times ranging from 47 sec to 13 min 15 sec post-contact (mean, 2 min 19 sec). With *P. acanthophoron*, host lysis was observed in 19 interactions out of a total of 22, with times ranging from 46 sec to 4 min 35 sec post-contact (mean, 2 min 11 sec).

For both *P. acanthophoron* and *P. oligandrum*, host tip growth stopped at the same time as disruption of *T. aureoviride* hyphae in all interactions involving contact between the mycoparasite hypha (side or tip) and the host apical compartment (side or tip).

Penetration of *T. aureoviride* hyphae was observed in 20 out of a total 23 interactions for *P. oligandrum*; in 4 cases the main hypha of the mycoparasite penetrated the host, but in the remaining cases penetration was by means of a branch formed close to the first point of contact. In 12 such interactions the branch was first seen just after disruption of the host, in 2 cases at the same time as host disruption and in the other 2 just beforehand (mean 1 min 7 sec before disruption). With *P. acanthophoron*, all penetrations were from branches formed close to the first point of contact, 15 of these branches being seen after disruption of the host hypha, and the remaining 6 at the same time as disruption. As with *F. oxysporum*, where penetration was not observed the mycoparasites were seen to branch prolifically in the spilled host contents.

Once the mycoparasites penetrated the host hyphae, they often continued to grow internally, causing disruption of subsequent compartments. The internal growth rates of the mycoparasites, shown in Table 6.5, were somewhat slower than the external growth rates, but not significantly so.

In summary of these interactions with non-*Pythium* hosts, *P. acanthophoron* was found to be as aggressively mycoparasitic as *P. oligandrum* and to cause identical types of damage at similar times post-contact. Moreover, the two tested isolates of *P. acanthophoron* showed similar aggressiveness to one another. Comparing the two hosts, *T. aureoviride* was apparently more susceptible than *F. oxysporum* because it was disrupted faster after contact events, but hyphae of both hosts were irreversibly damaged within means of *c.*1.5 to 5 min after contact events.

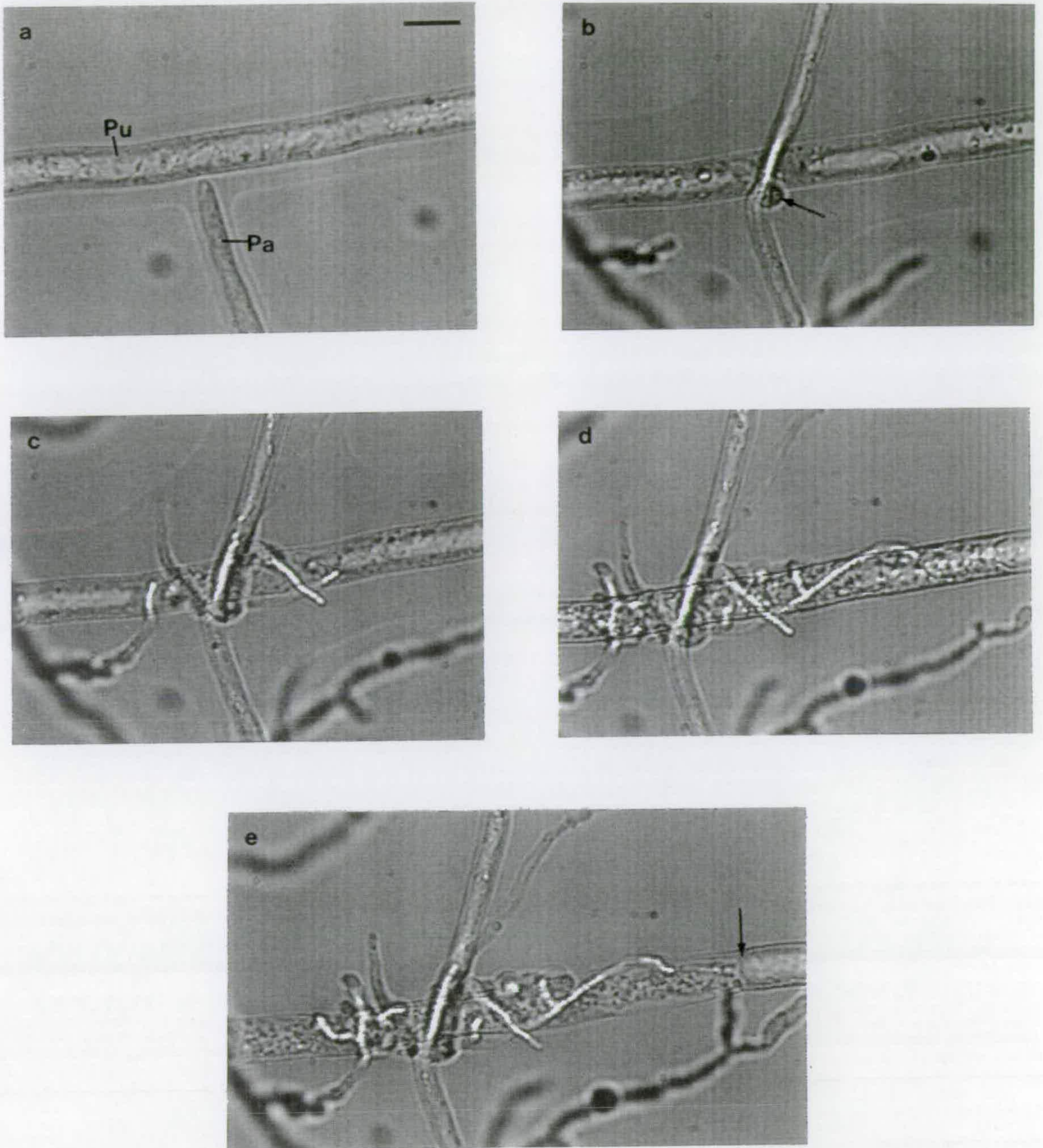


Figure 6.4a - e Stages in parasitism of *P. ultimum* (Pu) by *P. acanthophoron* (Pa), videotaped through a film of agar. Bar=10μm. (a) *P. acanthophoron* hyphal tip contacts side of a *P. ultimum* hypha (b) 55 min post-contact; *P. acanthophoron* branches (arrowed) and (c) begins to coil around *P. ultimum* hypha. (d) 1 h 38 min post-contact; coagulation of *P. ultimum* cytoplasm. (e) Barrier zone formed (arrowed); note the differences in the host cytoplasm on either side of this.

6.2.1.2 Interactions of *P. acanthophoron* and *P. oligandrum* with *Pythium* hosts

The interactions of *P. acanthophoron* and *P. oligandrum* with *Pythium* hosts (*P. ultimum* and *P. aphanidermatum*) were found to be different from those with non-*Pythium* hosts. Also, interactions with *P. ultimum* and *P. aphanidermatum* were seen to be different from each other, and will be dealt with separately.

6.2.1.2.1 *P. ultimum* as host

The characteristic pattern of attack of the two mycoparasites on the hyphae of *P. ultimum* was the same (Table 6.8), and can be described by a representative interaction (Figures 6.4a - e).

In this case a hyphal tip of *P. acanthophoron* (IMI 330 382) contacted the side of a *P. ultimum* hypha (Figure 6.4a) and continued to grow at an unaltered rate after contact. After 55 min a branch of *P. acanthophoron* formed at the initial point of contact (Figure 6.4b). This branch started to coil and branch further around the hypha of *P. ultimum* (Figure 6.4c) but had no initial effect on *P. ultimum*, as indicated by normal cytoplasmic streaming in the latter. A surge of cytoplasm in the *P. ultimum* hypha was observed at 1 h 36 min post-contact, resulting in coagulation of the cytoplasm (Figure 6.4d). However, this disruption of the *P. ultimum* hypha was seen to be contained by zones of densely coagulated cytoplasm, and cytoplasm of normal appearance and normal streaming was seen beyond these 'barrier zones' (Figure 6.4e).

In some interactions, but not in the one described above, a release of host (*P. ultimum*) cytoplasmic contents was seen from a point beneath the coiling hyphae of *P. acanthophoron* or *P. oligandrum*. The discharged cytoplasm normally had a globular appearance (Figure 6.5). Concurrent with this, there was a sudden rapid streaming of the cytoplasm of *P. acanthophoron* or *P. oligandrum* towards the interaction zone. 'Shifting' of the host cytoplasm was also observed after coagulation in many cases, and all these three observations were taken as evidence that *P. acanthophoron* or *P. oligandrum* had penetrated the hypha of *P. ultimum* or, at least, breached its wall. In later stages of some interactions, hyphae of *P. acanthophoron* and *P. oligandrum* were clearly seen inside the *P. ultimum* hyphae.

There was little difference in the incidence and timings of these events in interactions involving the two mycoparasites, *P. acanthophoron* and *P. oligandrum* (Table 6.8). However, compared to the interactions of *P. oligandrum* and *P. acanthophoron* with non-*Pythium* hosts, (*T. aureoviride* and *F. oxysporum*; Tables

Table 6.8 Incidence (in parentheses) and time (min, mean ± SEM) after contact of *P. ultimum* and mycoparasite hyphae when various mycoparasitic events occurred on films of water agar.

	Branch by parasite	Coiling	Cytoplasmic surging	Coagulation	Cytoplasmic discharge	Penetration
Pythium acanthophoron (IMI 330 382)	16 ± 7 (9/11)	n.d.* (10/11)	60 ± 3 (11/11)	61 ± 4 (10/11)	79 ± 4 (3/11)	n.d. (6/11)
Pythium oligandrum (P14.1.2)	8 ± 1 (10/11)	n.d. (10/11)	44 ± 8 (8/11)	54 ± 6 (9/11)	51 (1/11)	n.d. (2/11)
Pythium mycoparasiticum (AR7A)	32 ± 21 (5/9)	n.d. (3/9)	n.d. (0/9)	n.d. (0/9)	n.d. (0/9)	n.d. (0/9)

* n.d. = not determined

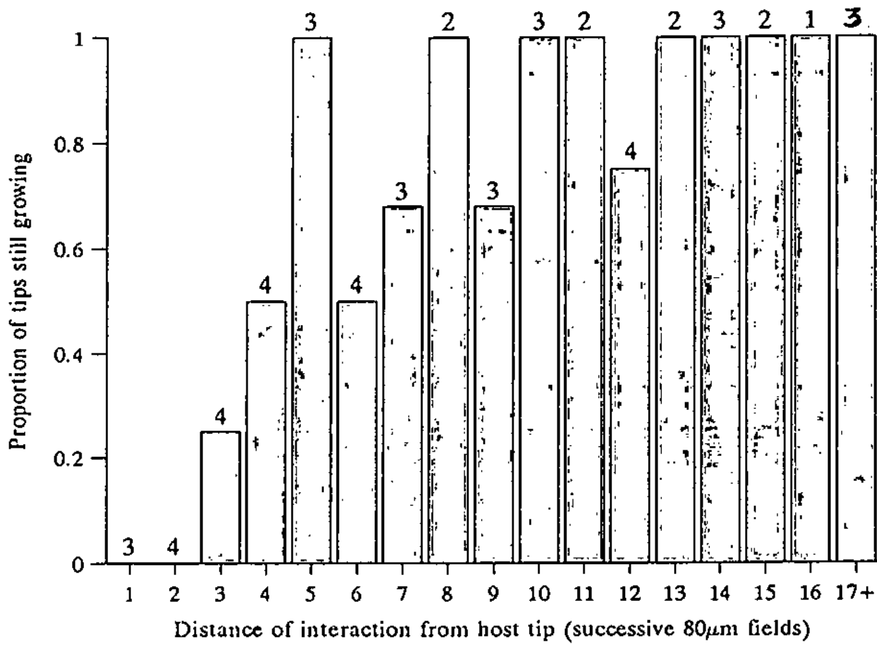
6.7 and 6.4), disruption of the *Pythium* host hyphae was much slower, and rapid 'explosive' lysis was never observed.

Eleven interactions were recorded for each of *P. oligandrum* and *P. acanthophoron* against *P. ultimum*. Eight interactions were between a *P. acanthophoron* hyphal tip and the side of a *P. ultimum* hypha; the remaining 3 were between the host hyphal tip and the side of *P. acanthophoron*. Of the 11 interactions involving *P. oligandrum*, 10 were between the mycoparasite tip and the side of a *P. ultimum* hypha. There was no obvious difference in the incidence or timings of events for 'parasite tip to host side' and 'host tip to parasite side' interactions for either *P. oligandrum* or *P. acanthophoron*, so the two types of interactions were pooled for analysis.

As shown in Table 6.8, in almost all interactions both mycoparasites branched and coiled round *P. ultimum*. Cytoplasmic surging in the host hyphae occurred after a considerable delay (mean 44 - 60 min) and led to coagulation of the host cytoplasm soon afterwards. However, discharge of cytoplasm from *P. ultimum* hyphae was seen in only 4 of the 22 interactions with *P. oligandrum* and *P. acanthophoron*. Internal hyphae of the mycoparasites in the hyphae of *P. ultimum* were seen in 8 of the total 22 interactions.

As was stated previously, in many cases the disruption of the *P. ultimum* hyphae was localised by apparent barrier zones of cytoplasm, and beyond these the host cytoplasm appeared normal (Figure 6.4e). Interactions behind the host tip would therefore not be expected to result in stoppage of the *P. ultimum* tip. The host might thus escape major parasitic damage unless interactions occurred close to its tip. In order to investigate this, interactions on water agar were scanned, with a X10 objective, for areas of mycoparasitic coiling near to the tips of *P. ultimum* hyphae, and were then observed with a X70 objective. Cases where the host cytoplasm had coagulated beneath the coils were selected, and the distance from the interaction site (point of initial contact) to the host tip was measured, with the host tip being scored as either growing or having been stopped (with coagulated cytoplasm). Fifty interactions for both *P. oligandrum* and *P. acanthophoron* were assessed. As shown in Figure 6.6, for both *P. oligandrum* and *P. acanthophoron* the further back the interaction site was located from the host tip, the greater was the frequency of continued growth of the hyphal tip of *P. ultimum*. *P. oligandrum* caused the stoppage of more *P. ultimum* hyphal tips than did *P. acanthophoron*, and also resulted in stoppage of *P. ultimum* from interactions further from the tips compared to

P. oligandrum



P. acanthophoron

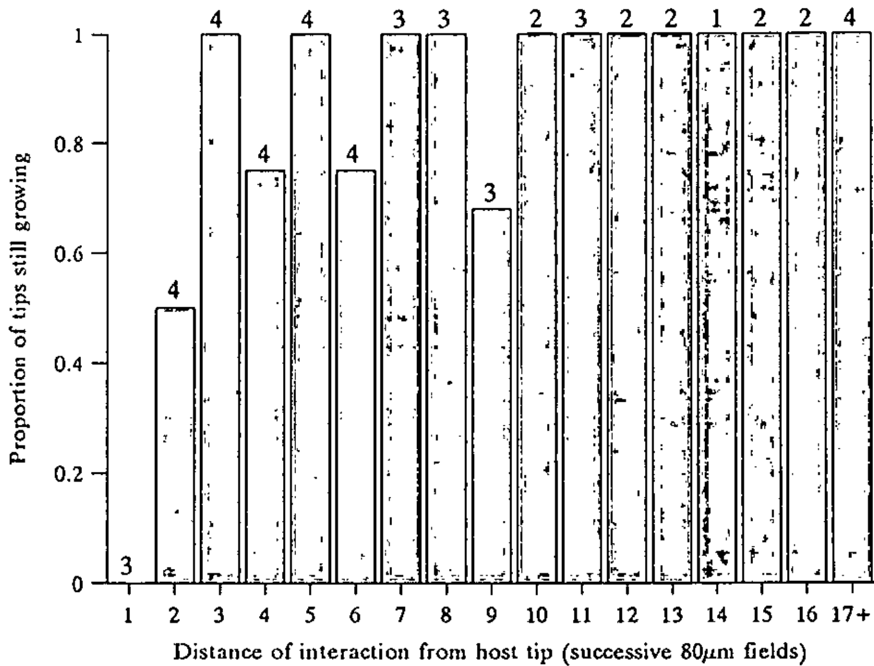


Figure 6.6 Proportion of tips of *P. ultimum* that were still growing when the hyphae had been parasitised by either *P. oligandrum* or *P. acanthophoron* at different distances behind the tip; numbers above the histograms refer to the number of replicate observations at each distance interval.

P. acanthophoron. However, interaction points further than 4 fields of view (one field of view= 80μm) from the host tip led to stoppage of the host tips in very few instances.

Videotaped interactions between *P. mycoparasiticum* and *P. ultimum* (Table 6.8) were different from those involving *P. oligandrum* or *P. acanthophoron*. Although *P. mycoparasiticum* often branched at point of contact with *P. ultimum* (5 of the total 9 interactions) and coiled in some (3) of them, this coiling by *P. mycoparasiticum* was not as extensive nor as tight as by *P. acanthophoron* or *P. oligandrum* (see Fig 6.7). Moreover, it never led to coagulation or lysis of the *P. ultimum* hypha, even after 3 h post-contact. Scanning of water agar films with X10 and X70 objectives to assess many further interaction sites similarly failed to give evidence of successful parasitism of *P. ultimum* by *P. mycoparasiticum*.

6.2.1.2.2 *P. aphanidermatum* as host

Interactions between the three mycoparasitic *Pythium* species and *P. aphanidermatum* (CBS 634.70) were different from those with *P. ultimum* as the host. In several cases *P. aphanidermatum* was found to be the aggressor in the interactions. The data from videotaped recordings of interactions between *P. aphanidermatum* and the three mycoparasites are shown in Table 6.9, and can be compared with Table 6.8 for the same mycoparasites with *P. ultimum*. Such comparison shows that the mycoparasites branched and coiled less frequently around *P. aphanidermatum* than around *P. ultimum*, and even when coiling occurred the disruption of *P. aphanidermatum* was never observed. When seen, the coiling of the mycoparasites round *P. aphanidermatum* hyphae was similar to that observed round *P. ultimum* (Figure 6.4a - c). In this respect, it is notable that only *P. acanthophoron* showed a high frequency of coiling round *P. aphanidermatum*, whereas both *P. acanthophoron* and *P. oligandrum* usually coiled round *P. ultimum*. In 6 of the total 27 recorded interactions, *P. aphanidermatum* coiled round the mycoparasite hyphae, and in all these 6 cases caused coagulation of the mycoparasite cytoplasm. However, penetration of the mycoparasite hyphae by *P. aphanidermatum* was only observed in one interaction, with *P. oligandrum*.

A common feature in interactions between mycoparasites and *P. aphanidermatum* was that *P. aphanidermatum* branched at the first point of contact with a mycoparasite hypha (in 10 of the total 27 interactions involving *P. acanthophoron*, *P. oligandrum* and *P. mycoparasiticum*; Table 6.9). However, this

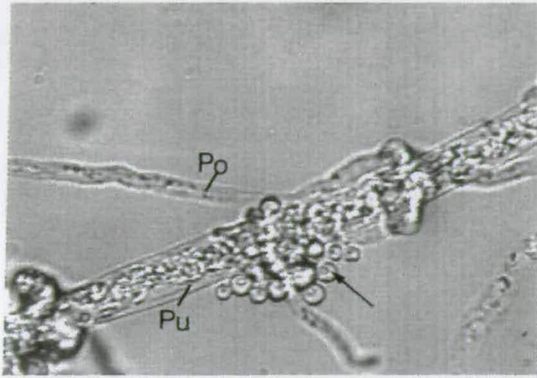


Figure 6.5 Cytoplasmic discharge of *P. ultimum* (Pu) hypha beneath a coiling branch of *P. oligandrum* (Po). Note the globular appearance of the cytoplasmic discharge (arrowed).

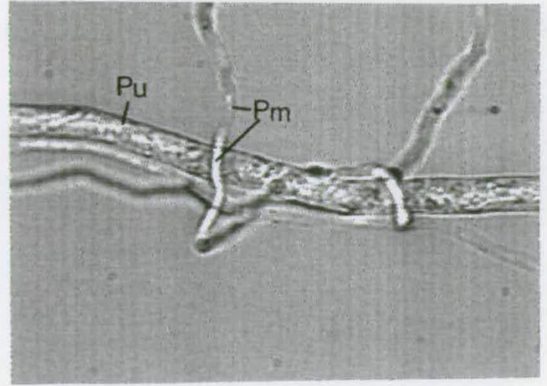
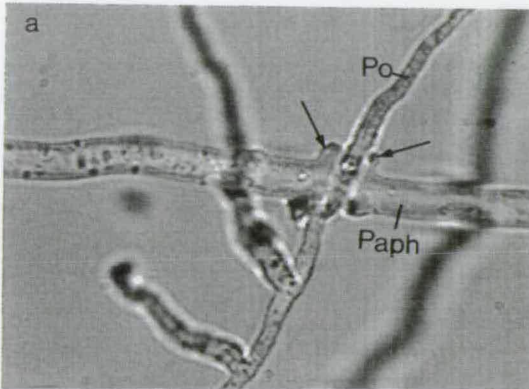


Figure 6.7 Coiling of *P. mycoparasiticum* (Pm) round a *P. ultimum* (Pu) hypha, 3h post-contact.



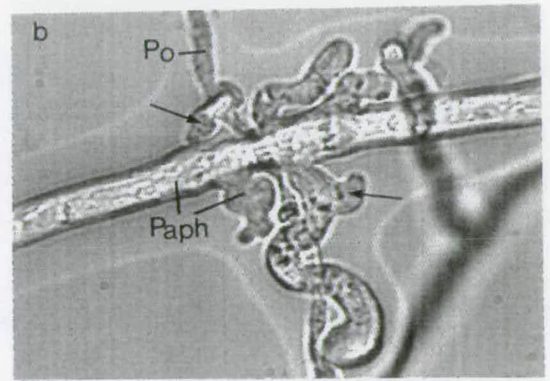


Figure 6.8 'Hyphal swellings' (arrowed) of *P. aphanidermatum* (Paph) against *P. oligandrum* (Po) at different stages: (a) initial formation, (b) swelling and development of lobes.

Table 6.9 Incidence (in parentheses) and time (min, means ± SEM) after contact of hyphae of *P. aphanidermatum* (Paph) and mycoparasitic *Pythium* spp. (myco) when various mycoparasitic events occurred on films of water agar.

	Branching of		Coiling of		Cytoplasmic surging in		Coagulation in		Cytoplasmic/ discharge from		Penetration by	
	myco	Paph	myco	Paph	myco	Paph	myco	Paph	myco	Paph	myco	Paph
Pythium acanthophoron	13 ± 3	47 ± 36	n.d.*	n.d.	112 ± 51	n.d.	112 ± 51	n.d.	n.d.	n.d.	n.d.	n.d.
(IMI 330 382)	(13/14)	(4/14)	(9/14)	(2/14)	(2/14)	(0/14)	(2/14)	(0/14)	(0/14)	(0/14)	(0/14)	(0/14)
Pythium oligandrum	3 ± 1	40 ± 13	n.d.	n.d.	80 ± 3	n.d.	90 ± 4	n.d.	n.d.	n.d.	n.d.	140
(P14.1.2)	(2/7)	(4/7)	(1/7)	(3/7)	(3/7)	(0/7)	(3/7)	(0/7)	(0/7)	(0/7)	(0/7)	(1/7)
Pythium mycoparasiticum	n.d.	24 ± 15	n.d.	n.d.	42	n.d.	57	n.d.	n.d.	n.d.	n.d.	n.d.
(AR7A)	(0/6)	(2/6)	(0/6)	(1/6)	(1/6)	(0/6)	(1/6)	(0/6)	(0/6)	(0/6)	(0/6)	(0/6)

* n.d. = not determined

branching took a peculiar form, because often more than one branch developed at the contact point, and these branches swelled into irregular lobate structures. Two examples of this with *P. oligandrum* are shown in Figure 6.8. The development of these branches was often slower than the development of branches of mycoparasites on contact with *Pythium* or non-*Pythium* hosts (compare Table 6.9 with Tables 6.8 and 6.4). They were slower to develop than the corresponding branches of the mycoparasites (where branches occurred) at points of contact with *P. aphanidermatum* (Table 6.8). Ultimately, the mycoparasitic hyphae were always disrupted (cytoplasmic coagulation) in the vicinity of these lobate branches of *P. aphanidermatum*. After this disruption it was sometimes observed that the branches of *P. aphanidermatum* coiled round hyphae of the mycoparasites, and occasionally *P. aphanidermatum* penetrated the mycoparasitic hypha from a pre-penetration swelling. The extent of damage in the mycoparasite hyphae was, however, always limited by 'barrier zones' of protoplasm, beyond which the cytoplasm was of normal appearance and showed cytoplasmic streaming.

6.2.2 Activity of P. aphanidermatum *against various fungi*

In view of the observations above it was of interest to extend the range of interactions involving *P. aphanidermatum*, to see if it could mimic the activities of a 'typical' mycoparasitic *Pythium* species.

6.2.2.1 Interaction with *P. periplocum*

Hockenhull *et al.* (1992) had reported that the mycoparasite *P. periplocum* antagonised *P. aphanidermatum*. However, videotaped interactions between *P. periplocum* (P9.8.4) and *P. aphanidermatum* (CBS 634.70) showed that *P. aphanidermatum* was the aggressor on films of water agar. On contact, *P. periplocum* was observed to branch and coil round *P. aphanidermatum* hyphae but never caused disruption of *P. aphanidermatum*. In contrast, *P. aphanidermatum* produced lobate hyphal swellings (but after some delay) at contact points with *P. periplocum* hyphae, and caused coagulation of the *P. periplocum* hyphae beneath these swellings. Thus all four of the mycoparasitic *Pythium* spp. tested against *P. aphanidermatum* caused no disruption of it but, sometimes, were disrupted by *P. aphanidermatum*.

6.2.2.2 Interactions with *T. aureoviride* and *F. oxysporum*

P. aphanidermatum was seen to antagonise *T. aureoviride* (Table 6.10). It did so in 8 of 15 recorded interactions, causing coagulation of the cytoplasm of *T. aureoviride* and less often penetrating the hyphae. But comparison of Table 6.10 with Table 6.7 shows that the speed of the antagonism was much slower than when the mycoparasitic *Pythium* spp. (*P. oligandrum* and *P. acanthophoron*) interacted with *T. aureoviride*. Also, in cases where *P. aphanidermatum* penetrated the hyphae of *T. aureoviride*, the growth rate of the internal hypha (2.9 ± 0.5 μm min^{-1}) was much slower than the normal (external) growth rate of hyphae (15.5 ± 1.0 μm min^{-1}; these values being for 5 replicates in each case). The internal growth rate of *P. aphanidermatum* was thus less than 20% of the 'external' growth rate, whereas internal hyphae of *P. oligandrum* and *P. acanthophoron* in *T. aureoviride* extended at more than 60% of the 'external' growth rate (see Table 6.5).

In a representative interaction (Figures 6.9a - f) a *T. aureoviride* hyphal tip contacted the side of a *P. aphanidermatum* hypha (Figure 6.9a) and continued to grow alongside the *P. aphanidermatum* hypha. After 2 min 45 sec, a jerk of the *T. aureoviride* cytoplasm was observed, resulting in stoppage of growth of the *Trichoderma* tip (Figure 6.9b). At 13 min 35 sec post-contact, two very narrow *P. aphanidermatum* branches were observed (locations marked in Figure 6.9b). A surge of *Trichoderma* host cytoplasm was then observed, 25 min after contact, and the *Trichoderma* cytoplasm coagulated (Figure 6.9c). Only the hyphal compartment in contact with *P. aphanidermatum* was affected; normal cytoplasmic streaming was observed in the adjacent compartment of *T. aureoviride*, beyond the first septum (Figure 6.9d). The hypha of *T. aureoviride* was seen to have been penetrated by *P. aphanidermatum* 51 min post-contact (Figure 6.9e). The internal hypha continued to grow, but slowly, and it eventually stopped before reaching the first septum (Figure 6.9f).

There was no obvious difference in the incidence or timings of such events between 'side to host tip' and 'tip to host side' interactions (Table 6.10).

A notable feature in several interactions that resulted in damage when hyphae of *T. aureoviride* contacted the hyphae of *P. aphanidermatum* was that *P. aphanidermatum* formed several narrow 'pin branches' in the zone of contact. These were so short and narrow that they were difficult to discern or photograph, but representative examples are shown in Figure 6.10 and Figure 6.9b. Usually (4 out of 5 videotaped cases) their development was followed by coagulation of the

Table 6.10 Incidence (in parentheses) and timings (sec, means ± SEM) of different disruptive events after contact between hyphae of *T. aureoviride* and *P. aphanidermatum* (CBS 634.70).

	Trichoderma tip stopped growing	Branch by *P. aphanidermatum*	Cytoplasmic surging in *Trichoderma*	Coagulation of *Trichoderma*	Lysis/ discharge	Penetration of *Trichoderma*
P. aphanidermatum tip to *Trichoderma* side	764 (1/7)	1995 (1/7)	1488 ± 607 (3/7)	1773 ± 816 (3/7)	n.d.* (0/7)	n.d. (2/7)
Trichoderma tip to *P. aphanidermatum* side	916 ± 460 (5/8)	307 ± 55 (3/8)	881 ± 402 (5/8)	1246 ± 601 (3/8)	n.d. (0/8)	n.d. (1/8)

* n.d. = not determined.

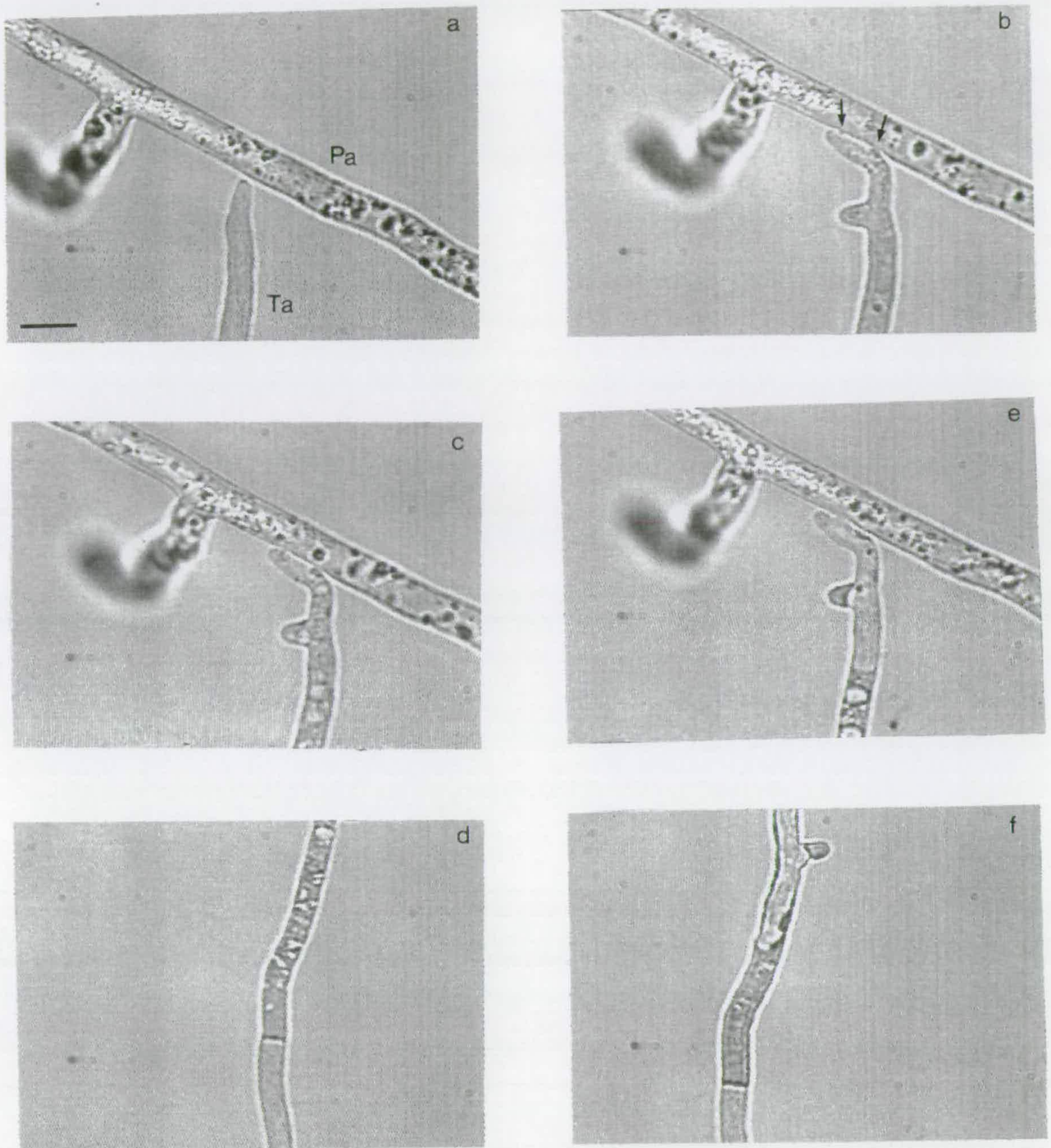
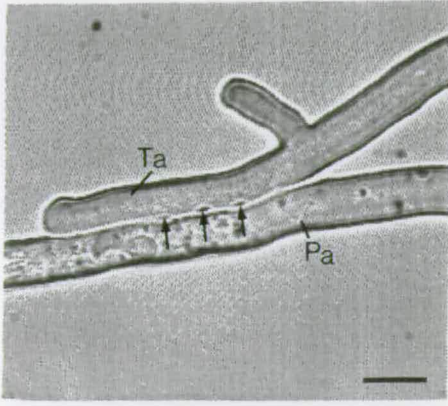
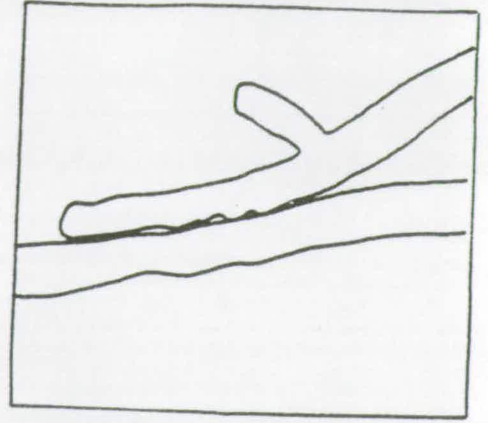
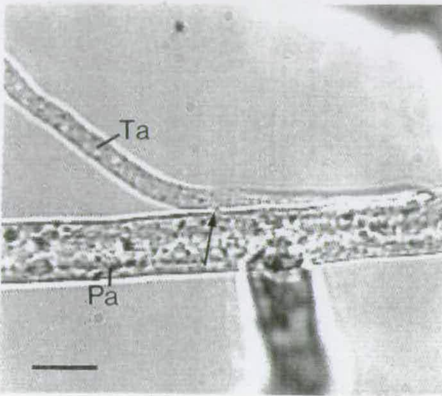
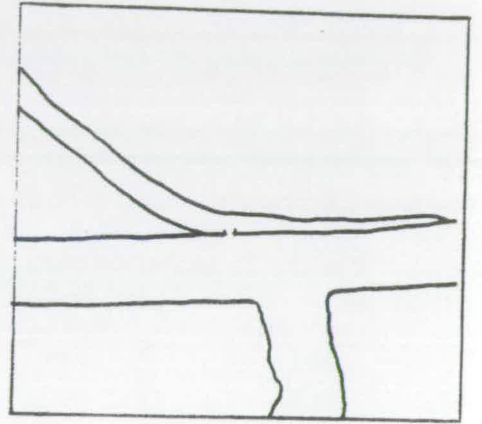


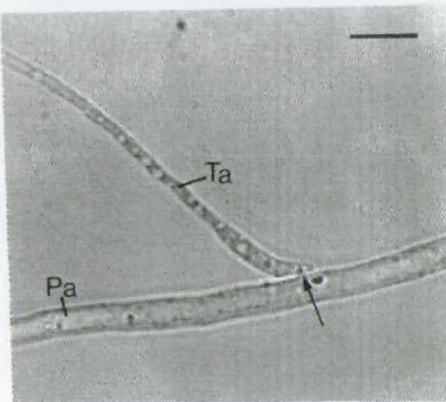
Figure 6.9a - f Stages in parasitism of *T. aureoviride* (Ta) by *P. aphanidermatum* (Pa), videotaped through a film of agar. Bar=10μm. (a) *T. aureoviride* hyphal tip makes contact with side of *P. aphanidermatum* hypha. (b) 2 min 49 sec post-contact; *T. aureoviride* hyphal tip has stopped growing. Note two very narrow *P. aphanidermatum* branches (arrowed). (c) 32 min 26 sec post-contact; coagulation of *T. aureoviride* cytoplasmic contents. (d) Coagulation is localised to compartment immediately in contact with *P. aphanidermatum* hypha; note the difference in cytoplasm on either side of the septum. (e) 55 min 10 sec post-contact; penetration by *P. aphanidermatum* observed. (f) 1 h 41 min post-contact; penetration branch stops growing before the first septum. Tip of the penetration branch is distorted.











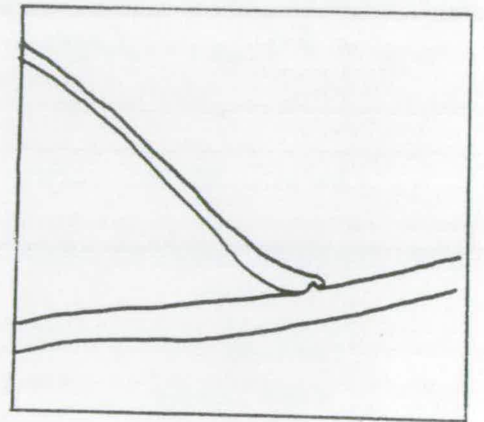


Figure 6.10 'Pin branches' (arrowed) of *P. aphanidermatum* (Pa) against *T. aureoviride* (Ta). Bar represents 10μm. For each video copy print a diagramatic representation of the general features is shown adjacent.

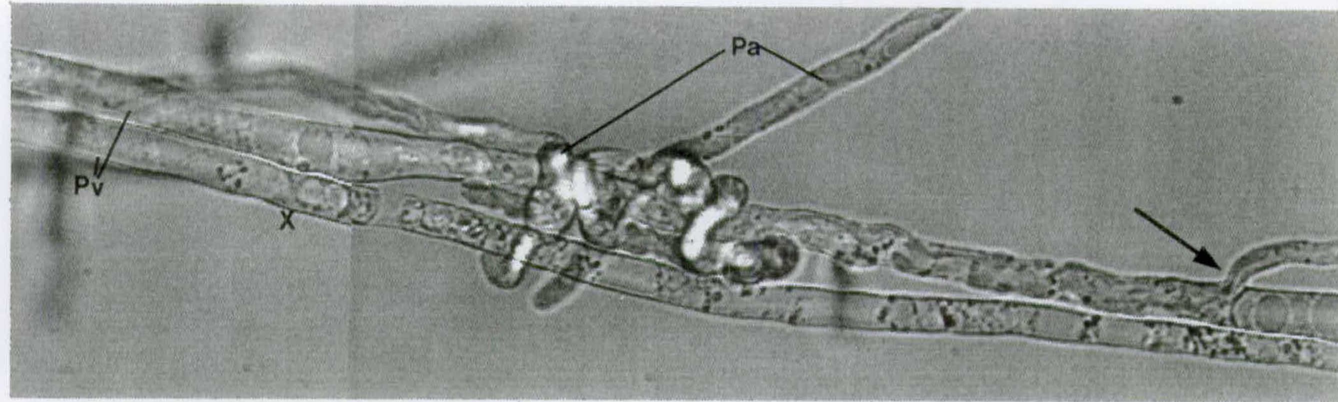


Figure 6.11 Composite photograph showing coiling by *P. aphanidermatum* around *P. vexans* (Pv) hypha resulting in coagulation and penetration of *P. vexans* hypha. Note exit of penetration hypha (arrowed) and normal cytoplasm beyond point 'x'.

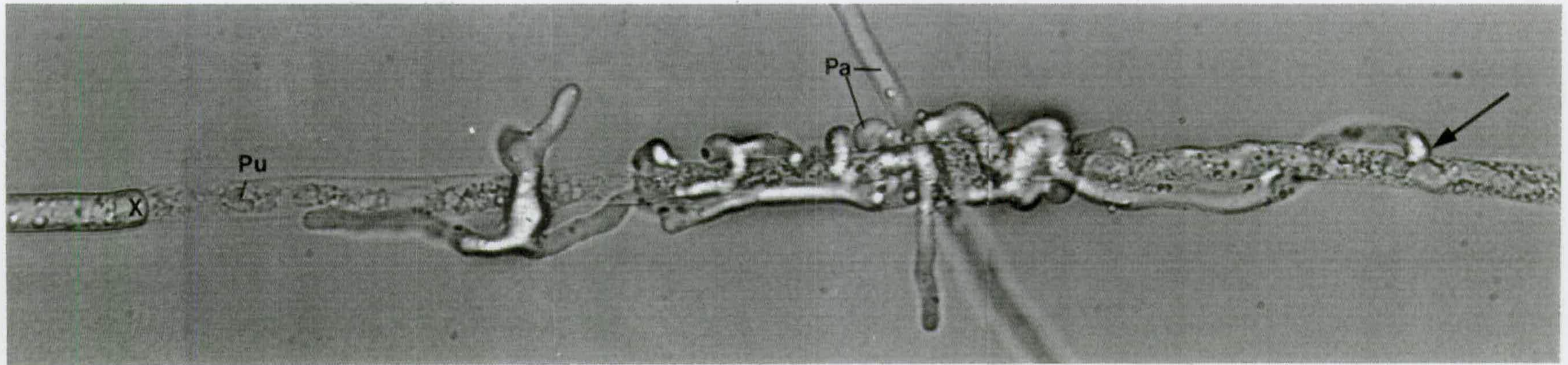


Figure 6.12 Composite photograph showing coiling by *P. aphanidermatum* (Pa) around *P. ultimum* (Pu) hypha resulting in coagulation and penetration of *P. ultimum* hypha. Note the cytoplasmic barrier zone (x) beyond which the *P. ultimum* cytoplasm is normal. Also shown is penetration point of *P. aphanidermatum* (arrowed).

cytoplasm of *T. aureoviride*. However, internal growth of *P. aphanidermatum* was never seen to develop from these 'pin branches'.

Identical types of interactions were observed between *P. aphanidermatum* (CBS 634.70) and *F. oxysporum* on water agar films, but these interactions were not videotaped individually. On scanning the agar films, there were several instances of cytoplasmic coagulation of *F. oxysporum*, and occasional penetration of its hyphae by *P. aphanidermatum*.

6.2.2.3 Interactions of *P. aphanidermatum* with other phytopathogenic *Pythium* spp

Five *Pythium* spp.- *P. graminicola* (IMI 91329), *P. catenulatum* (MS1), *P. dissotocum* (MS2), *P. vexans* (CBS 270.30) and *P. ultimum* (G39)- were opposed to *P. aphanidermatum* (CBS 634.70) on water agar films. Once the colonies had mingled the films were scanned, using a X10 objective, for contacts between individual hyphae. Then each contact event was examined using a X70 objective and recorded as either 'no effect', when the hyphae had not responded to one another, or 'effect' when *P. aphanidermatum* had coiled around the hypha of the other fungus. For each *Pythium* sp. 100 such contact events were assessed. There were no cases where the other fungi coiled around *P. aphanidermatum*.

As shown in Table 6.11, *P. aphanidermatum* coiled round some hyphae of each *Pythium* sp., but the incidence of this differed markedly between the species. Coiling was most often seen in contacts with *P. ultimum*, less often with *P. vexans*, *P. dissotocum* and *P. catenulatum*, and only rarely with *P. graminicola*. Chi-squared analysis of the data in Table 6.11 showed a very highly significant difference in incidence of coiling for the different species (X^2 40.6; 4 d.f.; $P < 0.001$). Even excluding *P. ultimum* from the analysis there was a significant difference (X^2 9.37; 3d.f.; $P < 0.05$). Of the three coiling interactions involving *P. graminicola*, coagulation of the 'host' cytoplasm was observed only once and penetration of the 'host' by *P. aphanidermatum* was never seen. In contrast, coagulation and penetration of *P. catenulatum*, *P. dissotocum* and *P. vexans* hyphae were often observed. Figure 6.11 shows one such case- the parasitism of *P. vexans* by *P. aphanidermatum*. It involved intense localised coiling of *P. aphanidermatum* around the 'host', followed by disruption of the host cytoplasm and penetration from a point (obscured) beneath the coils. Figure 6.11 also shows that the internal hypha of *P. aphanidermatum* grew irregularly within the hypha of *P. vexans* and subsequently left the host hypha, through the lateral wall. In addition, the extent of disruption of the host hypha was

limited. Beyond the point shown (x) in Figure 6.11, the host hypha had normal cytoplasmic appearance and normal cytoplasmic streaming.

Table 6.11 Numbers of coiling interactions (max. 100) by *P. aphanidermatum* against various *Pythium* hosts on films of water agar.

Host *Pythium* spp.	Number of positive interactions (max. 100)
P. graminicola	3
P. catenulatum	8
P. dissotocum	10
P. vexans	16
P. ultimum	34

A similar instance of interaction between *P. aphanidermatum* and *P. ultimum* is shown in Figure 6.12. Again, there is a clear demarcation between the damaged and normal cytoplasm of the host *Pythium*, and in this case at least one point of entry of *P. aphanidermatum* into the host hyphae is clearly seen (arrowed). In view of the fact that *P. ultimum* was the most susceptible of the host *Pythium* spp. to damage by *P. aphanidermatum*, the interactions between these fungi were studied in further detail, using timed video sequences.

As shown in Table 6.12, 6 of the 7 recorded interactions resulted in branching of *P. aphanidermatum* (mean, 4 min post-contact), but only once did this lead to coiling of *P. aphanidermatum* around a *P. ultimum* hypha. In this interaction (Figures 6.13a - f) a *P. aphanidermatum* hyphal tip contacted the side of a *P. ultimum* hypha (Figure 6.13a) and continued to grow across it with unaltered growth rate. *P. aphanidermatum* branched where it had crossed *P. ultimum* (Figure 6.13b), then formed further branches in the contact zone (Figure 6.13c) and the branches proliferated and 'coiled' round the *P. ultimum* hypha (Figure 6.13d). A surge of cytoplasm was observed in the *P. ultimum* hypha (42 min 44 sec post-contact) resulting in coagulation of the *P. ultimum* cytoplasm at *c.* 44 min

Table 6.12 Time after contact (min, means ± SEM) of *P. ultimum* and *P. aphanidermatum* hyphae when various mycoparasitic activities occurred on films of water agar; numbers in parentheses are numbers of interactions out of total in which each type of event occurred.

Branch by *P. aphanidermatum*	Coiling by *P. aphanidermatum*	Cytoplasmic surging in *P. ultimum*	Coagulation of *P. ultimum*	Lysis discharge from *P. ultimum*	Penetration of *P. ultimum*
5 ± 1 (6/7)	n.d.* (1/7)	42 (1/7)	44 (1/7)	54 (1/7)	n.d. (1/7)

* n.d. = not determined

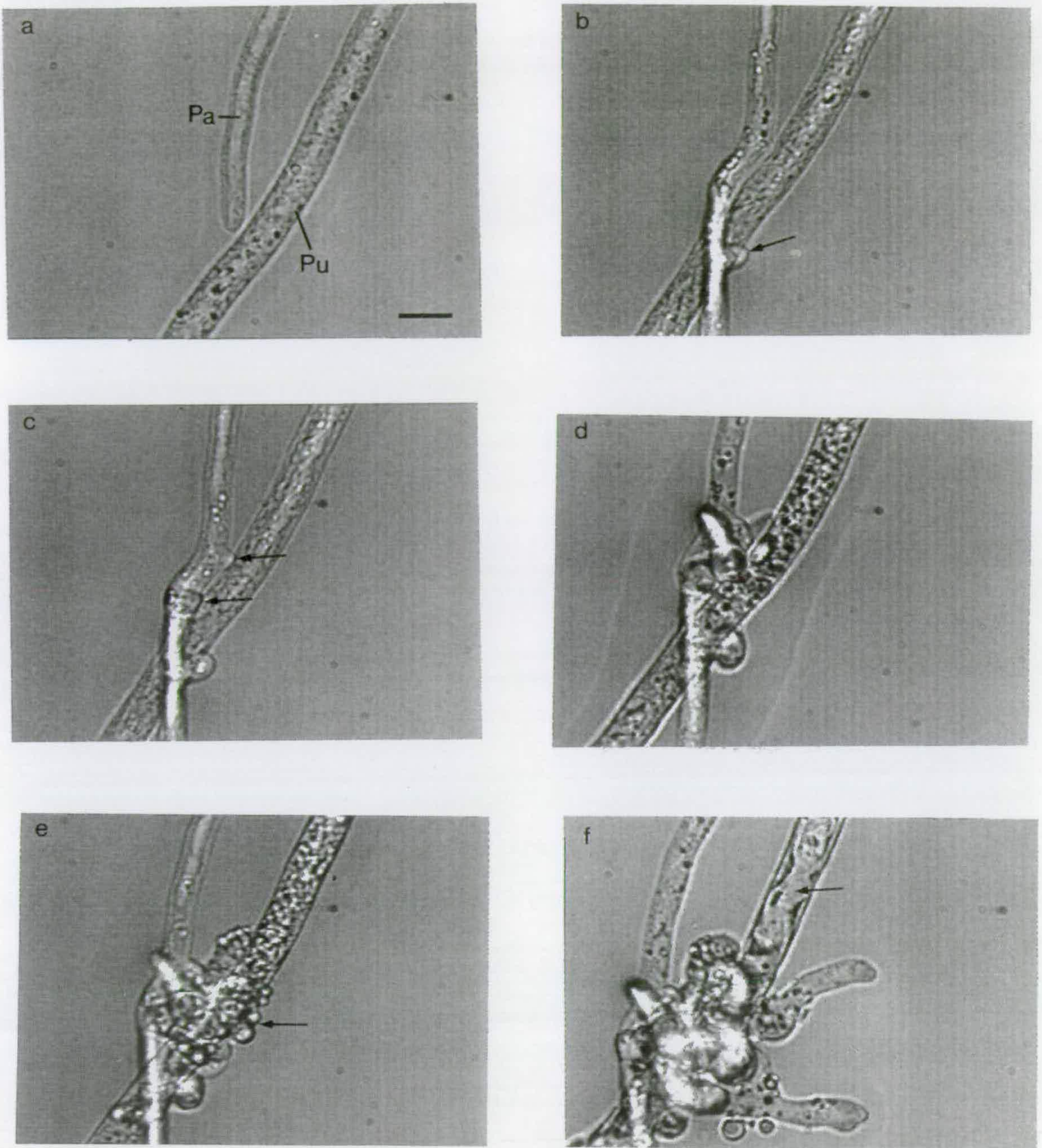


Figure 6.13a - f Stages in parasitism of *P. ultimum* (Pu) by *P. aphanidermatum* (Pa), videotaped through a film of agar. Bar=10μm. (a) *P. aphanidermatum* hyphal tip makes contact with side of a *P. ultimum* hypha. (b) 17min 41sec post-contact; *P. aphanidermatum* hypha has continued to grow across the *P. ultimum* hypha, and branched (arrowed) towards *P. ultimum* hypha. (c) 26min 55sec post-contact; *P. aphanidermatum* branched again towards *P. ultimum* hypha (arrowed) and began to 'swell'. (d) 44 min 13 sec post-contact; *P. aphanidermatum* branched more and 'coiled' round *P. ultimum* hypha; coagulation of *P. ultimum* cytoplasmic content is observed. Note the cytoplasmic difference in the hypha compared to the previous figure. (e) 55 min post-contact; cytoplasmic discharge or lysis from *P. ultimum* hypha (arrowed); note the globular appearance of the cytoplasmic discharge. (f) 1 h 46 min post-contact; penetration of *P. ultimum* hypha by *P. aphanidermatum* seen; internal hypha arrowed.

post-contact (Figure 6.13d). Subsequently (54 min post-contact; Figure 6.13e) there was localised release of cytoplasm from the *P. ultimum* hypha, the released cytoplasm having a globular appearance. As with interactions involving mycoparasitic *Pythium* spp. (e.g. *P. acanthophoron*) and *P. ultimum*, the disruption of the *P. ultimum* hypha was seen to be contained by 'barrier zones' of densely coagulated cytoplasm, normal cytoplasmic streaming being seen beyond these barrier zones. At later stages of this interaction *P. aphanidermatum* was observed to proliferate around the interaction site (Figure 6.13f) and an internal hypha of *P. aphanidermatum* was seen inside the disrupted *P. ultimum* hypha (arrowed). Even after disruption of this part of the hypha, the original *P. ultimum* hyphal tip continued to grow, being at this stage about 300μm from the interaction site.

In further tests, not reported in detail, identical events were seen in interactions between *P. ultimum* and five further isolates of *P. aphanidermatum* (IMI 332 885; IMI 335 084; IMI 334 887; PA4 and CBS 287.79). Each of these isolates coiled round hyphae of *P. ultimum*, causing coagulation and penetration of the host hyphae.

6.2.2.4 Interactions between *P. aphanidermatum* isolates

Interactions between different *P. aphanidermatum* isolates, were observed on water agar films using a X70 objective. *P. aphanidermatum* isolates had no adverse effects on each other, with no coiling or hyphal swellings observed.

6.2.4 Interactions between mycoparasitic Pythium species

Interactions between all the mycoparasitic *Pythium* spp. (*P. oligandrum*, *P. acanthophoron* and *P. mycoparasiticum*) were observed on water agar films using a X70 objective. The mycoparasitic *Pythium* species did not adversely affect each other, nor coil round each other.

6.3 Discussion

Of the three mycoparasites tested for ability to grow across PDA plates precolonised by other fungi, *P. oligandrum* grew across the largest number of fungal plates, *P. acanthophoron* grew across fewer, and *P. mycoparasiticum* grew across fewer still. *P. acanthophoron* was reported by Lodha & Webster (1990) to overgrow numerous fungi, including *Fusarium solani* and *Pythium myriotylum* on agar plates. Laing & Deacon (1990) also found that *P. oligandrum* grew over the widest range of host fungi on precolonised plates compared to either *P. mycoparasiticum* or *P. nunn*.

In their study, however, *P. mycoparasiticum* was reported to grow only across agar precolonised by *Phialophora* sp., whereas in this study *P. mycoparasiticum* was also found to grow across *B. cinerea*, which Laing & Deacon (1990) also tested, and *H. grisea*, which they did not test. The discrepancy regarding *B. cinerea* might be explained by methodology. Laing & Deacon (1990) took agar blocks from the mycoparasite-inoculated host-precolonised plates and transferred these blocks to PDA, to allow the mycoparasite to grow on the fresh PDA plates. In my experiment, however, the squares were placed on agar precolonised by *Phialophora* sp. to enable the mycoparasite, if present, to form characteristic oogonia. The presence of these was used to determine the extent of mycoparasite growth on the original precolonised plates. The detection method used by Laing & Deacon (1990) would have depended on the ability of the mycoparasite to grow onto fresh PDA in competition with *B. cinerea*, which grows much faster than *P. mycoparasiticum* so it is not surprising that *P. mycoparasiticum* remained undetected. In contrast to this, *Phialophora* sp. is slower-growing than *B. cinerea* and more nearly equivalent to *P. mycoparasiticum* in this respect, so it would be detected by either method. The finding that Sideris's isolate of *P. acanthophoron* was not seen to overgrow plates precolonised by *F. culmorum* can perhaps be explained in a similar way to that above. This isolate no longer produces reproductive structures and so had to be assessed by its ability to out-compete the fast-growing *F. culmorum* on fresh PDA.

In some cases, the mycoparasites were observed to grow part way across the host-precolonised plates in the first 7 days, but with no additional growth after further incubation. This initial growth was perhaps supported by nutrients from the mycoparasite inoculum block; after these nutrients had been exhausted, any further growth would have depended on nutrients obtained from the host fungus or residual nutrients that the host had not used. However, an additional possibility stems from the fact that the younger regions of host colonies are more susceptible to mycoparasitism than are older regions (Hoch & Fuller, 1977; Laing & Deacon, 1991). Since the mycoparasites were inoculated onto the youngest portions of the host fungal colonies, a mycoparasite might be able to obtain nutrients initially by antagonism and parasitism, but be progressively less able to colonise the older part of the colony.

The results of overgrowth of other fungal colonies on agar could not have been used to predict the aggressiveness of *P. acanthophoron* because it caused as rapid and consistent lysis of *F. oxysporum* and *T. aureoviride* on water agar films as did *P. oligandrum*, despite the difference in abilities of these *Pythium* spp. to overgrow *F.*

oxysporum and *T. aureoviride* on precolonised PDA plates. Similarly Laing & Deacon (1991) found that *P. mycoparasiticum* parasitised *F. oxysporum* and *T. aureoviride* on water agar films although it was much less effective against other fungi than was *P. oligandrum*. Relative growth rates of the different mycoparasites also cannot explain their different abilities to overgrow agar precolonised by other fungi. Indeed, *P. mycoparasiticum* almost completely overgrew the *Phialophora* sp.-precolonised plates by 7 days (Table 6.3) although it grows much more slowly than either *P. oligandrum* or *P. acanthophoron*. But relative growth rates of a mycoparasite compared with its host can be important in allowing a host to 'escape' parasitism by growing into fresh zones of substrate when appropriate experiments are done to test this (Laing & Deacon, 1990).

In videotaped interactions on water agar films *T. aureoviride* was somewhat more susceptible than *F. oxysporum*, in that it was disrupted faster in contact events by both *P. oligandrum* and *P. acanthophoron*. Yet on precolonised PDA plates the opposite seemed true- *F. oxysporum* appeared to be more susceptible, being overgrown faster and to a greater extent by both *P. oligandrum* and *P. acanthophoron*. Laing & Deacon (1990) suggested that on nutrient-rich agars, such as PDA, *T. aureoviride* produces growth inhibitors, such as non-volatile antibiotics, which *T. aureoviride* has been reported to produce (Dennis & Webster, 1971a). The nutrient status of the agar may thus perhaps affect the aggressiveness of the mycoparasite or the susceptibility of the host fungus. Bradshaw-Smith *et al.* (1991) reported that the aggressiveness of *P. oligandrum* towards different host fungi depended on the type of agar medium used. On cornmeal agar *P. oligandrum* was able to parasitise *Fusarium solani*, *Phoma medicaginis* and *Mycosphaerella pinodes*; on PDA however it was less aggressive, and on tap water agar even less aggressive. Whipps (1987) also found that the outcome of a mycoparasite-fungal host interaction depended on the agar medium used. *P. oligandrum* overgrew more host fungi when they were grown on tapwater agar or soil extract agar compared to when grown on PDA.

In summary of this comparison of methods for assessing the aggression or host ranges of mycoparasites, it is clear that the use of precolonised agar plates is an unreliable indicator of the ability of a mycoparaiste to parastise the hyphae of a fungus on water agar films. But it is still unclear why this should be so, because the two types of assessment method necessarily involved different agar media- a rich medium (PDA) so that the precolonising fungi would produce colonies for the

'overgrowth' studies, and a weak medium (water agar) to facilitate observations of individual hyphal interactions. Still, it is notable that *P. acanthophoron* was much less effective than *P. oligandrum* in overgrowing a range of fungi on precolonised plates and yet it was found to be as aggressive as *P. oligandrum* in inter-hyphal interactions. This might perhaps reflect an ecological difference between these fungi, so it merits further study.

In videotaped interactions *P. acanthophoron* had an identical mode of parasitism to that of *P. oligandrum* against the non-*Pythium* hosts, *T. aureoviride* and *F. oxysporum*. This parasitism involved branching at the point of contact and subsequent cytoplasmic disruption, lysis and penetration of host hypha. This is similar to the findings of Laing & Deacon (1991) who compared the modes of antagonism of *P. oligandrum*, *P. nunn* and *P. mycoparasiticum* on a range of non-*Pythium* host fungi. Likewise Lewis *et al.* (1989) and Bradshaw-Smith *et al.* (1991) reported that *P. oligandrum* parasitised various host fungi by causing lysis or coagulation of their cytoplasm and in many cases *P. oligandrum* was seen to penetrate the damaged hyphae. *P. acanthicum* also has a similar mode of action to *P. oligandrum* (Hoch & Fuller, 1977; Deacon & Henry, 1978). Hyphal interactions involving *P. periplocum* have not been reported in detail but *P. periplocum* was seen to behave similarly to *P. oligandrum* and *P. acanthicum* in colony interactions (Deacon & Henry, 1978). It would therefore seem that the six known mycoparasitic *Pythium* spp. have a similar, or even identical, mode of behaviour- a characteristic mode of parasitism. The only major point of contention in the published reports concerns pre-contact tropism. Lutchmeah & Cooke (1984), Lewis *et al.* (1989) and Bradshaw-Smith *et al.* (1991) all reported pre-contact tropism of hyphae of *P. oligandrum* towards hyphae of host fungi whereas Laing & Deacon (1991) found no evidence of this. There was also no evidence of it in the present study, where a high number of 'near-misses' between the individual hyphae was observed on water agar films, as reported by Laing & Deacon (1991). It is notable that both in this study and that in of Laing & Deacon (1991) water agar was used to study hyphal interactions, whilst in most of the other studies nutrient-rich agars or cellulose films were used. The host fungi may only produce the tropic factors in such conditions.

Hyphal tips of both *T. aureoviride* and *F. oxysporum* seemed to be more susceptible to parasitism by *P. oligandrum* and *P. acanthophoron* than were the sub-apical regions of the host hyphae. For example, interactions between the host hyphal tip and the side of a mycoparasite hypha most commonly resulted in lysis of

the host hyphae. Interactions further back from the host hyphal tip, on the other hand, resulted more often in coagulation of the host cytoplasm. Hoch & Fuller (1977) suggested that the hyphal tips may have a much weaker wall compared to the mature rigidified wall further back. This was reported to be the case by Wessels (1990), whereby the newly synthesised wall at the apex is relatively fragile, with plastic properties, and the wall polymers becoming progressively more cross-linked with time (distance back from the tips) so that the mature wall is rigid. He also reported that the newly synthesised chitin at the hyphal tips was extremely sensitive to chitinase, suggesting that the hyphal tips could be more sensitive to enzymic degradation by mycoparasites.

Parasitism by the mycoparasitic *Pythium* spp. has been suggested to involve the production of inducible enzymes by the mycoparasites (Elad *et al.* 1985; Lewis *et al.* 1989). However as discussed by Laing & Deacon (1991), the involvement of these in the earliest stages of parasitism is unproven. Elad *et al.* (1985) reported that *P. nunn* produced more types of inducible wall lytic enzymes compared to *P. oligandrum*, and also larger amounts of the enzymes that both parasites were found to produce. They argued that this correlated with the greater aggressiveness of *P. nunn* compared with *P. oligandrum* against other fungi. However, Laing & Deacon (1991) found *P. nunn* to be much less aggressive compared to *P. oligandrum* and this difference was convincingly (and statistically) demonstrated by recording the host ranges, incidence of parasitism and times of disruption in videotaped inter-hyphal interactions. In contrast to this, Elad *et al.* (1985) did not state the criteria that they used to assess mycoparasite aggressiveness, but they seem to have based this on incidence of hyphal coiling when interaction zones on agar were observed long after hyphal contacts had occurred. As Laing & Deacon (1991) point out, if this were so then Elad *et al.* (1985) and others (Lifshitz *et al.*, 1984c) who worked with *P. nunn* would not have seen the fastest mycoparasitic events that occur with susceptible host fungi; they would only have seen interactions (i.e. coiling) involving the most resistant host fungi or the weakest mycoparasite. The suggested relationship between mycoparasitic aggressiveness and enzyme production would thus be false. Irrespective of these points, lysis of the most susceptible host hyphae can occur very quickly, within 46 sec of contact in this study. As Laing & Deacon (1991) pointed out, this is too short a time for induction of synthesis of enzymes by mycoparasites to be realistically implicated in the mycoparasitic process. Instead Laing & Deacon (1991) suggested that host-derived wall lytic enzymes were activated by the mycoparasite to cause localised host lysis. The fungal hosts have all the necessary enzymes for degradation

of their walls, or at least for weakening of their walls during branch emergence (Gooday, 1983) and possibly (but see Wessels, 1990) for apical extension. Evidence for the presence of these enzymes as inactive forms within the walls was presented by Rosenberger (1979). These inactive forms were found to be capable of being activated (or released from bound sites in the walls) by relatively mild treatments. Adams & Ayers (1983) proposed a similar mechanism by which *Sporidesmium sclerotivorum* obtains nutrients from sclerotia of its fungal hosts, *Sclerotinia minor* or *Sclerotinia sclerotiorum. Sporidesmium sclerotivorum* was suggested to grow internally in sclerotia of its hosts, by scavenging nutrients that leaked from the sclerotial cells. In doing so, it was thought to create nutrient-stress conditions in the host cells such that reduction of host nutrient content would derepress the production of wall-lytic enzymes by the host sclerotia, releasing further nutrients for the mycoparasite.

In contrast to interactions of *P. oligandrum* and *P. acanthophoron* with non-*Pythium* hosts, interactions with *Pythium* hosts were much slower and involved conspicious coiling of the mycoparasites around the host hyphae. This was also observed by Drechsler (1943b, 1946) and subsequently by Walther & Gindrat (1987a), Lewis *et al.* (1989), Laing & Deacon (1991) and Berry *et al.* (1993). In interactions with the mycoparasitic *Pythium* spp., *P. aphanidermatum* behaved differently from all other hosts and so is considered separately. *P. ultimum* was seen to be parasitised by both *P. oligandrum* and *P. acanthophoron*, with no difference in their aggressiveness towards it. In contrast to interactions of these mycoparasites with *T. aureoviride* or *F. oxysporum*, explosive lysis was never observed in the *Pythium* hosts. Instead cytoplasmic coagulation was the normal outcome of parasitic interactions, and sometimes there was a small discharge of globular beads of cytoplasm, coinciding with a sudden rapid streaming of the cytoplasm of the mycoparasite towards the interaction site. This, along with a 'shifting' of the coagulated host cytoplasm, was taken to indicate penetration of the host hyphae by the mycoparasites. This was reported by Berry *et al.* (1993) but not by Laing & Deacon (1991) who reported *Pythium* hosts to be highly resistant to parasitism by the mycoparasites tested- *P. oligandrum*, *P. nunn* and *P. mycoparasiticum*. However their observations of inter-hyphal interactions were probably discontinued too early to detect these parasitic events (J. W. Deacon, pers. comm.).

Whilst both *P. acanthophoron* and *P. oligandrum* parasitised *P. ultimum*, this was not seen with *P. mycoparasiticum* - *P. ultimum* interactions. In fact *P. mycoparasiticum* did not coil extensively around the *P. ultimum* hyphae, and was

found not to grow to any appreciable distance across agar plates precolonised by *Pythium* hosts.

Damage to *P. ultimum* host hyphae was observed to be contained in many cases by what were termed barrier zones. These were zones of densely coagulated cytoplasm or they sometimes resembled septa, and beyond them normal cytoplasmic streaming was observed. They were also reported by Berry *et al.* (1993). Drechsler (1943b) reported what he termed septa, produced by *P. myriotylum* in response to parasitism by *P. acanthicum*, *P. periplocum* or *P. oligandrum*, and which seemed to localise the mycoparasitic attack. These 'septa' were also reported to check the growth of the internal hyphae of the mycoparasites, similar to what was observed in this study. These barrier zones may enable the host hyphal tips to continue to grow if the mycoparasitic interaction is some distance from the hyphal tip. This was tested by comparing the ability of *P. acanthophoron* and *P. oligandrum* to stop the growth of *P. ultimum* hyphal tips from interactions at different distances from these tips. *P. oligandrum* not only caused stoppage of more *P. ultimum* hyphal tips than did *P. acanthophoron* (15 compared with 8), but also stopped growth of the tips of *P. ultimum* from interactions further from the tips than did *P. acanthophoron*. Interactions further than *c.*300μm from the host tip, however, did not result in the stoppage of the host tips in most cases. Berry *et al.* (1993) suggested that the ability of the mycoparasites to stop the growth of the *Pythium* host tips depends on the extension rate of the host, relative to the time taken by the mycoparasite to disrupt the host cytoplasm at an interaction site near the tip, and the extent of the damage at these interaction sites. Therefore, faster growing *Pythium* hosts such as *P. graminicola* would be more able to escape parasitism than a slower growing host such as *P. vexans*. Thus containing the damage caused by the mycoparasitic *Pythium* spp. by means of these barrier zones may enable *Pythium* hosts in general to escape major damage by continued rapid extension of the host tips in the time (usually at least 1 h) before the mycoparasites cause disruption at the interaction site. But it should also be noted that the *Pythium* hosts differ, one from another, in terms of the number of contact events that resulted in coiling or disruption by the mycoparasites (Berry *et al.* 1993). These workers reported that only 6 of 11 contacts of *P. oligandrum* with *P. graminicola* led to coagulation of the host hyphae, compared with 11 of 12 contacts of *P. oligandrum* with *P. ultimum*. On this basis, *P. graminicola* can be considered more resistant to mycoparasitism than is *P. ultimum*. Such differences in susceptibility of *Pythium* hosts were previously reported by Foley & Deacon (1986b), based on whole colony, rather than inter-hyphal, interactions. But the differences were

considered to be relatively minor in relation to the relative resistance of *Pythium* spp. as a whole compared with the extreme susceptibility of non-*Pythium* hosts such as *F. oxysporum* and *T. aureoviride*. The present study confirms these points.

Although not reported in this thesis, the *Pythium* mycoparasites were also seen to coil around another Oomycete, *Phytophthora cinnamomi*. However disruption of the host cytoplasm was not observed so *P. cinnamomi* seemed to be resistant to parasitism by the mycoparasites. Lifshitz *et al.* (1984c) also reported that *P. cinnamomi* was less susceptible than *P. ultimum* to parasitism by *P. nunn* in that coiling of *P. nunn* around *P. cinammomi* was less extensive than around *P. ultimum* and occured more slowly.

P. aphanidermatum behaved differently from all the other *Pythium* hosts in interactions with the mycoparasitic *Pythium* spp. Disruption of the *P. aphanidermatum* hyphae was never recorded, and in some cases *P. aphanidermatum* was seen to antagonise the mycoparasites. In these cases *P. aphanidermatum* branched at the points of contact with the mycoparasite hyphae, but only after a considerable delay; often more than one branch was formed and they often swelled to produce lobate structures around the mycoparasitic *Pythium* hyphae. Cytoplasmic coagulation of the mycoparasite hyphae was seen in many cases, but penetration was only observed once, with *P. oligandrum*. Hoch & Fuller (1977) observed that in hyphal interactions between *P. aphanidermatum* and *P. acanthicum*, the latter showed signs of incompatibility. At the contact sites, the cytoplasm of *P. acanthicum* appeared moribund, and these workers considered *P. aphanidermatum* to be the aggressor in such interactions. However, penetration of *P. acanthicum* by *P. aphanidermatum* was never observed by Hoch & Fuller (1977). In the present study *P periplocum* also was seen to be antagonised by *P. aphanidermatum*. Hockenhull *et al.* (1992) had previously studied these fungi and reported that *P. aphanidermatum* was parasitised by *P. periplocum* although they also noted that *P. aphanidermatum* grew with apparent impunity through colonies of *P. periplocum*. This would seem to indicate that *P. aphanidermatum* was the more aggressive of the two fungi. Lifshitz *et al.* (1984c) reported that *P. nunn* coiled around hyphae of *P. aphanidermatum* but that *P. aphanidermatum* seemed more resistant to parasitism than was *P. ultimum*, because the mycoparasite coils only partially encompassed the hyphae of *P. aphanidermatum*.

The antagonistic behaviour of *P. aphanidermatum* in the present study was initially thought to be a possible defense response by *P. aphanidermatum* to attack by the mycoparasites. However, *P. aphanidermatum* was also found to antagonise other

Pythium hosts such as *P. ultimum* in a similar way, causing coagulation of the cytoplasm, and in some cases cytoplasmic discharge and penetration of *P. ultimum* hyphae. In response to damage by *P. aphanidermatum*, barrier zones were again seen in the 'host' hyphae and served to localise the damage. Growth of the internal hyphae of *P. aphanidermatum* were seen to be blocked by these barrier zones, and in many cases these internal hyphae were seen to exit the host hyphae at or before these barriers. The different phytopathogenic *Pythium* spp. were seen to differ in susceptibility to antagonism by *P. aphanidermatum*: *P. graminicola* was the most resistant whereas *P. ultimum* and *P. vexans* were the most susceptible. For *P. vexans*, *P. ultimum* and *P. graminicola*, the degree of susceptibility to antagonism by *P. aphanidermatum* corresponded to the degree of susceptibility to parasitism by *P. oligandrum* as reported by Berry *et al.* (1993).

In further tests on *P. aphanidermatum*, six isolates of this fungus from diverse origins all were found to antagonise *P. ultimum*. Evidently, therefore, this is a general property of the species. Furthermore, *P. aphanidermatum* was seen to antagonise *T. aureoviride* and *F. oxysporum*. Such interactions were much slower than those involving the mycoparasitic *Pythium* species against *T. aureoviride* and *F. oxysporum* but coagulation of the cytoplasm of *T. aureoviride* (the 'host' that was studied in detail) was often seen, and this was sometimes followed by penetration of the *Trichoderma* hyphae. Lysis of *T. aureoviride* hyphae was never seen, in contrast to the rapid lysis induced by *P. oligandrum* and *P. acanthophoron*. Formation of very thin branches or 'pin' branches was often seen approximately at the same time as the first disruption of the host hyphae was observed. Penetration was, however, not seen from these branches, but from branches formed after coagulation of *T. aureoviride*. Unlike the penetration hyphae of *P. oligandrum* and *P. acanthophoron*, internal hyphae of *P. aphanidermatum* grew considerably slower than the external hyphae. Also, unlike the case with the mycoparasitic *Pythium* spp., these internal hyphae never penetrated the host septa and often ceased growing before reaching these septa. All this evidence raises doubts that *P. aphanidermatum* behaves as a true mycoparasite, but it is an aggressive antagonist of other fungi. This behaviour of *P. aphanidermatum* could be an obvious advantage to it in competing with other soil fungi for ecological niches. Thus, unlike *P. ultimum* which has been shown to be displaced from organic substrates by mycoparasitic *Pythium* spp.(Martin & Hancock, 1986; Paulitz & Baker, 1987a), *P. aphanidermatum* might be able to prevent this by virtue of its antagonistic behaviour.

A final point of note is that the antagonistic behaviour of *P. aphanidermatum* towards other fungi (both *Pythium* spp. and non-pythiaceous species) quite closely resembled the antagonistic behaviour of the mycoparasites such as *P. oligandrum* and *P. acanthophoron*. In all cases the interactions involved branching at points of contact and subsequent disruption (eg. cytoplasmic coagulation) of non-pythiaceous hosts or coiling round other *Pythium* spp. This remarkable parallel in the behaviour of *P. aphanidermatum* and the typical mycoparasites suggests that the mechanism of mycoparasitism by the latter are not exclusive to them. By the same token, the differences in resistance of the various host fungi are expressed in relation to *P. aphanidermatum* in the same way as they are expressed in relation to *P. oligandrum* and the other mycoparasitic *Pythium* spp.

CHAPTER 7

CONCLUDING DISCUSSION

The five principal features of the work in this thesis were studies on the comparative nutrition of mycoparasitic *Pythium* species, attempts to optimise production and germination of oospores of these fungi, comparison of the modes of action of *P. oligandrum* and *P. acanthophoron*, selective isolation of the mycoparasites, especially *P. oligandrum* and *P. mycoparasiticum*, from soil, and the antagonistic attributes of *P. aphanidermatum*. All these points are relevant to understanding the activities of mycoparasitic *Pythium* spp. and to the practical exploitation of these fungi as potential biocontrol agents of economically important plant pathogens.

In terms of physiology and nutrition, *P. acanthophoron* was broadly similar to the five other known mycoparasitic *Pythium* species, insofar as it preferred complex, undefined media such as molasses to simple, defined media, it was non-pathogenic to plants, it showed, at best, weak cellulolytic activity, and it required organic nitrogen. But it was distinguishable nutritionally from all the other known mycoparasitic *Pythium* spp. in requiring organic nitrogen but not requiring thiamine for growth. This feature was shared by two isolates of *P. acanthophoron*: the recent isolate IMI 330 382, and the original isolate of Sideris (CBS 337.29) which is now sterile. It supports the taxonomic placement of the recent isolate within *P. acanthophoron* (Lodha & Webster, 1990) despite the fact that the original description (Sideris, 1932) was poor. Further nutritional and physiological differences between the mycoparasitic *Pythium* spp. were found with regard to growth on mannitol, responses to different sterols and tolerance of ethanol. But the ecological significance of such relatively minor physiological differences among the mycoparasitic *Pythium* spp. is unclear. It has also been shown here that there is physiological variation within the mycoparasitic species- for example, in germinability of oospores and mannitol utilisation. The fact that Martin & Hancock (1986) reported significant salt tolerance in isolates of *P. oligandrum* from irrigated Californian cotton crops but that this could not be confirmed for British isolates of *P. oligandrum* or the other mycoparasitic *Pythium* spp. suggests that strains of these fungi might become adapted to local environmental factors- a point of potential value in the selection of strains for biocontrol. In fact, one of the commonly recognised limitations of biocontrol is its unpredictability and inconsistency across sites and seasons (Becker & Schwinn, 1993). Deacon (1994)

argued that this is because most biocontrol inocula consist of single clonal strains which, inevitably, will be more suited to some seasonal or site conditions than to other. He proposed that a solution to this might be through development of mixed-strain inocula. Mixtures of two or more mycoparasitic *Pythium* spp., or strains of one species, merit investigation in this respect.

The mycelia of *Pythium* spp. are fragile, so for production of biocontrol inocula it would be necessary to produce oospores in large numbers. Various workers have achieved this for *P. oligandrum* and demonstrated biocontrol of seedling diseases when oospores are applied to seeds (e.g. Veselý, 1979; Al-Hamdani *et al.*, 1983; Walther & Gindrat, 1987; McQuilken *et al.*, 1990b). Other workers have implicated mycoparasitic *Pythium* spp. in naturally occurring biocontrol, and in these cases also the natural inoculum source is likely to have been oospores. In work here it has been shown that oospores of *P. acanthophoron* and *P. mycoparasiticum* can be produced in similar conditions to those used for *P. oligandrum*. It was also found that oospore production can be suppressed by the presence of some nutrient sources such as potato dextrose, even when other nutrients such as sunflower seed extract capable of supporting oospore production are present. This study also revealed a problem that has been encountered repeatedly (eg. Al-Hamdani *et al.*, 1983; Walther & Gindrat, 1987; Laing, 1989; McQuilken *et al.*, 1990a), namely that only a relatively low percentage of the oospores can be induced to germinate. In the case of *P. mycoparasiticum* the oospores consistently failed to germinate, for reasons that require further study. The oospores of both *P. oligandrum* and *P. acanthophoron* behaved similarly with respect to germination, with fairly consistent germination at *c*.20% in appropriate conditions, and maximum germination of just over 30% (after permanganate treatment) in any of the conditions tested for the isolates in this study. As in previous reports (Ayres & Lumsden, 1975), oospores were found to need a period of "maturation" after their production before they germinated. This was nutrient-independent as evidenced by the finding that oospores, once formed, matured at the same rate whether reincubated in molasses medium or in sterile distilled water. It is difficult to assess whether or not the oospores needed to remain attached to the mycelia during this maturation period, because some damage to the oospores themselves might have been caused by separating them from the mycelia, and the effect of this damage might have been enhanced during subsequent storage. Oospores stored wet, as oospore suspensions, lost germinability faster than did dry-stored oospores that remained attached to culture biomass. But storage progressively reduced the

germinability of all oospore preparations, and this could be a significant limitation to the use of oospore inocula in biocontrol.

Staining of oospores with tetrazolium bromide has been suggested to indicate their viability (Sutherland & Cohen, 1983). Using this technique, oospores of *P. oligandrum* and *P. acanthophoron* were indicated to be dormant, explaining the low germination observed. The failure of oospores of *P. mycoparasiticum* to germinate was consistent with the finding that when stained with tetrazolium bromide the oospores were apparently nonviable. The factors affecting germination of *P. mycoparasiticum* require further study, and further work might also focus on selection of isolates of the other mycoparasites with consistently high germination, especially after storage. One further observation here also merits detailed study: the fact that at least some oospores seemed to germinate from thin-walled antheridium-like appendages and not from the oospore *per se*. This was seen even from air-dried spores, indicating that the appendages were tolerant of air-drying, which might not be expected in view of the thin wall.

The existence of six known mycoparasitic *Pythium* spp. raises the question of their ecological relationships to one another: do they occur in different geographical regions or different types of habitats, or are they found together in soils? If the latter, then do they occupy different niches or do they compete with one another, with overlapping niches? The information to date on the occurrence and natural activities of these fungi does not enable these questions to be answered fully. The work of Dick & Ali-Shtayeh (1986) suggests some ecological or niche separation of *P. oligandrum* and *P. acanthicum*. On the other hand Ribeiro & Butler (1992) found *P. oligandrum*, *P. acanthicum* and *P. periplocum* together in some soils in California. A major problem to date is that different methods have been used by different workers to isolate or detect the presence of the mycoparasitic *Pythium* spp. in soils, so that direct comparative data are uncommon. In the present study *P. oligandrum* or *P. mycoparasiticum* were found quite commonly in four soils, whereas *P. periplocum* was detected only once. This supports the findings of Deacon & Henry (1978) and Foley & Deacon (1985), who used the same detection method- host-precolonised plates- and found *P. oligandrum* and *P. mycoparasiticum* commonly but *P. acanthicum* rarely, and they did not report the occurrence of *P. periplocum*. The precolonised plate method seems suitable for detecting all these fungi (Laing & Deacon, 1990) and for detecting *P. acanthophoron*, because these fungi could grow across host colonies from inoculum discs. So there is at least presumptive evidence

that the failure to detect any one species of mycoparasitic *Pythium* in a soil reflects the absence or rarity of that species in the soil sample. Of most interest here, there was clear and statistically supported evidence that *P. oligandrum* and *P. mycoparasiticum* can compete with one another on precolonised plates. In particular, the efficiency of detection of *P. mycoparasiticum* was enhanced when soils were diluted before addition to precolonised plates, but only when *P. oligandrum* was also commonly present in the soil. In two soils that had apparently low populations of *P. oligandrum*, the dilution of soil reduced the incidence of detection of *P. mycoparasiticum*. In such tests it seems that *P. oligandrum*, being faster growing than *P. mycoparasiticum*, competes better for colonisation of the precolonised agar. This competition could be regarded as an artifact of the detection method, but further evidence suggested that competition between these fungi might also occur in soils. Thus, when pieces of cellulose film were buried in soil that had been diluted to different degrees with sand, the incidence of colonisation of the film by *P. mycoparasiticum* was enhanced by soil dilution. In the particular conditions of incubation of the soil, therefore, it seems that *P. oligandrum* and *P. mycoparasiticum* compete with one another for the same substrate, and that this competition favours *P. oligandrum* over *P. mycoparasiticum*. However, the fact that *P. mycoparasiticum* can occur in soils despite the presence of *P. oligandrum* indicates that these two fungi must have different niches (i.e. be favoured by different factors), otherwise *P. mycoparasiticum* could not be expected to maintain its populations. In terms of biocontrol, it seems important to identify what these different niches or activities might be, and perhaps it would be valuable to use mixed biocontrol inocula, containing, for example, *P. oligandrum* and *P. mycoparasiticum*, if the poor germinability of oospores of the latter can be overcome. To what degree the presumed difference in niche of these mycoparasitic *Pythium* spp. is related to their differences in nutrition also merits further study.

The mode of action against representative host fungi on films of water agar was identical for *P. oligandrum* and *P. acanthophoron*, and in most cases the speed of action also was similar. Of interest, this was true also of the original isolate of *P. acanthophoron* which has now lost many of its characteristic morphological features such as the ability to produce a sexual stage. Detailed comparative studies of mode of action have now been reported for five of the six known mycoparasitic *Pythium* spp.- *P. oligandrum*, *P. acanthicum*, *P. acanthophoron*, *P. mycoparasiticum* and *P. nunn* (Hoch & Fuller, 1977; Lifshitz *et al.*, 1984c; Elad *et al.*, 1985; Lewis *et al.*, 1989; Laing & Deacon, 1991; Berry *et al.*, 1993), and the sixth species, *P. periplocum*, also seems to behave like the others. There is a remarkable degree of similarity in all their

reported modes of action- both against highly susceptible host fungi such as *T. aureoviride* and *F. oxysporum* and against the more resistant phytopathogenic *Pythium* spp. With the susceptible host fungi, the mycoparasites rapidly branch at points of initial contact and then cause cytoplasmic coagulation or lysis, and in some instances penetrate the host hyphae. With other *Pythium* spp. the mycoparasites branch at contact points after some delay and coil round the host hyphae before penetrating these or causing localised cytoplasmic coagulation. Although this disruption can be quite common in some *Pythium* hosts, such as *P. ultimum,* its effect is always localised and in a quantitative survey of interactions on water agar films it was found to disrupt tip growth of the *Pythium* hosts only if parasitism was initiated relatively close to the tip.

On the basis of the results here and those reported by others (Hoch & Fuller, 1977; Lifshitz *et al.*, 1984c; Elad *et al.*, 1985; Lewis *et al.*, 1989; Laing & Deacon, 1991; Berry *et al.*, 1993), it is clear that the differences in mycoparasitism by the six mycoparasitc *Pythium* spp. relate mainly to the speed of disruption of hosts and to the range of hosts that are affected. In contrast, the mechanism of mycoparasitism seems to be the same for all these mycoparasites. The only possible exception concerns *P. nunn*, which is reported to produce diffusible inhibitory metabolites in some conditions (Elad *et al.*, 1985). The other mycoparasitic spp. show no pre-contact effects that could be attributed to diffusible factors. It should, however, be noted that Lifshitz *et al.* (1984c) reported an entirely different order of host susceptibility to P. *nunn* compared with the report by Laing & Deacon (1991). The likely reason for this, as Laing & Deacon (1991) noted, is that these workers misinterpreted the evidence for mycoparasitism- they apparently believed that hyphal coiling is a sign of successful aggressive parasitism of a susceptible host, whereas the evidence suggests, as confirmed here, that hyphal coiling does not occur on the most susceptible host hyphae.

One of the major findings in the present study was that *P. aphanidermatum* was resistant to parasitism by the mycoparasitic *Pythium* spp. and was even the aggressor in these interactions. This was observed in studies with *P. oligandrum*, *P. acanthophoron*, *P. mycoparasiticum* and *P. periplocum*. It supports the suggestion of Hoch & Fuller (1977) that *P. aphanidermatum* was the aggressor in interactions with *P. acanthicum*, although these workers could not be sure of their interpretation, which was based on electron microscopy where it was sometimes difficult to distinguish between the hyphae of the two fungi. In contrast Hockenhull *et al.* (1992) reported

that *P. aphanidermatum* was parasitised by *P. periplocum*, although actual evidence of parasitism was not presented by them. In detailed exploration of the activities of *P. aphanidermatum* it was shown here that *P. aphanidermatum* antagonised a range of different fungi, including *T. aureoviride, F. oxysporum* and both mycoparasitic and plant-pathogenic *Pythium* spp. Indeed, its mode of action against them strongly resembled the mode of action of the mycoparasitic *Pythium* spp. themselves. It branched at points of hyphal contact with these fungi and led, sooner or later, to cytoplasmic coagulation of the other hyphae. In some cases, also, it penetrated the other hyphae. The parallels with behaviour of mycoparasitic *Pythium* spp. extended to the fact that *P. aphanidermatum* typically coiled round the hyphae of other *Pythium* spp. but not round hyphae of other fungi like *T. aureoviride* and *F. oxysporum*. But there were notable differences between the behaviour of *P. aphanidermatum* and the mycoparasitic *Pythium* spp. In particular, *P. aphanidermatum* always responded more slowly, e.g. by branching, after hyphal contact than did the mycoparasites, it never caused explosive hyphal lysis of other fungi, and its internal growth in other hyphae was always restricted in extent. A further interesting feature was its unusual branching behaviour at points of contact with other fungi: often several branches formed and swelled into lobate structures.

It is difficult to interpret all these events, and certainly too early to suggest that *P. aphanidermatum* is a parasite of other fungi. It shows mycoparasite-like behaviour but its slow response to other fungi compared with that of the mycoparasitic *Pythium* spp. and its seemingly poor ability to make sustained growth inside other hyphae do not suggest a mycoparasitic lifestyle. Instead, *P. aphanidermatum* might benefit from the activities reported here by displaying a degree of resistance against mycoparasitic *Pythium* spp., and even antagonising potential fungal competitors for the substrates such as host tissues that it has colonised by its aggressive phytopathogenicity. In other words, *P. aphanidermatum* might employ its limited aggressive activities for defence of its substrates. If this can be shown to occur in nature it could be significant because the mycoparasitic *Pythium* spp. such as *P. oligandrum* were first reported as secondary colonisers of diseased plant tissues, growing in association with aggressive phytopathogens in the lesions (Drechsler, 1930, 1943a). Subsequently both *P. oligandrum* and *P. nunn* have been implicated in disease-suppression in soils and have been shown to colonise fresh plant remains to the detriment of the phytopathogenic *Pythium* spp which had previously colonised these remains (Martin & Hancock, 1986; Paulitz & Baker, 1988b). From the evidence in this thesis it might be predicted that *P. aphanidermatum* is less susceptible to displacement from host tissues by

mycoparasitic *Pythium* spp. than are some other phytopathogens such as *P. ultimum*. Berry *et al.* (1993) demonstrated such an effect *in vitro* by showing that the production of reproductive propagules by *P. aphanidermatum* was unaffected by *P. oligandrum* when the two fungi were opposed on films of agar containing sunflower seed extract. In contrast to *P. aphanidermatum*, *P. vexans* was adversely affected by *P. oligandrum* in such conditions. Here there was an indication that root pieces precolonised by *P. aphanidermatum*, as opposed to *P. ultimum* were less conductive to invasion by *P. oligandrum* or *P. mycoparasiticum* when the root pieces were buried in soil. This topic merits further study in natural soil conditions; not all phytopathogenic *Pythium* spp. are necessarily amenable to biocontrol by the mycoparasitic *Pythium* spp.

APPENDIX 1

Data for Figure 3.1 (means of 5 replicates ± SEM).

Supplement to basal Medium *	Mycelial dry weight (mg) after different incubation times (days).*2					
	1	2	3	7	10	14
None	4.3 ± 0.8[a]	4.6 ± 1.7[a]	9.0 ± 1.3[a]	14.6 ± 1.3[a]	18.0 ± 3.3[a]	18.0 ± 4.8[a]
Glucose & Calcium	5.3 ± 0.8[a]	15.1 ± 1.7[b]	18.6 ± 2.4[b]	21.4 ± 4.0[a]	20.6 ± 4.3[a]	22.6 ± 5.1[a]
Glucose & Cholesterol	7.7 ± 1.1[a]	9.7 ± 1.9[ab]	14.0 ± 1.3[ab]	23.4 ± 4.0[a]	19.2 ± 3.6[a]	19.6 ± 4.4[a]
Glucose, Calcium & Cholesterol	6.5 ± 0.8[a]	12.5 ± 0.7[b]	18.4 ± 1.5[b]	21.6 ± 3.5[a]	20.0 ± 5.0[a]	20.0 ± 4.5[a]

* Basal medium contained mineral salts, L-asparagine, glucose and thiamine.

*2 Values, within a column, not followed by the same letter differ significantly ($P < 0.01$) by one way analysis of variance.

REFERENCES

ADAMS, P. B. (1971). *Pythium aphanidermatum* oospore germination as affected by time, temperature and pH. *Phytopathology* **61**, 1149-1150.

ADAMS, P. B. & AYERS, W. A. (1983). Histological and physiological aspects of infection of sclerotia of two *Sclerotinia* species by two mycoparasites. *Phytopathology* **73 (7)**, 1072-1076.

ADAMS, P. B., AYERS, W. A. & MAROIS, J. J. (1985). Energy efficiency of the mycoparasite *Sporidesmium sclerotivorum in vitro* and in soil. *Soil biology and biochemistry* **17 (2)**, 155-158.

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 (3)**, 619-621.

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. (1985). *Pythium* populations in middle Eastern soils relative to different cropping practices. *Transactions of the British Mycological Society* **84 (4)**, 695-700.

ANN, P. J. & KO, W. H. (1987). A method for inducing high frequency of oospore germination of *Phytophthora parasitica*. *Phytopathology* **77 (12)**, 1759 (Abst.)

APINIS, A. E. (1964). Concerning occurrence of Phycomycetes in alluvial soils of certain pastures, marshes and swamps. *Nova Hedwigia* **8**, 103-126.

AYERS, W. A. & LUMSDEN, R. D. (1975). Factors affecting production and germination of oospores of three *Pythium* species. *Phytopathology* **65 (10)**, 1094-1100.

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

BARAK, R., ELAD, Y., MIRELMAN, D. & CHET, I. (1985). Lectins: A possible basis for specific recognition in the interaction of *Trichoderma* and *Sclerotium rolfsii*. *Phytopathology* **75 (4)**, 458-462.

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

BARTON, R. (1957). Germination of oospores of *Pythium mamillatum* in response to exudates from living seedlings. *Nature* **180**, 613-614.

BARTON, R. (1958). Occurrence and establishment of *Pythium* in soils. *Transactions of the British Mycological Society* **41 (2)**, 207-222.

BARTON, R. (1960). Saprophytic activity of *Pythium mamillatum* in soils. I. Influence of substrate composition and soil environment. *Transactions of the British Mycological Society* **43 (3)**, 529-540.

BARTON, R. (1961). Saprophytic activity of *Pythium mamillatum* in soils. II. Factors restricting *P. mamillatum* to pioneer colonization of substrates. *Transactions of the British Mycological Society* **44 (1)**, 105-118.

BEAKES, G. W., EL-HAMALAWI, Z. A. & ERWIN, D. C. (1986). Ultrastructure of mature oospores of *Phytophthora megasperma* f. sp. *medicaginis*: preparation protocols and effects of MTT vital staining and permanganate pre-treatment. *Transactions of the British Mycological Society* **86 (2)**, 195-206.

BECKER, J. O. & SCHWINN, F. J. (1993). Control of soil-borne pathogens with living bacteria and fungi, status and outlook. *Pesticide Science* **37**, 355-363.

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.

BLOMBERG, A. & ADLER, L. (1993). Tolerance of fungi to NaCl. In *Stress tolerance of fungi*. pp. 209-232 Edited by D. H. Jennings, Marcel Dekker Inc., New York.

BOOSALIS, M. G. (1964). Hyperparasitism. *Annual Review of Phytopathology* **2**, 363-377.

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 (7)**, 861-865.

BRUSHABER, J. A., CHILD, J. J. & HASKINS, R. H. (1972). Effects of cholesterol on growth and lipid composition of *Pythium* sp. PRL 2142. *Canadian Journal of Microbiology* **18**, 1059-1063.

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

CAMPBELL, W. A. & HENDRIX, F. F. (1967). *Pythium* and *Phytophthora* species in forest soils in the Southeastern United States. *Plant Disease Reporter* **51 (11)**, 929-932.

CHESTERS, C. G. C. & HICKMAN, C. J. (1944). On *Pythium violae* n.sp. and *P. oligandrum* Drechsler from cultivated viola. *Transactions of the British Mycological Society* **44**, 55-62.

CHILD, J. J., DÉFAGO, G. & HASKINS, R. H. (1969a). The influence of carbon and nitrogen nutrition on growth and sterol-induced sexuality of *Pythium* species PRL 2142. *Mycologia* **61 (6)**, 1096-1105.

CHILD, J. J., DÉFAGO, G. & HASKINS, R. H. (1969b). The effect of cholesterol and polyene antibiotics on the permeability of the protoplasmic membrane of *Pythium* PRL 2142. *Canadian Journal of Microbiology* **15**, 599-603.

CHRISTIAS, C. & LOCKWOOD, J. L. (1973). Conservation of mycelial constituents in four sclerotium-forming fungi in nutrient-deprived conditions. *Phytopathology* **63**, 602-605.

COTHER, E. J. & GILBERT, R. L. (1993). Comparative pathogenicity of *Pythium* species associated with poor seedling establishment of rice in Southern Australia. *Plant Pathology* **42**, 151-157.

DAVISON, E. M. & BUMBIERIS, M. (1973). *Phytophthora* and *Pythium* spp. from pine plantations in South Australia. *Australian Journal of Biological Sciences* **26**, 163-169.

DEACON, J. W. (1976). Studies on *Pythium oligandrum*, an aggressive parasite of other fungi. *Transactions of the British Mycological Society* **66 (3)**, 383-391.

DEACON, J. W. (1979). Cellulose decomposition by *Pythium* and its relevance to substrate-groups of fungi. *Transactions of the British Mycological Society* **72 (3)**, 469-477.

DEACON, J. W. (1988). Biocontrol of soil-borne plant pathogens with introduced inocula. *Philosophical Transactions of the Royal Society of London* **318**, 249-264.

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. (1992). Current issues in rhizosphere biology, with specific reference to cereals. In *Barley Genetics VI.* Vol. 2, pp. 1023-1034. Edited by L. Munck, Munksgaard International Publishers Ltd., Copenhagen, Denmark).

DEACON, J. W. (1994). Rhizosphere constraints affecting biocontrol organisms applied to seeds. BCPC monographs No 57: *Seed treatments Progress and Prospects*. pp. 315-325.

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. Papavisaz & R. J. Cook, Plenum Publishing Corporation, New York.

DEACON, J. W. & BERRY, L. A. (1993). Biocontrol of soil-borne plant pathogens: concepts and their application. *Pesticide Science* **37**, 417-426.

DEACON, J. W. & HENRY, C. M. (1978). Mycoparasitism by *Pythium oligandrum* and *Pythium 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 (1)**, 25-39.

DENNIS, C. & WEBSTER, J. (1971b). Antagonistic properties of species-groups of *Trichoderma*. III Hyphal interaction. *Transactions of the British Mycological Society* **57 (3)**, 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 (1)**, 49-62.

DRECHSLER, C. (1930). Some new species of *Pythium*. *Journal of the Washington Academy of Sciences* **20 (16)**, 398-418.

DRECHSLER, C. (1943a). Antagonism and parasitism among some oomycetes associated with root rot. *Journal of the Washington Academy of Sciences* **33 (1)**, 21-28.

DRECHSLER, C. (1943b). Two species of *Pythium* occurring in Southern States. *Phytopathology* **33 (4)**, 261-299.

DRECHSLER, C. (1946). Several species of *Pythium* peculiar in their sexual development. *Phytopathology* **36 (10)**, 781-864.

ELAD, Y., BARAK, R. & CHET, I. (1983). Possible role of lectins in mycoparasitism. *Journal of Bacteriology* **154 (3)**, 1431-1435.

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.

EL-HAMALAWI, Z. A. & ERWIN, D. C. (1986). Physical enzymic and chemical factors affecting viability and germination of oospores of *Phytophthora megasperma* f. sp. *medicaginis*. *Phytopathology* **76 (5)**, 503-507.

ERWIN, D. C. (1968). The effect of calcium on mycelial growth of *Phytophthora megasperma* and *Phytophthora cinnammomi*. *Mycologia* **60**, 1112-1116.

FEDERATION OF BRITISH PLANT PATHOLOGISTS (1973). A guide to the use of terms in plant pathology, *Phytopathological Papers*. No 17.

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.

FLOWERS, R. A. & LITTRELL, R. H. (1972). Oospore germination of *Pythium aphanidermatum* as affected by caesin, gallic acid and pH levels in a selective agar medium. *Phytopathology* **62**, 757 (Abst.).

FOLEY, M. F. (1983). *Studies on* Pythium oligandrum *and other suspected mycoparasites*. Ph.D thesis, Edinburgh University, Edinburgh.

FOLEY, M. F. & DEACON, J. W. (1985). Isolation of *Pythium oligandrum* and other necrotrophic mycoparasites from soil. *Transactions of the British Mycological Society* **85 (4)**, 631-639.

FOLEY, M. F. & DEACON, J. W. (1986a). Physiological differences between mycoparasitic and plant pathogenic *Pythium* species. *Transactions of the British Mycological Society* **86 (2)**, 225-231.

FOLEY, M. F. & DEACON, J. W. (1986b). Susceptibility of *Pythium* species and other fungi to antagonism by the mycoparasite *Pythium oligandrum*. *Soil Biology and Biochemistry* **18 (1)**, 91-95.

GALE, E. F., CUNDLIFFE, E., REYNOLDS, P. E., RICHMOND, M.H. & WARING, M.J. (1981). *The molecular basis of antibiotic action*. John Wiley & Sons, London.

GARRAWAY M. O. & EVANS R. C. (1984). *Fungal nutrition and Physiology*. Wiley-Interscience Publications, New York.

GARRETT, S. D. (1970). *Pathogenic root-infecting fungi*. Cambridge University Press, Cambridge.

GOODAY, G. W. (1983). The hyphal tip. In *Fungal differentiation. A Contemporary Synthesis*. pp. 315-356. Edited by J. E. Smith.

HAN, D. Y. & NELSON, E. B. (1993). Responses of propagules of two root-infecting *Pythium* species to seed and root exudates from turfgrasses. Conference abstracts, 6th International Congress of Plant Pathology, Montreal, Canada. p. 279

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

HASKINS, R. H., TULLOCH, A. P. & MICETICH, R. G. (1964). Steroids and the stimulation of sexual reproduction of a species of *Pythium*. *Canadian Journal of Microbiology* **10**, 187-195 .

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.

HENDRIX, F. F. & CAMPBELL, W. A. (1973). *Pythium* as plant pathogens. *Phytopathology* **11**, 77-98.

HENDRIX, F. F., CAMPBELL, W. A. & MONCREIF, J. B. (1970). *Pythium* species associated with golf turfgrasses in the south and southeast. *Plant Disease Reporter* **54 (5)**, 419-421.

HENDRIX, F. F., CAMPBELL, W. A. & CHIEN, C. Y. (1971). Some Phycomycetes indigenous to soils of old growth forests. *Mycologia* **63**, 283-289.

HENDRIX, F. F. & KUHLMAN, E. G. (1965). Factors affecting direct recovery of *Phytophthora cinnamomi* from soil. *Phytopathology* **55**, 1183-1187.

HENDRIX, J. W. (1964). Sterol induction of reproduction and stimulation of growth of *Pythium* and *Phytophthora*. *Science* **144**, 1028-1029.

HENDRIX, J. W. (1965). Influence of sterols on growth and reproduction of *Pythium* and *Phytophthora* spp. *Phytopathology* **55**, 790-797.

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

HENDRIX, J. W. (1974). Physiology and biochemistry of growth and reproduction in *Pythium*. *Proceedings of the American Phytopathological Society* **1**, 207-210.

HOCH, H. C. & FULLER, M. S. (1977). Mycoparasitic relationships. 1. Morphological features of interactions between *Pythium acanthicum* and several fungal hosts. *Archives of Microbiology* **111**, 207-224.

HOCKENHULL, J., JENSEN, D. F. & YUDIARTI, T. (1992). The use of *Pythium periplocum* to control damping-off of cucmber seedlings caused by *Pythium aphanidermatum*. In *Biological control of plant diseases: Progress and Challenges for the future*. pp. 203-206. Edited by E. S. Tjamos, G. Papavisaz & R. J. Cook, Plenum Publishing Corporation, New York.

INBAR, J. & CHET, I. (1992). Biomimics of fungal cell-cell recognition by use of lectin-coated nylon fibers. *Journal of Bacteriology* **174 (3)**, 1055-1059.

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

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

JOHNSON, L. F. (1988). Effects of atmospheric gases and light on changes in thickness of oospore walls and in germinability of oospores of *Pythium ultimum*. *Phytopathology* **78 (4)**, 435-439.

JOHNSON, L. F. & ARROYO, T. (1983). Germination of oospores of *Pythium ultimum* in the cotton rhizosphere. *Phytopathology* **73 (12)**, 1620-1624.

JOHNSON, L. F., QIAN, P. & FERRISS, R. S. (1990). Soil matric potential effects on changes in wall morphology, germination and lysis of oospores of *Pythium ultimum*. *Phytopathology* **80**, 1357-1361.

KERWIN, J. L. & DUDDLES, N. D. (1989). Reassessment of the role of phospholipids in sexual reproduction by sterol-auxotrophic fungi. *Journal of Bacteriology* **171 (7)**, 3831-3839.

KILPATRICK, R. A. (1968). Seedling reaction of barley, oats and wheat to *Pythium* species. *Plant Disease Reporter* **52 (3)**, 209-212.

KLEMMER, H. W. & LENNEY, J. F. (1965). Lipids stimulating sexual reproduction and growth in *Pythiaceous* fungi. *Phytopathology* **55**, 320-323.

KLEMMER, H. W. & NAKANO, R. Y. (1964). Distribution and pathogenicity of *Phytophthora* and *Pythium* in pineapple soils of Hawaii. *Plant Disease Reporter* **48 (11)**, 848-852.

LAING, S. A. K. (1989). *Comparative studies on mycoparasitic* Pythium *species*. Ph. D thesis, Edinburgh University, Edinburgh.

LAING, S. A. K. & DEACON, J. W. (1990). Aggressievness and fungal host ranges of mycoparasitic *Pythium* species. *Soil Biology and Biochemistry* **22 (7)**, 905-911.

LAING, S. A. K. & DEACON, J. W. (1991). Video microscopical comparison of mycoparasitism by *Pythium oligandrum*, *Pythium nunn* and an unnamed *Pythium* species. *Mycological Research* **95 (4)**, 469-479.

LENNEY, J. F. & KLEMMER, H. W. (1966). Factors controlling sexual reproduction and growth in *Pythium graminicola*. *Nature* **209**, 1365-1366.

LEONIAN, L. H. & LILLY, V. G. (1938). Studies on the nutrition of fungi. 1 thiamine, its constituents and the source of nitrogen. *Phytopathology* **28**, 531-548.

LEWIS, D. H. & SMITH, D. C. (1967). Sugar alcohols (polyols) in fungi and green plants. *New phytologist* **66**, 143-184.

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, and R. D. Lumsden, Cambridge University Press, Cambridge.

LIDDELL, C. M., DAVIS, R. M., NUNEZ, J. J. & GUERARD, J. P. (1989). Association of *Pythium* spp. with carrot root dieback in the San Joaquin Valley of California. *Plant disease* **73**, 246-249.

LIFSHITZ, R., STANGHELLINI, M. E. & BAKER, R. (1984a). A new species of *Pythium* isolated from soil in Colorado. *Mycotaxon* **20 (2)**, 373-379.

LIFSHITZ, R., SNEH, B. & BAKER, R. (1984b). Soil suppressiveness to a plant pathogenic *Pythium* spp. *Phytopathology* **74 (9)**, 1054-1061.

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

LODHA, B. C. & WEBSTER, J (1990). *Pythium acanthophoron*, a mycoparasite, rediscovered in India and Britain. *Mycological Research* **94 (7)**, 1006-1008.

LUARD, E. J. (1982). Growth and accumulation of solutes by *Phytophthora cinnamomi* and other lower fungi in response to changes in external osmotic potential. *Journal of General Microbiology* **128**, 2583-2590.

LUMSDEN, R. D. (1981). Ecology of mycoparasitism. In *The fungal community, its organisation and role in the ecosystem*. pp. 295-317. Edited by D. T. Wicklow and G. C. Carroll, Marcel Dekker Inc., New York.

LUMSDEN, R. D. & AYERS, W. A. (1975). Influence of soil environment on the germinability of constitutively dormant oospores of *Pythium ultimum*. *Phytopathology* **65 (10)**, 1101-1107.

LUTCHMEAH, R. S. & COOKE, R. C. (1984). Aspects of antagonism by the mycoparasite *Pythium oligandrum*. *Transactions of the British Mycological Society* **83 (4)**, 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.

McQUILKEN, M. P., WHIPPS, J. M. & COOKE, R. C. (1990a). Oospores of the biocontrol agent *Pythium oligandrum* bulk-produced in liquid culture. *Mycological Research* **94 (5)**, 613-616.

McQUILKEN, M. P., WHIPPS, J. M. & COOKE, R. C. (1990b). 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). Nutritional and environmental factors affecting biomass and oospore production of the biocontrol agent *Pythium oligandrum*. *Enzyme Microbial Technology* **14**, 106-111.

McQUILKEN, M. P., WHIPPS, J. M. & COOKE, R. C. (1992b). Effects of osmotic and matric potential on growth and oospore germination of the biocontrol agent *Pythium oligandrum*. *Mycological Research* **96 (7)**, 588-591.

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

MANOCHA, M. S., CHEIN, Y. & RAO, N. (1990). Involvement of cell surface sugars in recognition, attachment, and appressorium formation by a mycoparasite. *Canadian Journal of Microbiology* **36**, 771-778.

MARTIN, F. N. & HANCOCK, J. G. (1984). The use of *Pythium oligandrum* for the biological control of *Pythium ultimum*. *Phytopathology* **74 (7)**, 835 (Abst.).

MARTIN, F. N. & HANCOCK, J. G. (1986). Association of chemical and biological factors in soils suppressive to *Pythium ultimum*. *Phytopathology* **76 (11)**, 1221-1231.

MARTIN, F. N. & HANCOCK, J. G. (1987). The use of *Pythium oligandrum* for biological control of pre emergence damping-off caused by *Pythium ultimum*. *Phytopathology* **77 (7)**, 1013-1020.

MINISTRY OF AGRICULTURE, FISHERIES AND FOODS PUBLICATION (1984). Tomato production. In *Hydroponic growing systems*. Booklet 2249.

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.

MULLIGAN, D. F. C. & DEACON, J. W. (1992). Detection of presumptive mycoparasites in soil placed on host-colonised agar plates. *Mycological Research* **96 (7)**, 605-608.

PAPAVIZAS, G. C. & LEWIS, J. A. (1981). Side effects of pesticides on soil-borne plant pathogens. In *Soil-borne plant pathogens*. pp. 483-505. Edited by B. Schippers and W. Gams, Academic Press London.

PAPAVIZAS, G. C., DUNN, M. T., LEWIS, J. A. & BEAGLE-RISTAINO, J. (1984). Liquid fermentation technology for experimental production of biocontrol fungi. *Phytopathology* **74 (10)**, 1171-1175.

PAPENDICK, R. I. & CAMPBELL, G. S. (1981). Theory and measurement of water potential. In water potential relations in soil microbiology. pp. 1-20. Edited by J. F. Parr, W. R. Gardner & L. F. Elliott, Soil Science of America, WI.

PARK, D. (1975). A cellulolytic Pythiaceous fungus. *Transactions of the British Mycological Society* **65 (2)**, 249-257.

PARK, D. (1977). *Pythium fluminum* sp. nov. with one variety and *Pythium uladhum* sp. nov. from cellulose in fresh-water habitats. *Transactions of the British Mycological Society* **69 (2)**, 225-231.

PAULITZ, T. C. & BAKER, R. (1987a). Biological control of *Pythium* damping-off of cucumbers with *Pythium nunn*: Population dynamics and disease suppression. *Phytopathology* **77 (2)**, 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 (2)**, 341-346.

PAULITZ, T. C. & BAKER, R. (1988a). The formation of secondary sporangia by *Pythium ultimum*: The influence of organic amendments and *Pythium nunn*. *Soil Biology and Biochemistry* **20 (2)**, 151-156.

PAULITZ, T. C. & BAKER, R. (1988b). Interactions between *Pythium nunn* and *Pythium ultimum* on bean leaves. *Canadian Journal of Microbiology* **34**, 947-951.

PAULITZ, T. C., AHMAD, J. S. & BAKER, R. (1990). Integration of *Pythium nunn* and *Trichoderma harzianum* isolate T-95 for the biological control of *Pythium* damping-off of cucumber. *Plant and Soil* **121**, 243-250.

PIECZRAKA, D. J. & ABAWI, G. S. (1978). Populations and biology of *Pythium* species associated with snap bean roots and soils in New York. *Phytopathology* **68**, 409-416.

PRATT, R. G. & JANKE, G. D. (1980). Pathogenicity of three species of *Pythium* to seedlings and mature plants of grain sorghum. *Phytopathology* **70**, 766-771.

QIAN, P. & JOHNSON, L. F. (1987). Chemical and physical soil characteristics related to lysis of oospores of *Pythium ultimum*. *Phytopathology* **77** **(7)**, 1062-1066.

QUIMIO, T. H. & ABILAY, L. E. (1977). *Pythiums* from Philippine soils. *Philippine Phytopathology* **13**, 54-73.

RIBEIRO, W. R. C. & BUTLER, E. E. (1992). Isolation of mycoparasitic species of *Pythium* with spiny oogonia from soil in California. *Mycological Research* **96** **(10)**, 857-862.

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

ROBERTSON, G. I. (1973). Occurrence of *Pythium* species in New Zealand soils, sands, pumices, and peat, and on roots of container-grown plants. *New Zealand Journal of Agricultural Research* **16**, 357-365.

ROSENBERGER, R. F. (1979). Endogenous lytic enzymes and wall metabolism. In *Fungal walls and hyphal growth*. pp. 265-277. Edited by J. H. Burnett & A. P. J. Trinci, Cambridge University Press, Cambridge.

ROSENDAHL, C. N. & OLSON, L. W. (1991). Importance of light for synchronous germination of *Pythium ultimum* Trow. oospores. Conference abstracts. *New approaches in Biological Control of soil borne diseases*. Copenhagen, Denmark.

RUBEN, D. M. & STANGHELLINI, M. E. (1978). Ultrastructure of oospore germination in *Pythium aphanidermatum*. *American Journal of Botany* **65** **(5)**, 491-501.

RUBEN, D. M., FRANK, Z. R. & CHET, I. (1980). Factors affecting behaviour and developmental synchrony of germinating oospores of *Pythium aphanidermatum*. *Phytopathology* **70**, 54-59.

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

SCHMITTHENNER, A. F. (1964). Prevalence and virulence of *Phytophthora, Aphanomyces, Pythium, Rhizoctonia* and *Fusarium* isolated from diseased alfalfa seedlings. *Phytopathology* **54**, 1012-1018.

SCHMITTHENNER, A. F. (1972). Effect of light and calcium on germination of oospores of *Pythium aphanidermatum*. *Phytopathology* **62**, 788 (Abst.).

SIDERIS, C. P. (1932). Taxonomic studies in the family Pythiaceae. II *Pythium*. *Mycologia* **24**, 14-61.

SIETSMA, J. H. & HASKINS, R. H. (1968). The incorporation of cholesterol by *Pythium* spp. PRL 2142, and some of its effects on cell metabolism. *Canadian Journal of Biochemistry* **46**, 813-818.

STANGHELLINI, M. E. (1974). Spore germination, growth and survival of *Pythium* in soil. *Proceedings of the American Phytopathological Society* **1**, 211-214.

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

STANGHELLINI, M. E. & BURR, T. J. (1973b). Effect of soil water potential on disease incidence and oospore germination of *Pythium aphanidermatum*. *Phytopathology* **63**, 1496-1498.

STANGHELLINI, M. E. & KRONLAND, W. C. (1985). Bioassay for quantification of *Pythium aphanidermatum* in soil. *Phytopathology* **75 (11)**, 1242-1245.

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

STASZ, T. E. & MARTIN, S. P. (1988). Insensitivity of thick-walled oospores of *Pythium ultimum* to fungicides, methyl bromide and heat. *Phytopathology* **78 (11)**, 1409-1412.

SUSSMAN, A. S. (1976). Activators of fungal spore germination. In *The fungal spore: form and function*. pp. 101-137. Edited by D. J. Weber & W. M. Hess, John Wiley and Sons Inc., New York.

SUSSMAN, A. S. & HALVORSON, H. O. (1966). *Spores, their dormancy and germination*. Edited by Harper & Row Publishers, New York and London.

SUTHERLAND, E. D. & COHEN, S. D. (1983). Evaluation of tetrazolium bromide as a vital stain for fungal oospores. *Phytopathology* **73**, 1532-1535.

THINGGARD, K., LARSEN, H. & HOCKENHULL, J. (1988). Antagonistic *Pythium* against pathogenic *Pythium* on cucumber roots. *Bulletin O.E.P.P./E.P.P.O.* **18**, 91-94.

TRAPERO-CASAS, A., KAISER, W. J. & INGRAM, D. M. (1990). Control of *Pythium* seed rot and pre emergence damping-off of chickpea in the U.S. Pacific North west and Spain. *Plant Disease* **74 (8)**, 563-569.

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** **(3)**, 457-466.

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**, 436-435.

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

VAN DER PLAATS-NITERINK, A. J. (1981). Monograph of the Genus *Pythium*. *Studies in Mycology No 21.*

VESELÝ, D. (1978a). Biological protection of emerging sugar-beet against damping-off established by mycoparasitism in non-sterilized soil. *Zentralblatt fuer Bakteriologie* II **133**, 436-443.

VESELÝ, D. (1978b). Parasitic relationships between *Pythium oligandrum* Drechsler and some other species of the Oomycetes class. *Zentralblatt fuer Bakteriologie* II **133**, 341-349.

VESELÝ, D. (1979). Use of *Pythium oligandrum* to protect emerging sugar-beet. In *Soil-borne plant pathogens* pp. 593-595. Edited by B. Schippers and W. Gams, Academic Press, London.

VESELÝ, D. (1987). Germinating power of oospores of *Pythium oligandrum* in a powder preparation. *Folia Microbiologica* **32** (6), 502 (Abst.)

VESELÝ, 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 für 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.

WASTIE. R. L. (1962). Mechanism of action of an infective dose of *Botrytis* spores on bean leaves. *Transactions of the British Mycological Society* **45** **(4)**, 465-473.

WATERHOUSE, G. M. (1968). The Genus *Pythium* Pringsheim. *Mycological papers No 110.*

WEBB, S. J., CORMACK, D. V. & MORRISON, H. G. (1964). Relative humidity, inositol and the effect of radiations on air-dried micro-organisms. *Nature* **201**, 1103-1105.

WESSELS, J. G. H. (1990). Role of cell wall architecture in fungal tip growth generation. In *Tip growth in plant and fungal cells*. pp. 1-29. Edited by Heath, I. B. Academic Press, San Diego.

WHIPPS, J. M. (1987). 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. (1992). Concepts in mycoparasitism and biological control of plant diseases. In *New approaches in Biological control of soil-borne diseases*. pp. 54-59. Edited by D. F. Jensen, J. Hockenhull & N. J. Fokkema, Proceedings Workshop, Copenhagen, Denmark.

WHIPPS, J. M., LEWIS, K. & COOKE, R. C. (1988). Mycoparasitism and plant disease control. In *Fungi in Biological Control Systems*. pp. 161-178. Edited by M. N. Burge, Manchester University Press, Manchester.

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

WHITE, J. G. (1992). The effect of previous cropping and fungicides on field populations of *Pythium oligandrum*. *Phytoparasitica* **20**, 117-120

WHITE, J. G., HENN, A. J. & PETCH, G. M. (1993). Deleterious effect of soil-applied metalaxyl and mancozeb on the mycoparasite *Pythium oligandrum*. *Biocontrol Science and Technology* **2**, 335-340.

WOLF, A. V., BROWN, M. G. & PRENTISS, P. G. (1990). *C.R.C. Handbook of Chemistry and Physics*. ppD-221 - D-261. Edited by R. C. Weast, D. R. Lide, M. J. Astle & W. H. Beyer, C.R.C. Press Inc., Florida, USA.