

DEVELOPMENT OF PYTHIUM OLIGANDRUM DRECHSLER FOR BIOLOGICAL  
CONTROL OF FUNGAL SOIL-BORNE DISEASES

BY

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TO MUM AND DAD

"Biological control is much neglected, not because it does not work but because not enough research is done on it. Biological control has worked, is working and can if we desire it, greatly extend its success"

ORDISH (1967)

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## SUMMARY

The nutritional and environmental requirements for mycelial growth, oospore production and germination of Pythium oligandrum were examined. Optimum temperatures for growth of several isolates were in the range of 20 - 30°, with little growth occurring below 10° or above 35°. Oospore germination occurred over the range of 10-35°. Both growth and oospore germination occurred over the range of pH 4.5 - 9.0 and were optimum between pH 6.0 - 7.5. Growth was reduced markedly below -1.0 to -1.5 MPa osmotic potential and ceased at approximately -2.5 to -3.5 MPa; similar results were obtained for oospore germination. Growth and oospore germination were affected more by low matric than by low osmotic potentials. Oospore production required an exogenous supply of sterols; it was also increased by the presence of calcium and affected by the C:N ratio.

Semi-solid, static and aerated culture systems were developed for bulk production of P. oligandrum oospores. A liquid cane molasses medium was particularly convenient and efficient. A range of formulations were prepared using oospores produced mainly in this medium. Formulations were evaluated against pathogens causing damping-off in cress and the level of biocontrol in artificially infested sand was not as good as that obtained in naturally infested soil. Alginate pellets and a perlite preparation survived well in laboratory storage at 5-25° for at least 24 wk.

Seeds of cress and sugar beet were coated with oospores using commercial seed-pelleting and film-coating procedures. Both types of seed treatment reduced damping-off of cress caused by P. ultimum in

artificially infested sand and potting compost, and by Rhizoctonia solani in artificially infested sand. In general, pelleting of P. oligandrum on cress gave better control than film-coating treatments. P. oligandrum also reduced damping-off of sugar beet in soil naturally infested with Aphanomyces cochlioides and Pythium spp.. Control was equivalent to that achieved with hymexazol fungicide seed-coating treatments and was related to the inoculum potential of A. cochlioides in the soil.

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CHAPTER I

GENERAL INTRODUCTION

## CHAPTER I

### GENERAL INTRODUCTION

The historical importance of plant diseases, and their consequent economic and social effects are well documented (Large, 1941; Carefoot & Sprott, 1969). The most recent comprehensive assessment of global losses due to diseases is that of Cramer (1967) who indicated that about 10-15% of potential crop yield is lost through attack by plant pathogens. Soil-borne diseases cause about 50% of the total estimated annual losses of economic crops due to plant diseases (James, 1981). It is conservatively estimated that in the U.S.A. alone, annual losses on crops due to soil-borne fungal pathogens amount to at least \$4 billion (Papavizas, 1984). Since most of the damage to plants by soil-borne diseases is below ground, or results from below ground infection, estimations of crop losses from such diseases are greatly underestimated.

Fungicides are currently used to control many fungal diseases. However, control is not always effective, and the intensive use of fungicides increases environmental pollution, health hazards and sometimes induces phytotoxicity. To reduce the deleterious effects of fungicide application, alternative methods are required. Public concern about the use of chemicals in farming and food production has resulted in an increased demand for biologically-based products, non toxic to animals as well as humans, harmless to crops and non-hazardous to the environment. This highlights the importance of biological control of plant pathogens.

There is much disagreement on what constitutes biological control. Some plant pathologists, such as Baker & Cook (1974), have adopted an extremely broad view of biological control and include, for example, plant breeding as a form of biological control against plant pathogens. They defined it as 'the reduction of inoculum density or disease-producing activities of a pathogen or parasite in its active or dormant state by one or more organisms accomplished naturally or through manipulation of the environment, host or antagonist, or by mass introduction of one or more antagonists. An acceptable definition must be one that outlines an approach to control disease. However, Baker & Cook's definition merely excludes some cases of chemical control. I propose to accept the definition suggested by Garrett (1970) which covers both pests and pathogens of plants. He defined it as 'the practice in which, or process whereby, the undesirable effects of an organism are reduced through the agency of another organism that is not the host plant, the pest or pathogen, or man'. The definition implies that a 'third party' is involved. In the case of microbial control this is a micro-organism.

Biological control, often shortened to biocontrol, using fungal antagonists against fungal plant pathogens has gained considerable attention and appears to be promising as a suitable supplement or alternative to chemical control (Papavizas & Lewis, 1981; Cook & Baker, 1983; Papavizas 1985). At present, there are very few fungicides effective against root and damping-off diseases (Papavizas, 1984; Lynch, 1988). There is also a lack of plant resistance to soil-borne pathogens (Sidhu, 1983). Consequently, there are great commercial opportunities in developing biocontrol agents for use in this area.

Considerable research has gone into the study of biocontrol of plant pathogens over the past 25-30 yr, but to date, there are only three fungal microbial agents registered for commercial plant disease control in Europe. The most successful in terms of a long market life and consistency of effect has been Peniophora gigantea. The antagonist has been used to control Heterobasidion annosum of pine since 1963 (Rishbeth, 1963, 1975, 1979, 1988). It is distributed as oidia in tablets or fluid suspensions and has been used with success on more than 62,000 ha of pine plantations in East Anglia (Greig, 1976). Strains of Trichoderma viride applied as spore suspensions to pruning wounds of plum trees protected the trees from silver leaf disease caused by Chondrostereum purpureum (Grosclaude, 1970; Grosclaude, Ricard & Dubos, 1973). A curative treatment for silver leaf disease has also been reported whereby T. viride-impregnated wood dowels are implanted into the trunks of plum trees (Corke, 1978). A commercial preparation based on T. viride and T. polysporum was registered subsequently for use in France and the U.K. (Ricard, 1981). A commercial preparation of Pythium oligandrum (Polygandron) grown on a grain substrate was recently registered for use in Czechoslovakia. Control of damping-off diseases of sugar beet in the field with this preparation has so far been quite promising (Vesely, 1989). Several other fungal biocontrol agents are undergoing exploratory commercial development or are used on a limited or local non-commercial scale.

Considering the limited number of commercial fungal biocontrol products available, biocontrol has not progressed as rapidly as originally anticipated. Commercially successful fungal biocontrol agents of soil-borne plant pathogenic fungi are undoubtedly

insignificant components of the modern plant protection weaponry. The likely explanation as to why this might be lies in the difficulties which challenge researchers during the development of a putative biocontrol agent.

The development of a biocontrol agent is a lengthy process in which many factors need to be considered. These factors include elementary aspects of strain isolation and selection, the use of suitable in vitro screening systems, efficient inoculum production, formulation, storage ability and choosing acceptable methods for application (Whipps, 1986, Whipps, Lewis & Cooke, 1988; Lewis, Whipps & Cooke, 1989; Lumsden & Lewis, 1989). Each one of these factors has its own inherent problems and difficulties which must be overcome if effective disease biocontrol is to be established. It is also essential to have a detailed understanding of mode of action, nutrition and ecophysiology of the biocontrol organism. Such information has important implications for appropriate targeting, devising suitable inoculum production techniques, enhancing formulations and improving subsequent biocontrol efficacy in the field (Whipps, 1986; Whipps et al., 1988; Lewis et al., 1989). It is vitally important to examine the compatibility of the biocontrol agent with existing agrochemicals and other pest and pathogen control strategies (Papavizas, 1984; Baker & Scher, 1987; Lynch, 1988; Papavizas & Lewis, 1988). Furthermore, there may be the potential for genetic manipulation of the biocontrol agent to create genetically superior strains that can perform better than the original wild type isolate selected from the environment (Papavizas, 1984; Linderman, 1985; Napoli & Staskawicz, 1985; Lumsden & Papavizas, 1989). Finally, commercialization of the biocontrol agent must be considered,

a process which has its own innate difficulties (Lumsden & Lewis, 1989; Powell & Faull, 1989).

No attempt is made here to cover the enormous literature accumulated on the subject of biocontrol, rather the technical aspects relevant to developing and using biocontrol fungi against soil-borne fungal diseases are highlighted with a slight emphasis on the problems encountered therein. Some of the more important aspects from the research findings on P. oligandrum reported by different researchers are summarized. Also, the experimental objectives of the research undertaken in this study are mentioned.

As indicated above, an understanding of the mode of action of potential biocontrol agents is of importance. There are three direct modes of action known for biocontrol agents of fungal plant pathogens. They include hyperparasitism, antibiosis and competition, and it is unlikely that any of these act independently. Further indirect modes of action known as induced resistance and cross protection are also known (de Wit, 1985). Recent reviews have covered all these modes of action in some detail (Whipps, 1986; Lewis et al., 1989) and only aspects relevant to biocontrol fungi of soil-borne fungal diseases are mentioned here.

Hyperparasitism is the parasitism or predation of one organism by another, and includes mycoparasitism which is probably the most important of the hyperparasitic interactions that can occur in nature. Mycoparasitism takes place when one fungus exists in intimate association with another from which it derives some or all of its nutrients while conferring no benefit in return. Examples of mycoparasitism in natural ecosystems can be found among all groups of

fungi from the chytrids to the higher basidiomycetes (De Vay, 1956). Mycoparasites can be divided into biotrophs and necrotrophs. The biotrophs have a persistent contact with, or occupation of, living cells whereas the necrotrophs kill host cells, often in advance of contact and penetration. Mycoparasitism as a mode of action in the biocontrol of plant diseases has been reviewed extensively in the last few years (Ayers & Adams, 1981; Lumsden, 1981; Whipps et al., 1988). The majority of mycoparasites of plant pathogenic fungi are necrotrophs and can parasitize hyphae, spores and other fungal structures, most notably sclerotia.

Examples of mycoparasites parasitizing hyphae have been shown mostly from in vitro studies, and includes species of Trichoderma and Gliocladium as well as P. nunn. Trichoderma spp. showed directed growth towards hyphae of P. ultimum, Rhizoctonia solani and Sclerotium rolfsii, and, after contact, various amounts of coiling were found, sometimes accompanied by penetrations (Chet, Harman & Baker, 1981; Elad et al., 1983). When grown on host cell walls as carbon sources, Trichoderma spp. produced the cell wall degrading enzymes chitinase,  $\beta$ 1-3 glucanase and cellulase in culture (Elad, Chet & Henis, 1982). It is likely that these enzymes are produced at the site of interaction. Gliocladium spp. have been shown to kill host cells by direct hyphal contact without penetration as with Gliocladium catenulatum on Sclerotium rolfsii and Fusarium spp. (Huang, 1978). Others can form appressoria, penetrate host hyphae and develop intracellular hyphae as with G. roseum on hyphae of Botrytis allii (Walker & Maude, 1975). P. nunn has shown a wide range of hyphal interactions with a number of fungi (Lifshitz, Stanghellini & Baker, 1984). For example, it coiled

around and lysed hyphae of P. ultimum and P. vexans. After appressorium formation, it penetrated and subsequently parasitized hyphae of R. solani, P. aphanidermatum and Phytophthora parasitica. It was not shown to be mycoparasitic against Fusarium oxysporum f. sp. cucumerinum and was killed by T. harzianum and T. viride. The mycoparasite has been shown to produce chitinases, glucanases and proteases which facilitates penetration of host cell walls. However, these enzymes are inducible rather than constitutive, their release being triggered by contact only with susceptible target species (Elad, Lifshitz & Baker, 1985).

Mycoparasitism of spores is quite a common phenomenon. Oospores of Aphanomyces, Phytophthora and Sclerospora are attacked by a wide range of Chytridiomycetes, Oomycetes and Hyphomycetes (Kenneth, Cohn & Shahar, 1975; Hoch & Abawi, 1979; Wynn & Epton, 1979). The cytosori of Polymyxa betae contained within the roots of sugar beet have been shown to be degraded by T. harzianum in vitro (D'Ambra & Mutto, 1986). As P. betae is the vector of beet necrotic yellow vein virus, this could have important implications for the indirect control of viral 'rhizomania' disease. In vitro studies have also shown that Gliocladium species can infect conidia of some soil-borne plant pathogens but, as the majority of conidia in soil lose their viability through the process of mycolysis, the contribution of mycoparasitism to their disappearance in nature seems likely to be negligible (Whipps et al., 1988).

A wide range of fungi have been recorded as sclerotial mycoparasites. They may be either facultatively or obligately mycoparasitic. The facultative mycoparasites such as Gliocladium and Trichoderma species occur widely in soil. In contrast, the obligate

mycoparasites have only been isolated from infected sclerotia and include the Coelomycete Coniothyrium minitans and Hyphomycetes Laterispora brevissima, Sporidesmium sclerotivorum and Teratosperma oligocladum. These mycoparasites are highly destructive of healthy sclerotia and, in at least some field conditions, they may play a major role in reducing diseases caused by sclerotium-forming fungi. They penetrate the tough melanised protective rind of sclerotia and quickly colonize the medullary tissues. T. harzianum and C. minitans destroy sclerotia by the production of glucanases and chitinases (Jones, Gordon & Bacon, 1974; Elad, Chet & Henis, 1982a, b). The mycoparasites then sporulate upon or within the degraded tissues (Tu, 1980, 1984; Adams & Ayers, 1983; Phillips & Price, 1983). An interesting feature of S. sclerotivorum is that it possesses some biotrophic characteristics compared with the other sclerotial mycoparasites which are solely necrotrophic. This mycoparasite does not produce glucanases like the others but stimulates release of these enzymes by host sclerotium tissues leading to their autodigestion (Adams & Ayers, 1983). Furthermore, an electron microscope study has shown that S. sclerotivorum produces haustoria within hyphae of sclerotia of Sclerotinia minor (Bullock *et al.*, 1986).

Antibiosis is where antagonistic micro-organisms secrete metabolites which are harmful to other organisms. As a possible mode of action for biocontrol fungi, it is by far the easiest to demonstrate in vitro. In this regard, many agar plate tests have been devised to detect the production of volatile and non-volatile antibiotics by fungal antagonists and to quantify their effect on target pathogens (Whipps, 1987a). Well known biocontrol fungi including species of Gliocladium

and Trichoderma have been shown to produce a range of antibiotics which are active against target pathogens in vitro (Dennis & Webster, 1971a, b; Bell, Wells & Markham, 1982; Howell & Stipanovic, 1983; Claydon et al., 1987). Consequently, antibiotic production has been suggested as a mode of action for these antagonists. Unfortunately, evidence for antibiotic production in the soil has not been forthcoming, and the presence of such compounds within the soil microbiome rests largely on assumption. It is possible, however, that the detection techniques used are insensitive, that antibiotics are rapidly degraded or that they are bound to soil particles preventing detection. Nevertheless, evidence does exist for antibiotic production by fungi in sites of high nutrient content such as the rhizosphere, seeds and decaying organic matter (Williams, 1982).

Competition can be defined simply as the active demand by two or more individuals for the same resource, which includes both space and nutrients. It is probably the single most important type of interaction in nature, and occurs between micro-organisms when space or nutrients are limiting. In the rhizosphere, there is some evidence for the involvement of competition in biocontrol. This evidence is mainly associated with competition for substrates. For instance, one property of a successful rhizosphere biocontrol agent would be the ability to maintain a high population on the rhizosphere and so provide protection of the whole root for the duration of its life (Lewis et al., 1989). In this regard, rhizosphere competent isolates of T. harzianum have been obtained by mutagenesis (Ahmad & Baker, 1987a). Results from a further study suggested that the rhizosphere competence of these isolates was associated with their capacity to utilize cellulose

substrates associated with the root through increased cellulase production (Ahmad & Baker, 1987b). Likewise, it was found that Microdochium bolleyi and Phialophora graminicola utilize senescing cortical cells of cereal roots and, in glasshouse conditions, gave control of take-all (Kirk & Deacon, 1987a, b). It has been suggested that these fungi compete with the take-all pathogen Gaeumannomyces graminis var. graminis for the senescing root cortices and so reduce the level of take-all. Competition for substrates also occurs naturally, outside the rhizosphere, especially within soil regions enriched with organic matter. The best example is the suppression of Fusarium spp. in the Chateaufrenard soil in France (Alabouvette, Couteadier & Louvet, 1985; Alabouvette, 1986). This suppressive soil was shown to have a greater microbial biomass present compared with a conducive soil. It was suggested that this led to greater nutrient competition and consequent inhibition of Fusarium spp..

Induced resistance is a plant response to challenge by micro-organisms or abiotic agents such that following the inducing challenge de novo resistance to pathogens is shown in normally susceptible plants (de Wit, 1985). This mechanism can be localized or systemic and involves many different processes. Cross protection is also mediated through the host plant. It differs from induced resistance in that following inoculation with avirulent strains of pathogens or other micro-organisms, both inducing micro-organisms and challenge pathogens occur on or within the protected tissues (de Wit, 1985). Some well known examples of cross protection occur with the biocontrol of vascular pathogens. Control of Verticillium wilt of tomato has been obtained by dipping roots in a suspension of an

avirulent strain of Verticillium albo-atrum prior to planting (Matta & Garibaldi, 1977). Fusarium wilt of sweet potato has been decreased by dipping freshly cut ends of cuttings into suspensions of bud cells of non-pathogenic isolates of F. oxysporum before planting (Ogawa & Komada, 1985). Also, Rhizoctonia damping-off of cotton, radish and wheat has been depressed with avirulent strains of R. solani (Ichielevich-Auster et al., 1985).

Before even contemplating the use of a biocontrol agent against a soil-borne pathogen, sufficient details about the pathogen's life-cycle and its host crop must be known (Whipps et al., 1988). A decision can then be made as to which stage of the pathogen's life-cycle is to be targeted. This in turn determines whether the biocontrol agent is to function in the infection court or at some distance away from it, or whether the aim is to prevent infection or reduce carry-over following infection. The success of any biocontrol strategy will then depend on the use of rationally designed screening procedures and the ability to deliver the chosen biocontrol agent to the plant pathogen's habitat at the correct time, in sufficient quantities, and also in a suitable state of activity (Whipps et al., 1988).

The first stages in the development of a biocontrol agent for disease control involve its isolation and selection. Two distinct approaches have generally been taken. In the first, isolations have been made from habitats in which the target pathogen was known to be present, but disease was not expressed (e.g. a suppressive soil). Using the same approach, isolations have also been made from habitats in which the target pathogen has previously caused disease but was absent at the sampling time (e.g. the rhizosphere), and from baited

fungal structures such as hyphae or sclerotia buried in the soil. Any potential antagonists isolated from these habitats are likely to be well adapted to the same environmental conditions as the target pathogen (Rouxel, Alabouvette & Louvet, 1979; Scher & Baker, 1980; Whipps, 1986; Whipps et al., 1988). This feature is important because an antagonist adapted to the optimum environment of the target pathogen has a better chance for controlling disease (Cook & Baker, 1983; Baker & Scher, 1987). The second approach has involved isolating biocontrol agents from a wide range of habitats irrespective of whether these harboured the target pathogen or not (Cook, 1985). There is the obvious possibility that biocontrol agents isolated in this way may prove difficult to establish if introduced into habitats which are alien to them (Whipps, 1986; Whipps et al., 1988). The latter approach resembles the systems used by commercial companies for screening chemicals for biological activity and is dependent upon an able test system. Until such a costly and efficient marketable system is established, the former more scientifically sound approach should be favoured (Whipps, 1986).

Biocontrol fungi have been isolated successfully from soils, disease suppressive soils, baited hyphae or sclerotia buried in soil, and sclerotia taken directly from the soil or from infected plant material. Some examples are noted here.

Whipps (1987a) isolated the antagonist G. roseum from glasshouse soil under routine cropping with lettuce and celery. A collection of antagonistic Trichoderma spp. were isolated from soil sampled from commercial radish fields and from rhizosphere soil of radishes grown in these fields (Mihuta Grimm & Rowe, 1986). Likewise, T. harzianum was

isolated from a soil normally cropped with sunflowers and known to contain Sclerotinia sclerotiorum (Zizzerini & Tosi, 1985).

Disease suppressive soils are generally difficult to recognize. However, several such examples are known (Burke, 1965; Baker & Cook, 1974; Broadbent & Baker, 1974; Alabouvette, Rouxel & Louvet, 1979; Furuya, Owada & Ui, 1979; Lin & Cook, 1979; Schneider, 1982; Cook & Baker, 1983; Linderman et al., 1983; Alabouvette, 1986). In the past, these have been valuable sources of fungal antagonists. For example, T. hamatum has been commonly isolated from soils naturally suppressive to R. solani, and has induced suppressiveness in soils when introduced to those conducive to Pythium spp. and Sclerotium rolfsii as well as R. solani (Chet & Baker, 1981). Similarly, the mycoparasite P. nunn has been isolated from a grassland soil suppressive to P. ultimum (Lifshitz, Stanghellini & Baker, 1984). Suppression of cucumber damping-off caused by P. ultimum was induced when inoculum of P. nunn was added subsequently to soil (Lifshitz, Sneh & Baker, 1984). More recently, a Pythium-suppressive soil was identified in the traditional chinampa agroecosystem in the Valley of Mexico (Lumsden et al., 1987). Isolates of F. solani, T. harzianum and Gliocladium spp. from this soil were capable of reducing disease caused by Pythium spp. in glasshouse experiments.

Baiting with fungal structures has proved an effective technique for isolating antagonistic fungi. T. harzianum, for example, has been isolated from baited hyphae of R. solani in soil (Ridout, Coley-Smith & Lynch, 1986). Many antagonists, especially mycoparasites, have been isolated from soil by baiting with sclerotia of sclerotium-forming soil-borne plant pathogens. For example, C. minitans and species of

Trichoderma and Gliocladium were isolated from sclerotia of Phymatotrichum omnivorum buried in soil in nylon bags (Kenerley et al., 1987). Similar antagonistic fungi were isolated from baited sclerotia of Sclerotinia spp. either buried in soil or placed on the soil surface (Zizzerini & Tosi, 1985; McCredie & Sivasithamparam, 1985). Baiting with sclerotia of Sclerotinia spp. in soil resulted in the recognition of obligately mycoparasitic fungi, most notably the Hyphomycetes Sporidesmium sclerotivorum (Adams & Ayers, 1981; Adams, 1987; Sansford, Coley-Smith & Parfitt, 1987), Teratosperma oligocladum (Parfitt, Coley-Smith & Jeves, 1983) and L. brevirama (Parfitt, Coley-Smith & McHale, 1984).

Sclerotia taken directly from pathogen-infested soils and infected crop material have been good sources of fungal antagonists. Nine isolates of T. harzianum were isolated from dead sclerotia of Sclerotium cepivorum collected from soil (Mousa, Khalil & Mona, 1987). Also, C. minitans has been isolated from sclerotia of S. sclerotiorum removed from infected sunflowers (Trutmann, Keane & Merriman, 1980).

Pure cultures of potential antagonistic fungi are normally obtained after isolation prior to identification by established biosystematic approaches (Knutson, 1981). Once isolated antagonists have been identified, they are then screened for biocontrol potential. Reliable bioassay screening systems are required to determine which few isolates are to be developed for biocontrol and which of the vast majority are to be discarded (Spurr, 1985). Such ideal systems are not as yet available.

Present in vitro screening systems used to detect and select antagonistic fungi frequently include agar plate tests. These tests

have the advantages of being relatively quick and easy to perform. As noted earlier, such tests can also give a possible insight into the mechanisms of action including the potential of an antagonist to produce volatile and non-volatile antibiotics, or to parasitize the pathogen (Whipps, 1986; Whipps, 1987a; Lewis et al., 1989). They do not, however, always relate to biocontrol in the field. Isolates that have shown strong zones of inhibition or evidence of mycoparasitic behaviour in dual culture, for example, have not given biocontrol when introduced into the soil (Linderman et al., 1983). Conversely, some of the more promising fungi used for biocontrol in the soil have not produced obvious interactions on culture media.

The inability of agar plate tests to predict accurately the behaviour of antagonists in vivo has been partly attributed to performing these tests under controlled and often ecologically unrealistic nutrient conditions (Whipps et al., 1988). Since the work by Dennis & Webster (1971a, b, c) on the evaluation of the antagonistic properties of Trichoderma spp., several agar plate tests have been performed to assess the relative activity of antagonists against target pathogens, and to elucidate their possible modes of action (Huang & Hoes, 1976; Skidmore & Dickinson, 1976; Bell, Wells & Markham, 1982; Chand & Logan, 1984; De Oliveira, Bellei & Borges, 1984; Henis, Lewis & Papavizas, 1984; Reyes, 1984; Vesely & Hejdanek, 1984; Vakili, 1985; Zazzerini & Tosi, 1985; Webber & Hedger, 1986; Lynch, 1987; Sreenivasaprasad & Manibhushanrao, 1990). On the whole, these have followed the same principles and have been carried out on one or two nutrient-rich media including malt extract agar (MEA) and potato dextrose agar (PDA). Under these nutrient-rich conditions, fungal

growth and development is generally unrestricted and antibiotic production can be maximal (Bu'lock, 1975). Similarly, opposition of fungi on nutrient-rich culture media can result in mycoparasitism being expressed by species which are probably not mycoparasitic in nature (Rudakov, 1978). Apart from some nutrient-rich conditions in crop residues, biocontrol of soil-borne plant pathogens is mainly required to operate under nutrient-poor soil conditions. Consequently, screening tests carried out on nutrient-rich media do not imitate the soil conditions under which antagonist-pathogen interactions are likely to occur and may therefore be of little significance (Whipps, 1987a; Whipps & Magan, 1987). In view of this, some researchers have designed tests to reflect the natural ecology of the pathogen and antagonist. For example, Lutchmeah & Cooke (1984) investigated the activity of P. oligandrum towards P. ultimum, R. solani and Mycocentrospora acerina on tap water agar (TWA) to simulate the low nutrient conditions that occur in soil. Another example includes a study of hyphal interactions between P. oligandrum and a range of important plant pathogenic fungi on cellulose film overlaying distilled water agar (Lewis, 1988; Lewis et al., 1989). Nevertheless, use of nutrient-rich media can be appropriate, where, in a few cases, biocontrol is required to operate in nutrient-rich plant residues. In some instances, the use of both rich and poor media can be relevant for testing fungi such as G. roseum. The fungus is a soil resident able to colonize plant debris (Mueller & Sinclair, 1986) and is a known antagonist of other fungi (Barnett & Lilly, 1962; Papavizas, 1985). Hence, it is worth assessing the ability of the fungus to produce antibiotics and parasitize target pathogens on both rich and poor media.

Whatever approach is taken in using agar plate tests as initial screens, greater consideration must be given to designing tests which reflect the natural ecology of the antagonists and target pathogens. Ideally, tests need to be designed so that they assess the relative activity of antagonists under a range of nutrient conditions, and so attempt to simulate all possible antagonist-pathogen interactions that can occur in nature. Tests should therefore be carried out on a range of media of different nutrient status or on at least a nutrient-rich and a nutrient-poor medium. A few researchers have studied interactions on more than one medium. One example includes the use of three agar media to observe the effect of a range of antagonists against soil-borne glasshouse pathogens (Whipps, 1987a). In another example, Kuter (1984) studied the interactions between R. solani and some antagonistic Verticillium spp. on at least two agar media, always including TWA and PDA to give a comparison between a nutrient-poor and nutrient-rich medium. Whipps (1987a) has expressed some doubt in using TWA and PDA because of their undefined nature. In future, it may be imperative to develop well defined media to conduct such plate tests with accuracy for a logical interpretation of results.

Many antagonist-pathogen interactions are known to be influenced by temperature (Tronsmo & Dennis, 1977; 1978; Phillips, 1986), pH (Sy et al., 1984) and water potential (Whipps & Magan, 1987). In accordance with these findings, it is important to carry out tests under a range of physical environmental conditions as well as on different nutrient media. Tests carried out in this way may be used to determine the range of environmental conditions under which optimal use of the antagonist and control of the pathogen can be obtained. More attention

must also be paid to designing tests which consider the effects of fluctuating temperature and water potential regimes similar to those in the sub-surface soil layers. Unfortunately, there are difficulties in simulating these natural environmental conditions.

A major problem relating to screening with agar plate tests is that the interactions observed may depend upon the isolates used (Whipps, 1987a; Whipps et al., 1988). Variation in the effectiveness of isolates has been reported by a number of researchers (Bell et al., 1982; Artrigues, Davet & Roure, 1984; Henis et al., 1964; Whipps, 1987a). Similarly, there is also a danger that some target pathogen isolates may be more resistant than others or may act as antagonists themselves (Upadhyay, Rai & Gupta, 1983; Burton & Coley-Smith, 1985; Vajna, 1985; Whipps, 1987a). In the past, it has been a common practice to assess the activity of only one potential antagonist isolate against one isolate of the target pathogen. This should be avoided if practically possible, and more consideration given to comparing a range of isolates of both the antagonist and target pathogen.

Finally, plate tests may be of little value for assessing the relative activity of newly isolated mycoparasites against some sclerotium-forming soil-borne plant pathogens (Whipps, 1987a; Whipps & Magan, 1987) and is related to the life-cycle and ecological behaviour of these pathogens. For example, S. sclerotiorum survives between crops as sclerotia either in soil or crop residues and infects aerial plant parts where active mycelial growth usually occurs. The pathogen shows little ability to grow as mycelium through the soil in nutrient-poor conditions, except when it infects myceliogenically by

direct contact with decaying crop material (Lynch & Ebben, 1986). Therefore, plate tests with mycelium of this pathogen on nutrient-poor media may be irrelevant although tests with sclerotia on such media could be beneficial. Tests with mycelium of other pathogens of this kind may be of some use if they have a mycelial stage in soil as with S. minor on lettuce (Whipps, 1990). Even so, the poor success rate of plate tests for selection of mycoparasites of these particular pathogens must cast some doubt on their value.

Some rational screening systems have been recently reported for the selection of sclerotial mycoparasites of S. sclerotiorum. These have been designed to evaluate the ability of a range of mycoparasites to infect sclerotia or prevent sclerotial formation of the pathogen under specific environmental conditions. With a view to using a novel approach for the biocontrol of Sclerotinia disease in glasshouse crops (e.g. lettuce and celery), Whipps (1987b) designed an in vitro plant-tissue-based system to assess the ability of fungi antagonistic to S. sclerotiorum to inhibit the formation of sclerotia and to grow through plant tissue from cut surfaces. The system successfully demonstrated the inability of P. oligandrum to grow through plant tissue or inhibit sclerotium formation, and the ability of Gliocladium and Trichoderma strains to do so. More recently, Whipps & Budge (1990) devised a system in order to improve the ability of ten known mycoparasites to infect sclerotia of S. sclerotiorum using five combinations of inoculum form and substrate type. The sclerotia were produced on agar medium and the environmental conditions employed were similar to those in U.K. glasshouses. In every combination, G. virens and C. minitans, both originally isolated from sclerotia of

Sclerotinia, infected and decreased viability of sclerotia more frequently than the other mycoparasites tested. When interpreting results from a screening system of this kind, researchers should be aware that sclerotia produced on agar medium are known to differ in their physiological properties and resistance to biocontrol agents compared with those produced on living material (Turner & Tribe, 1976; Trutmann et al., 1980; Coley-Smith, 1985). However, the sclerotia obtained in this manner are of uniform size and contamination-free thus minimizing chances of variability which can affect screening systems (Whipps & Budge, 1990).

Before leaving the subject of screening, it is important to remember that the techniques used may only infer potential for biocontrol, and nothing can replace the results obtained from antagonist-pathogen interactions in a natural ecosystem (Lumsden & Lewis, 1989).

Following in vitro screening, simple small-scale pot assays are usually carried out to test putative antagonists for biocontrol under controlled conditions (Whipps, 1986). At this stage, attention is mainly focused on determining whether the antagonist has any potential to give biocontrol by using very basic formulations. Antagonists are usually incorporated into the pathogen-infested growing medium which can then be planted with susceptible test plants or coated onto seeds. The assays are usually standardized with a known amount of pathogen inoculum to give a suitable level of disease which does not overwhelm the antagonist. These assays allow easy manipulation of environmental factors such as temperature, pH, light, water potential and humidity as well as variation in timing of application of both antagonist and pathogen. Moreover, they can also usually detect any phytotoxic

effects from prospective biocontrol agents.

Once isolated fungi have shown antagonistic properties and a potential to give biocontrol, it is very important to deposit them in a reputable culture collection store for preservation (Lumsden & Lewis, 1989). Several culture collections including the one at the International Mycological Institute (Kew) accept biocontrol fungi. Working cultures derived from single spore isolations should also be stored in-house in liquid nitrogen, silica gel or low-temperature freezers. Researchers can then gain easy access to genetically stable, pure cultures for future use in their biocontrol studies.

Once a biocontrol fungus has shown potential for disease control, the next stage in its development involves the adequate production of an effective biomass, preferably by using a commercially compatible system. The biomass can be in the form of spores, mycelia or mixtures of both. Liquid and semi-solid culture systems are used for biomass production, as an outgrowth of the commercial microbial product industry.

Recent adaptations of existing liquid fermentation technology have resulted in the production of bacterial and fungal biomass for use as biocontrol insecticides (Dulmage, 1981; Soper & Ward, 1981) and herbicides (Churchill, 1982; Templeton & Heiny, 1989). Unfortunately, similar technology with regard to production of biocontrol fungi effective against soil-borne plant pathogens is almost non-existent. This is obviously an obstacle to the advancement of biocontrol research. In most industrialized countries, commercial liquid fermentation systems are already in place. Consequently, the use of liquid culture systems for the production of biocontrol fungal biomass

is desirable (Churchill, 1982). Many of the liquid fermenters available can provide a wide range of culture conditions (Knight, 1988). Culture conditions can therefore be set to meet the growth requirements of each individual biocontrol fungus.

The first step in the production of a biocontrol fungus involves the development of a suitable liquid medium using readily available inexpensive agricultural by-products and food processing wastes with an appropriate nutrient balance (Kenney & Couch, 1981). Suitable ingredients include molasses, corn steep liquor, whey, brewers' yeast and cotton seed and soy flours (Lisansky, 1985). Constituents of the final medium must be investigated carefully ; ingredients and their concentrations can affect biomass quantity and the nature of the final propagules produced (Lewis & Papavizas, 1983). The temperature and pH optima for both vegetative growth and sporulation of the biocontrol fungus have to be ascertained, as there are occasions when the optimum environmental conditions for growth and sporulation do not coincide. Usually, the starting pH of the medium is 5.0 to 6.5, and temperatures are maintained in the 20 to 30° range.

Following the development of a suitable liquid medium, it is imperative before scaling-up the culture system to larger equipment to have a basic understanding as to how culture conditions affect growth. Simple experiments to compare growth in aerated, shake and static culture conditions are easily carried out in the laboratory. Information from these simple experiments can then be used to optimize growth and spore production in large-scale culture systems, where, in many cases, aeration and agitation are essential requirements. In some cases, however, it may not be desirable to maintain optimum nutrient,

aeration and pH levels since unfavourable conditions can promote spore production (Kenney & Couch, 1981).

Large batches of biomass of G. virens, T. hamatum, T. harzianum, T. viride and Talaromyces flavus have been produced in 20 l aerated vessels simulating industrial conditions by utilizing a molasses-brewers' yeast medium (Papavizas et al., 1984). Maximum biomass yields of T. harzianum, T. viride and G. virens were obtained after 15 d of agitated incubation and that of T. hamatum at 10 d. The biomass of G. virens and the Trichoderma spp. consisted mainly of mycelia and chlamydospores and some conidia. In contrast, biomass of Talaromyces flavus consisted of mycelia only. Recently, a liquid culture system has been reported for producing abundant conidia of Trichoderma spp. (Tabachnik, 1988). The biomass produced in liquid culture systems such as these is separated from spent medium usually by filtration or centrifugation. Harvested biomass is normally dried and then milled prior to incorporation into various formulations.

Other additional factors need to be considered when using liquid culture systems. For instance, the rate at which an effective biomass is produced affects cost of production as well as the chance of culture contamination and the viability of the final propagules produced (Lisansky, 1985). It is more economic to obtain the optimum amount of biomass in the shortest time. Satisfactory biomass quantities of Trichoderma, Gliocladium, and Talaromyces were obtained in 6-7 d, but this time period is still relatively long compared to that for bacteria (Papavizas et al., 1984). Increased periods of growth can also reduce the viability of some fungal propagules and increases the risk of contamination (Churchill, 1982).

Despite the rapid advances in large-scale liquid culture systems demonstrated by industrialized nations, most of the research on mass production of biocontrol fungi has been concentrated on the use of semi-solid culture systems (Aidoo, Hendry & Wood, 1982). These systems involve culturing the fungus on solid materials moistened with water or a nutrient liquid. The solid materials include various grain seeds and meals, bagasse, straws, wheat bran, sawdust and peat; these have been used successfully for producing biocontrol inoculum of fungi in the genera Coniothyrium, Gliocladium, Penicillium and Trichoderma (Papavizas & Lewis, 1981; Lewis & Papavizas, 1983; Papavizas, 1985; Lynch & Ebben, 1986). Semi-solid culture systems are easy to set up in the laboratory and do not usually require any sophisticated equipment apart from autoclaving facilities. They are especially useful for producing biocontrol fungi which are unable to sporulate in liquid culture (Lisansky, 1985). For example, conidia of the mycoparasite Sporidesmium sclerotivorum have been produced on vermiculite moistened with a liquid medium (Adams & Ayers, 1982; Adams, Marois & Ayers, 1984).

Although semi-solid culture systems are useful in some circumstances, there are, however, some disadvantages and problems associated with their use (Lumsden & Lewis, 1989). The final preparations are generally bulky and may be subject to contamination. They may also have to be dried and milled with the undesirable formation of dusts containing spores. Furthermore, they may require specialized equipment for their application. Finally, commercial semi-solid culture facilities are very limited in some countries such as N. America (Kenney & Couch, 1981). The advent of liquid culture

fermentation technology for the production of antibiotics has resulted in practically all existing facilities being liquid culture. In Japan, however, enormous quantities of enzymes required for traditional soybean fermentation have resulted in the availability of large, fully automated, commercial semi-solid substrate culture facilities.

In order to control soil-borne diseases, biocontrol fungi produced in either liquid or semi-solid culture systems must be formulated to permit effective delivery and introduction into the soil microbiome. Ideally, a biocontrol formulation should possess several desirable characteristics such as ease in preparation and application, stability, adequate shelf life, abundant viable propagules and low cost (Churchill, 1982; Lisansky, 1985).

Two basic strategies have been used for delivering biocontrol fungi to the soil microbiome. In the first, biocontrol inoculum is localized around the microbiome which is to be protected. This can be achieved by techniques such as seed-coating or pelleting, or treating materials used in plant propagation. It is envisaged that development of the plant and biocontrol fungus will result in the latter occupying the spermosphere, rhizoplane and rhizosphere, although perhaps only during the initial stages of plant growth (Whipps *et al.*, 1988). In the second, biocontrol inoculum is distributed evenly throughout the bulk growing medium. This strategy allows protection close to the plant as it develops, and may also bring about destruction of pathogenic propagules at some distance from it.

There are many reports of seed treatment with biocontrol fungi to control disease (Kommedahl & Windels, 1981). In the main, biocontrol fungi have been either coated onto seeds with adhesives or incorporated

into seed pelleting or seed-coating materials. For example, propagules of Trichoderma spp. have been coated onto seeds using the adhesives carboxymethyl cellulose (Locke, et al., 1979; Wu, 1982; Wu & Lu, 1984; Mihuta-Grimm & Rowe, 1986) and polyvinyl alcohol (Hadar et al., 1984) to control various soil-borne diseases. Similarly, P. oligandrum oospores have been commercially pelleted with seed in a clay carrier to control Pythium-induced damping-off in sugar beet and Mycocentrospora acerina infection of carrot seedlings (Lutchmeah & Cooke, 1985). Many seed treatments can control seed and seedling diseases. However, protection beyond the seedling stage requires establishment of the biocontrol fungus around the hypocotyl and proliferation within the ever-growing rhizosphere. In general, however, biocontrol fungi applied to seeds have failed to colonize the rhizosphere (Kommedahl & Windels, 1981; Lewis & Papavizas, 1984; Chao et al., 1986). An exception to this finding includes Verticillium biguttatum which is able to spread from coated potato seed tubers to sprouts and stolons, protecting them from infection by R. solani and producing a 60% reduction in sclerotium production by the pathogen (Velvis & Jager, 1983; Jager & Velvis, 1984, 1985). Furthermore, it has been shown that benomyl-tolerant mutants of T. harzianum are rhizosphere competent (Ahmad & Baker, 1987a).

Biocontrol activity of T. koningii and T. harzianum against Pythium-induced damping-off of pea has been increased by adding various compounds to seed treatments (Nelson, Harman & Nash, 1988). Organic acids were most promotive to the activity of T. koningii whereas polysaccharides and polyhydroxy alcohols were most promotive to T. harzianum. Recently, improvement in biocontrol efficacy has been

reported by Harman & Taylor (1988) using a solid matrix priming system for seed application. With this technique, seeds were hydrated to a controlled level by mixing with a moistened organic carrier to allow pre-germinative metabolic activity to occur but without allowing the emergence of the radicle. Biocontrol activity of T. harzianum against Pythium-induced damping-off was markedly increased with solid matrix priming when applied onto cucumber and tomato seeds with coal dust.

Another method for treatment of seed involves the incorporation of the biocontrol fungus into semi-liquid gels used for fluid-drill seeding (Conway, 1986). Selected gels used in this method include Natrosol (a hydroxyethyl cellulose), Polytran N (a glucan gum), Laponite 508 (a synthetic magnesium silicate) and Viterra 332 (a potassium propenoate-propenamide copolymer). Mihuta-Grimm & Rowe (1986) evaluated four delivery systems, including fluid-drill seeding and coated seed treatments, for applying Trichoderma spp. in the field for biocontrol of R. solani on radish. The fluid drilling technique was superior to the others in reducing disease. Nevertheless, it has been suggested that costs and laborious methods of application may preclude the use of gels for large-scale application of biocontrol fungi to field crops (Jones, Pettit & Taber, 1984).

Solid-substrate and granular preparations of biocontrol fungi have been distributed throughout the soil either broadcast or applied in the seed furrow. C. minitans grown on a barley-rye-sunflower seed mixture applied to sunflower seed rows reduced Sclerotinia wilt by 30% over a 2 yr period (Huang, 1980). Similarly, in glasshouse tests, soil treatment with a C. minitans preparation grown on milled rice decreased the incidence of white rot on onion seedlings (Ahmed & Tribe, 1977).

Backman & Rodriguez-Kabana (1975) used a diatomaceous earth granule impregnated with molasses for delivery of T. harzianum to peanut fields. With this carrier system, significant reductions in Sclerotium rolfsii damage and increases in yield were recorded over the 3 yr test period. Similarly, Jones et al. (1984) evaluated a lignite stillage carrier system, formulated with Trichoderma and Gliocladium into granules, against R. solani-induced damping-off of peanuts. Researchers must be aware that formulations of this kind can be detrimental if the nutrients stimulate growth of the pathogen. For example, Kelley (1976) used clay granules impregnated with molasses as a carrier for T. harzianum to control damping-off of pine caused by Phytophthora cinnamomi, but disease was favoured, especially if the soil was also water-saturated. The molasses was stimulatory to P. cinnamomi. Furthermore, if using in-furrow or broadcast preparations, the cost, rate and ease of application with existing agricultural machinery must all be considered. The amounts of biocontrol preparation to be applied as broadcast applications are often agriculturally unrealistic (Wells, Bell & Jaworski, 1972; Backman et al., 1975). For example, rates are commonly 250-500 kg/ha. In contrast, applications to the seed furrow are often much less and hence more economical.

More recently, a number of practicable formulations have been developed using dried biomass produced in liquid culture. Dusts have been produced from air-dried biomass of Gliocladium and Trichoderma by milling, and mixing the resulting powder with a pyrophyllite clay carrier. After addition to soil, the biocontrol fungi proliferated as shown by a rise in the number of colony forming units (cfu's) present

per unit weight of soil (Papavizas et al., 1984). A similar dust formulation prepared from ground biomass of several Trichoderma spp. and G. virens isolates, and mixed with a pyrophyllite clay carrier reduced Rhizoctonia diseases of potato and cotton when applied to field plots (Beagle-Ristaino & Papavizas, 1985). An innovative approach to formulation and delivery of biocontrol fungi involves the encapsulation of biomass within alginate gel pellets. Encapsulation is based on the ability of aqueous solutions of sodium alginate to react with certain metal cations (e.g.  $Ca^{2+}$ ) to form gels. The method was used first for encapsulating chemical herbicides and mycoherbicides (Walker & Connick, 1983). Briefly, the method consists of mixing biomass and a carrier with a sodium alginate solution. The mixture is then dripped into a calcium chloride solution to form insoluble gelatinous beads which dry into stable, hard pellets of uniform size. A further refinement of this technique is the incorporation in the pellet of appropriate food bases, selective nutrients or stimulants for the biocontrol fungus.

A wide range of biocontrol fungi have been successfully encapsulated into alginate pellets including Talaromyces flavus, G. virens and Trichoderma spp. (Fravel et al., 1985; Lewis & Papavizas, 1985, 1987; Papavizas & Lewis, 1989). Addition of alginate pellets containing several biocontrol fungi to soils has resulted in the reduction of many soil-borne diseases (Lewis & Papavizas, 1987; Lumsden & Locke, 1989). Moreover, biocontrol fungi have proliferated in soils following delivery to these in alginate pellets (Lewis & Papavizas, 1984; 1985; Papavizas, Fravel & Lewis, 1987).

Before evaluating new formulations of biocontrol fungi under normal agricultural and horticultural conditions, it is common to test them

against prospective target pathogens under controlled environmental conditions in growth chambers. When such tests are performed, attempts should be made to simulate as closely as possible the environment in which biocontrol is expected to work. Naturally infested soils should be used (Linderman, et al., 1983) or if appropriate, artificially infested commercially used soil mixes or soilless potting products (Lumsden & Locke, 1989). As with the simple small-scale pot assays for screening putative biocontrol fungi, care must also be taken here to use an appropriate amount of pathogen inoculum to give a suitable level of disease which does not overpower the biocontrol fungus. The pathogen inoculum should also be as 'natural' as possible. For example, Lumsden et al., (1990) used sporangia of P. ultimum to artificially infest commercial potting mix.

A number of important factors need to be considered for successful field testing of fungal biocontrol formulations (Scher & Castagno, 1986). The biocontrol formulation should be evaluated under normal agronomic conditions at several representative sites. For example, where the targets for biocontrol are glasshouse soil-borne pathogens, testing should be carried out, if possible, in glasshouses. Soils should be naturally infested with the pathogen at sufficient inoculum density to ensure uniform disease development (Ayers & Adams, 1981). Where this does not occur, artificial infestation is often necessary. For instance, to evaluate solid substrate preparations of C. minitans and G. roseum against Sclerotinia disease of glasshouse lettuce, sclerotia of S. sclerotiorum produced on artificially infected pot-grown lettuce plants were used to infest plots of glasshouse soil on two separate sites (Lynch & Ebben, 1986). Similarly, Adams & Ayers

(1982) successfully infested a previously uninfested field plot with S. minor. The pathogen was grown on autoclaved oats and this inoculum was then used to inoculate mature lettuce plants in field plots. Once the pathogen was established, a solid preparation of the mycoparasite Sporidesmium sclerotivorum was tested for ability to control lettuce drop.

The success of any biocontrol fungus in the field will depend upon its persistence and the effects which it has on the target pathogen population. Consequently, it is important to monitor the populations of both the introduced biocontrol fungus and target pathogen as well as the crop for levels of disease (Whipps, 1986; Whipps et al., 1987). Information of this kind from field testing can assess whether the biocontrol fungus is successful and also to some extent how it works allowing further improvements in its formulation to be made.

During field testing it is also important to assess the effect of the resident microbial population on the introduced biocontrol fungus. T. hamatum has been shown to be ineffective against P. ultimum root rot of pea in certain regions as a result of antagonism of the biocontrol fungus by fluorescent Pseudomonads (Hubbard, Harman & Hadar, 1983). Furthermore, the effects of introduced biocontrol fungi on beneficial micro-organisms such as mycorrhizal fungi must also be evaluated (Ross & Ruttencutter, 1977).

Most biocontrol formulations consist of a single strain of a biocontrol fungus. It has been suggested by various researchers that use of mixtures of strains belonging in a single species, or even mixtures of biocontrol fungi belonging to different species or genera, may be more effective in biocontrol than single strains (Papavizas,

1985).

A biocontrol formulation must have an adequate shelf life especially if commercialization is anticipated. The physical, chemical and biological integrity of the preparation must be maintained from initial formulation until final use (Bowers, 1982). Hence, the formulation must be stable for 6 months to 2 yr at -5 to 30°, and should have the ability to withstand high temperatures (c. 55°) for several hours during transportation (Powell & Faull, 1989; Lumsden & Lewis, 1989). These requirements could be modified in some cases.

Suitable formulations and the storage temperature of such formulations have been shown to extend propagule survival and shelf life. For example, a dust formulation of T. harzianum containing conidia mixed with pyrophyllite carrier survived better than conidia alone at temperatures between -5 to 30°; the best temperatures to prolong shelf life of conidia in pyrophyllite were -5 and 5°, and the worst were 25 and 30° (Papavizas et al., 1984). Shelf life of formulations may also be extended further by the incorporation of various food bases and nutrients (Lumsden & Lewis, 1989).

The activity of biocontrol fungi can be influenced profoundly by extrinsic factors of the environment (Baker & Scher, 1987). Ideally, a biocontrol fungus should be active over a range of environmental conditions or, at least, in an environment conducive for the target pathogen. It is unfortunate that most biocontrol fungi are only active under particular environmental conditions. Consequently, biocontrol of diseases in environmentally controlled covered structures, such as glasshouses and plastic tunnels, would seem most promising (Whipps,

1986). Conditions in these often coincide with those insuring optimum activity of biocontrol fungi.

The main physical environmental factors affecting the activity of biocontrol fungi include pH and temperature. Biocontrol by Trichoderma spp. is not easily established in alkaline soils, since optimum pH for spore germination and vegetative growth is between pH 5.0 and 6.5 (Sivan et al., 1984; Lewis & Papavizas, 1987). When a biocontrol fungus is selected for use in a particular location, the temperature range of the fungus must be considered. Most Trichoderma spp. have a relatively high optima of 25-30° but develop poorly at low temperatures (Tronsmo & Dennis, 1977; Gladders & Coley-Smith, 1980). In the field Trichoderma spp. may function in warm climates but may be ineffective in cool soils. In this regard, coating pea and radish seed with conidia of T. harzianum did not reduce the incidence of Pythium or Rhizoctonia damping-off at temperatures unfavourable for growth of the biocontrol fungus (Harman, Chet & Baker, 1981). However, at temperatures near the optimum for growth, almost complete disease control was achieved. Similarly, C. minitans develops optimally at 20-25° with significantly slower growth at 15° (Turner & Tribe, 1976; Ayers & Adams, 1981). Thus an application of C. minitans to soil at a low temperature in winter did not reduce sclerotial inoculum of S. sclerotiorum (Trutmann et al., 1980). In another example, the biocontrol activity of P. nunn against P. ultimum-induced damping-off of cucumbers has been shown to be markedly affected by both temperature and pH (Paulitz & Baker, 1987). Disease was significantly reduced at 26° but not at 22 or 17°. Also, disease was reduced at pH 6.7 but not at 5.0 or 6.0. Finally, soil water potential has important

implications for biocontrol (Cook & Baker, 1983) but as yet there has been very little research on the effect of this physical factor on the activity of biocontrol fungi. Future progress on biocontrol of fungal pathogens through ecological manipulations to favour introduced biocontrol fungi will depend on more research within this area.

Once biocontrol fungi have shown potential under field conditions, genetic improvement may be carried out to enhance their efficacy. This may involve conventional genetic crossing of strains when applicable, the inducement of mutations by mutagenic agents or, perhaps, the use of some of the relatively new techniques discovered through advances in molecular biology and genetic engineering. Papavizas, Lewis & Abd-El Moity (1982) induced mutation in Trichoderma spp. with U.V. light and selected strains tolerant to methyl benzimidazole carbamate (MBC) fungicides. Some of the mutants were more effective than the wild types in reducing Rhizoctonia rot of carnation. As mentioned before, mutation and selection of strains of T. harzianum for ability to colonize plant rhizospheres has already been achieved (Ahmad & Baker, 1987a). Genetic recombination through conventional sexual crosses is potentially a much more powerful method for developing superior strains of biocontrol fungi than selection or mutation. Here, strains expressing desirable attributes could be used as parents in crosses with other strains expressing other desirable traits to produce progeny with combinations of desirable traits. Unfortunately, sexual stages are rare or lacking in many biocontrol fungi such as Trichoderma spp., and so conventional crosses cannot be used to genetically manipulate these fungi. However, it has been suggested that it may be possible to combine desirable traits from various parental strains to produce

superior strains of biocontrol fungi using protoplast fusion (Papavizas, 1987; Hocart & Peberdy, 1989). Advances in this area have already been made. Protoplasts obtained from two strains of T. harzianum have been successfully fused and also readily regenerated (Stasz, Harman & Weeden, 1988). Genetic characterization and biocontrol activity of the new strains is currently under investigation.

Many soil-borne diseases are difficult to control. Success in reducing losses from these diseases is likely to depend on the development of an effective integrated control system possibly using biocontrol as one of its components. If biocontrol fungi are to be integrated effectively with fungicides to control single or multiple soil-borne diseases, they must have natural resistance to these fungicides or be manipulated genetically to develop such resistance.

Some of the biocontrol fungi possess natural tolerance to several commercially used fungicides including chloroneb, captan, PCNB and metalaxyl (Abd-El Moity, Papavizas & Shatla, 1982). In this regard, Chet et al. (1979) integrated the use of T. harzianum and low dosages of pentachloronitrobenzene (PCNB) to manage damping-off disease of bean seedlings caused by Sclerotium rolfsii. Other researchers integrated the same two components to manage damping-off of eggplant and radish seedlings caused by R. solani (Henis, Ghaffar & Baker, 1978; Hadar, Chet & Henis, 1979). When Henis et al. (1978) applied wheat-bran cultures of T. harzianum to soil together with PCNB it had an additive effect on disease control. Similarly, application of a T. harzianum bran culture preparation in association with prothiocarb to a commercial nursery rooting mixture infested with R. solani and P.

aphanidermatum prevented damping-off of Gypsophila cuttings to a greater extent than either of the single components (Sivan et al., 1984). Another example of the use of a fungicide together with an introduced biocontrol fungus includes the combination of T. hamatum and the fungicide metalaxyl against Pythium blight on turf (Rasmussen-Dykes & Brown, 1982). Nevertheless, not all biocontrol fungi are tolerant to commonly used fungicides, and almost all of them are very sensitive to the methyl benzimidazole carbamate (MBC) group of fungicides.

As mentioned earlier, there is now considerable interest in applying genome modifications to improve the efficacy of biocontrol fungi and their tolerance to fungicides. Considerable progress has already been made in developing strains of Trichoderma and Talaromyces resistant to MBC fungicides by physical and chemical mutagenesis (Papavizas et al., 1982; Papavizas & Lewis, 1983; Papavizas, 1987).

Integrating biocontrol fungi with fungicides is not the only possible integrated approach to reduce losses from soil-borne plant pathogens. Beneficial interactions may also be obtained with combinations of cultural practices and biocontrol fungi. However, there are very few reports in the literature on integrated control involving introduced biocontrol fungi and other cultural practices such as soil cultivation, steam sterilization or fertilizer application. Some of the few examples are noted here.

Fruit rot (soil rot) caused by R. solani is a serious disease causing heavy losses on cucumber and tomato especially in warm humid areas of the U.S.A.. It has been shown that the use of the biocontrol fungi, Laetisaria arvalis and T. harzianum, could be effectively integrated with mechanical ploughing to reduce disease (Lewis &

Papavizas, 1980). Furthermore, the control obtained was as good as that achieved with commercial fungicides registered for the control of this disease. A benomyl-resistant strain of T. viride applied as conidia to a soil mix following steam sterilization rapidly colonized the mix, prevented reinvasion by the pathogen, and provided control of Fusarium wilt of chrysanthemum (Marois & Locke, 1985). The level of control was equivalent to that obtained with a commercial integrated procedure that involves application of benomyl and fertilizers, but not biocontrol fungi. Glasshouse studies have shown that applications of urea fertilizer combined with a wheat-bran preparation of T. harzianum reduced the viability of sclerotia of Sclerotium rolfsii in soil more effectively than when either component was used alone (Maiti & Sen, 1985).

The final factor to consider is the possible commercial adoption of a biocontrol fungus. Many of the aspects relevant to the commercialization of biocontrol agents are reviewed by Lumsden & Lewis (1989) and Powell & Faull (1989). For industry to become interested in developing any biocontrol agent, it must be clearly demonstrated that biocontrol not only works but that the eventual product can be profitable. One of the commercial advantages of biocontrol agents is that the registration costs relative to chemical pesticides are cheaper.

Biocontrol is usually slow acting compared with the use of pesticides. Consequently, re-education of the user not to expect immediate control may be a major problem once a biocontrol agent has reached the commercial market.

The prospective biocontrol fungus P. oligandrum has been studied by

a number of researchers for several years. It was first described as the cause of pea root-rot in the U.S.A. (Drechsler, 1930) and has since been isolated from mainly subterranean parts of many other plants (Middleton, 1943; Drechsler, 1946). The fact that the fungus was frequently found in association with the aggressive plant pathogens, P. debaryanum and P. ultimum, led Drechsler (1946) to suggest that it is perhaps not a primary plant pathogen, but is, instead, a secondary invader of diseased tissues and partly parasitic on these pathogens. Findings from more recent research have confirmed that P. oligandrum is an aggressive mycoparasite with a wide fungal host range (Deacon & Henry, 1978; Vesely, 1978; Vesely & Hejdanek, 1984; Lutchmeah & Cooke, 1984; Foley & Deacon, 1986a; Whipps, 1987a; Lewis, 1988; Lewis et al., 1989). Laboratory studies involving co-inoculations of P. oligandrum and a range of cellulolytic fungi have shown that both mycoparasitism and nutrient competition can occur (Tribe, 1966; Deacon, 1976; Al-Hamdani & Cooke, 1983). In view of these findings, some researchers have suggested a role for substrate competition in disease control in the soil and spermosphere (Martin & Hancock, 1986, 1987). However, it has been concluded that mycoparasitism is the major mechanism of biocontrol affected by P. oligandrum (Lewis, 1988; Lewis et al., 1990). In addition, findings from an in vitro study have shown the ability of the fungus to exhibit directed growth towards potential host fungi, secrete cell wall degrading enzymes ( $\beta$ -1, 3-D-glucanase, lipase and protease) and to produce some inhibitory metabolites (Lewis, 1988).

P. oligandrum has shown biocontrol activity against a number of important soil-borne plant pathogens in small-scale experiments under controlled as well as field conditions. Oospore preparations applied

either as seed treatments or directly to sowing sites have been shown to reduce the incidence of Pythium-induced damping-off in cacti, cress, cucumber and sugar beet, M. acerina on carrot and Phoma betae in sugar beet (Vesely, 1977, 1979; Al-Hamdani, Lutchmeah & Cooke, 1983; Starling & Butler, 1983; Vesely & Hejdanek, 1984; Lutchmeah & Cooke, 1985; Martin & Hancock, 1987; Walther & Gindrat, 1987a; Thinggaard, Larsen & Hockenhull, 1988).

Though there are a number of reports on the biocontrol potential of P. oligandrum, the information is fragmentary as no systematic attempt appears to have been made to elucidate in detail the biocontrol attributes of the antagonist. In view of this, the present investigation was carried out to identify nutritional and environmental factors favouring growth, oospore production and germination. The study also emphasized the development of a suitable technique for bulk production of oospores and their formulation for use against pathogens causing damping-off of cress. The survival of the different oospore formulations was also monitored. Finally, seeds of cress and sugar beet were coated with oospores using commercial seed-pelleting and film-coating procedures, and tested against a number of soil-borne plant pathogens.

CHAPTER II

ORIGIN AND MAINTENANCE OF CULTURES

## CHAPTER II

### ORIGIN AND MAINTENANCE OF CULTURES

Details of the fungi used during this study and their origin are listed in Tables 1 and 2. All fungi were placed in ampoules made from polypropylene drinking straws and were stored in liquid nitrogen in the IHR (Littlehampton) culture collection according to the method described by Challen & Elliott (1986).

For experiments Pythium spp. were maintained on Oxoid cornmeal agar (CMA) and Rhizoctonia solani on Oxoid potato dextrose agar (PDA) at 25°C in darkness. Both agar media were prepared according to the manufacturer's instructions (Anon, 1982) and were dispensed in c. 18 ml aliquots into 9 cm diam Petri dishes.

All solid and liquid media utilized were autoclaved at 120° and 103.4 kPa for 15 min prior to use unless stated otherwise.

Table 1

Origin of Pythium oligandrum Drechsler isolates

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IMI number	IHR culture collection number	Source
133857	R228	<u>Nasturtium</u> sp. seedlings, in soil studies, Birmingham
-	R331/MPMP05	Garden, South Yorkshire (17Ga)*
-	R333/MPMP04	Garden, Surrey, site 2 (16Gii)
-	R334/MPMP03	Agricultural soil, Derbyshire, site 3 (3A3)
-	R335/MPMP02	Garden, Surrey, site 1 (15Gi)
-	R336/MPMP01	Agricultural soil, North Yorkshire, site 2 (7Aii)

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\* Isolates followed by a code number in parentheses were isolated by Lewis (1988).

Table 2

Origin of cultures

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Species	IHR culture collection number	Source
<u>Pythium aphanidermatum</u> (Edson) Fitzpatrick	R330	Dr. G. M. McPherson, ADAS, Leeds. Cucumber ( <u>Cucumis</u> <u>sativus</u> L.) root grown in rockwool at Stockbridge House, EHS, Selby
<u>Pythium ultimum</u> Trow	R329/MPM02	Damped-off cress ( <u>Lepidium sativum</u> L.) seedlings grown in soil from Sheffield University Experimental Garden
<u>Pythium ultimum</u> Trow	R328/MPM01	Damped-off sugar beet ( <u>Beta vulgaris</u> L.) seedlings grown in soil from Broom's Barn Experimental Station
<u>Rhizoctonia solani</u> Kühn Anastomosis group 2, type 1	R230(RS21)	Professor J. R. Coley-Smith, Hull, isolated from lettuce ( <u>Lactuca sativa</u> L.)

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CHAPTER III

NUTRITIONAL AND ENVIRONMENTAL FACTORS  
AFFECTING GROWTH

## CHAPTER III

### NUTRITIONAL AND ENVIRONMENTAL FACTORS

#### AFFECTING GROWTH

The use of P. oligandrum as a biocontrol agent against a number of important soil-borne plant pathogens, both in the laboratory and in the field, has already been highlighted. Biological treatments with this antagonist have, in the past, not usually performed as quickly and efficiently as their fungicide-chemical counterparts (Vesely, 1979; Martin & Hancock, 1987). This may in part be due to a lack in the understanding as to how ecological, environmental and nutritional factors affect the growth and biocontrol efficiency of the antagonist under natural conditions. Environmental factors such as fluctuations in pH, temperature, and water potential can all play a major role in restricting the activity of biocontrol agents (Cook & Baker, 1983). There is also a need to determine the nutritional and environmental requirements of P. oligandrum to enable optimal production of inoculum using novel growth media for use in the field. Furthermore, there is a general lack of information on the nutritional requirements of P. oligandrum . Only with a greater understanding of the nutrition of P. oligandrum and its interaction with the environmental conditions can

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further advances in its use be made.

Various nutritional aspects of P. oligandrum were therefore studied together with the effect of different temperatures, pH and water potentials on growth. Also it was considered that such information would be of use in the development of large-scale production techniques for biocontrol inoculum. The results obtained should be treated with caution as it is a well known fact that temperature, water potential, pH and other factors optimal for growth in vitro are often considerably different from those most favourable in nature.

### 3.1 Growth in a defined liquid medium

A preliminary time-course study in a defined liquid medium was carried out to determine a suitable harvest time for use in further experiments. Biomass production in relation to incubation period was followed using a basal medium containing (per l) 50 mg  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 30 mg cholesterol (1.5% w/v solution in 95% v/v ethanol), 1 mg  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ , 1.23 g  $\text{KH}_2\text{PO}_4$ , 170 mg  $\text{K}_2\text{HPO}_4$ , 0.4 mg  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$  and 0.1 mg thiamine hydrochloride. To this was added L-asparagine (1.5 g) and D-glucose (10 g). Batches of basal medium, L-asparagine and D-glucose were combined after autoclaving separately. Thiamine hydrochloride was added after filter-sterilization through a 0.2  $\mu\text{m}$  Whatman cellulose nitrate filter. Medium was adjusted to pH 6.5 with sterile N-NaOH and was dispensed in 20 ml aliquots into sterile 100 ml Erlenmeyer flasks. Each flask was then inoculated with a 5 mm diam mycelial disc cut from a 2-d-old colony of P. oligandrum (IMI 133857) on CMA and incubated at 25°. This isolate was used in all further experiments unless stated otherwise. At 3 d intervals over a 3 wk period, four flasks were

removed and biomass harvested by vacuum filtration using pre-weighed, oven-dried Whatman GF/A filter paper (5 cm diam). Dry weights were determined after oven-drying at 60° for 36 h. Treatment variances and those in all subsequent experiments were tested for equality by the Fmax test (Parker, 1979) and the analysis of variance performed where appropriate.

Results are shown in Fig. 1. and Appendix 1.1. Biomass production increased over the incubation period to reach a maximum at 15 d, after which time yields gradually declined. On the basis of these results, all cultures grown in this defined medium in further experiments were harvested after 12 d incubation.

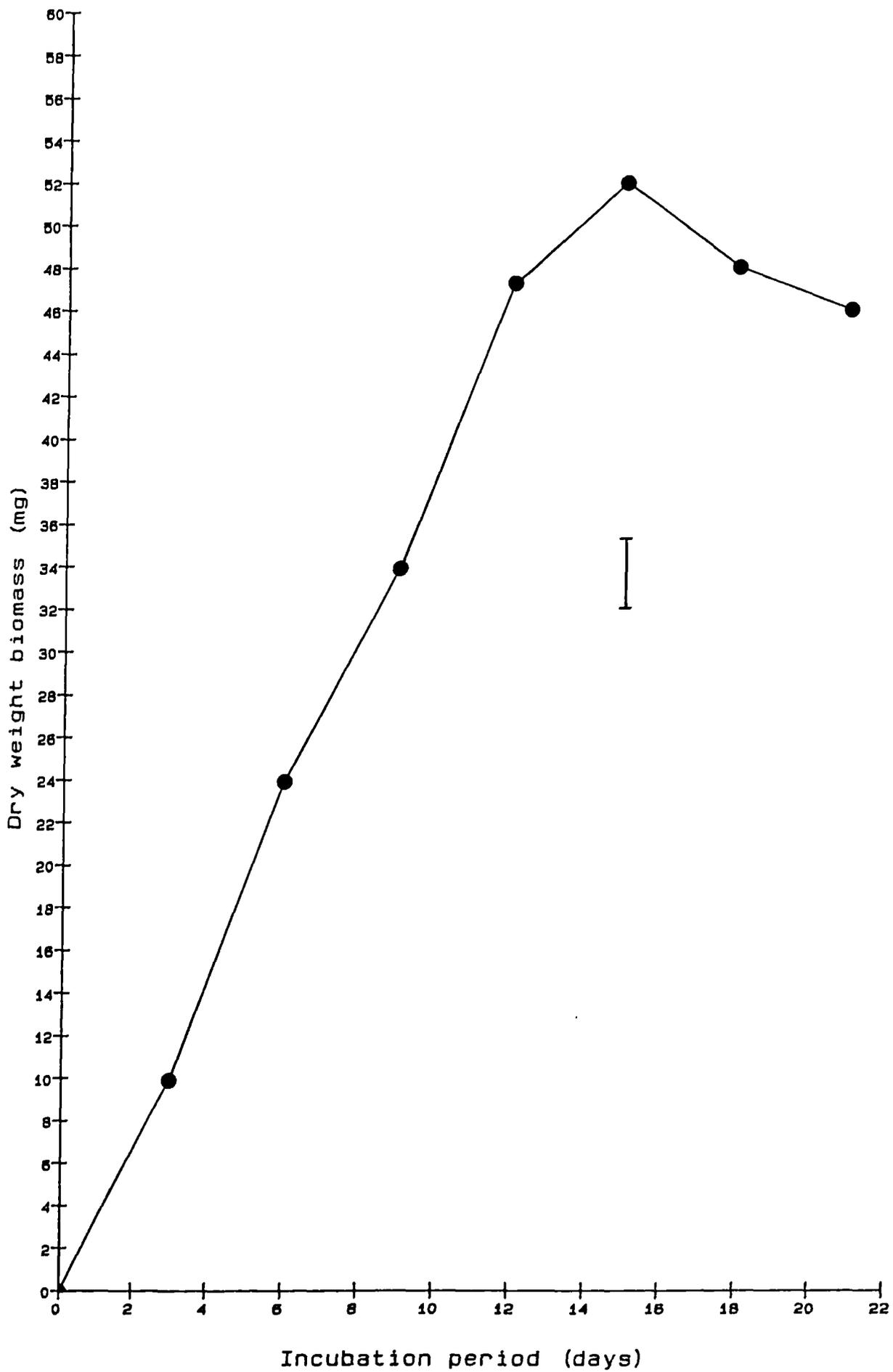
### 3.2 Effect of carbon sources on growth

Apart from a study reported by Foley & Deacon (1986b) there is little information available in the literature on the influence of carbon source on growth of P. oligandrum. In view of this, the ability of the fungus to utilize a range of carbon sources was tested in a basal medium with L-asparagine as the nitrogen source. The different carbon sources tested were:-

1. Monosaccharides and related compounds
  - i. Hexoses  
D-Glucose, L-Rhamnose
  - ii. Pentoses  
D-Arabinose, D-Xylose
  - iii. Sugar alcohols  
D-Mannitol, Sorbitol, myo-Inositol
2. Oligosaccharides

Figure 1

Effect of incubation period on the biomass yield (mg) of Pythium oligandrum in glucose-asparagine liquid medium at 25°. Points are mean values of four replicates. For SEM, see Appendix 1.1. The bar indicates the least significant difference at P=0.05.



i. Disaccharides

Sucrose, Lactose, Maltose, Trehalose

ii. Trisaccharide

Raffinose

3. Polysaccharides

Cellulose (Whatman microgranular cellulose powder)

Starch (soluble), Dextrin

Autoclaved basal medium (as in 3.1) was supplemented with sterile L-asparagine (1.5 g/l). Carbon sources were autoclaved separately and added individually to batches of sterile medium, except for oligosaccharides which were added to medium after filter-sterilization. The total amount of carbon added in each case was 4 g/l. Control medium consisted of basal medium plus L-asparagine. Media were adjusted to pH 6.5 with sterile N-NaOH or N-HCl and each was dispensed in 20 ml aliquots into four replicate 100 ml Erlenmeyer flasks. General procedures were the same as for those in the previous experiment, all cultures being harvested after 12 d incubation.

Results are presented in Table 3. The control medium alone supported some growth, so that the criterion for the utilization of a carbon source was that it supported more growth than the control medium. D-glucose, maltose and trehalose were all utilized and supported substantial growth. Biomass yields on D-glucose and maltose were comparable, but both were greater than on trehalose. The remainder of the carbon sources tested were utilized very poorly or not at all.

Table 3

Effect of various carbon sources on the growth of Pythium oligandrum in a basal liquid medium containing L-asparagine as nitrogen source. Each figure is the mean of four replicates with SEM.

Carbon sources	Dry weight biomass* (mg)
Control medium	5.8±0.5
Monosaccharides	
D-Glucose	54.5±1.9
D-Arabinose	10.0±0.4
L-Rhamnose	6.8±0.6
D-Xylose	10.0±1.2
Dissaccharides	
Sucrose	12.8±0.5
Lactose	12.2±0.9
Maltose	54.2±1.5
Trehalose	30.5±1.3
Trisaccharide	
Raffinose	7.8±0.8
Polysaccharides	
Cellulose	5.5±0.6
Starch (soluble)	15.2±0.5
Dextrin	14.8±0.8
Alcohols	
D-Mannitol	13.5±0.6
Sorbitol	9.2±0.8
<u>myo</u> -Inositol	9.0±0.7
LSD (P=0.05)	2.7

\* Dry weight biomass after 12 d incubation at 25°.

### 3.3 Effect of nitrogen sources on growth

Previous investigations on the ability of P. oligandrum to utilize nitrogen compounds are few in number and lacking in quantitative results (Leonian & Lilly, 1938; Foley & Deacon, 1986b). It was therefore thought to be useful to compare growth on a range of individual nitrogen sources.

The following compounds, incorporating inorganic and organic nitrogen, were tested as sole nitrogen sources:-

#### 1. Inorganic nitrogen

Ammonium nitrate, Potassium nitrate

#### 2. Organic nitrogen

##### i. Simple sources

L-Asparagine, L-Arginine, L-Aspartic acid

L-Glutamic acid, L-Alanine, Glycine

L-Isoleucine, L-Phenylalanine, L-Proline

L-Serine, L-Valine, Urea

##### ii. Complex sources

Waste yeast (King & Barnes Brewery, Horsham,  
W.Sussex)

Autolysed yeast (The Distillers Co., Surrey)

Mycological peptone (Oxoid)

Malt extract (Oxoid)

Autoclaved basal medium (as in 3.1) was supplemented with sterile D-glucose (10 g/l). All inorganic and simple organic sources were autoclaved separately and added to batches of sterile medium to give a nitrogen level of 0.28 g/l; sterile complex organic sources being added at a rate of 2 g/l. As urea is heat-labile it was added after filter-sterilization. Control medium consisted of basal medium plus D-glucose, and general procedures were the same as those in previous experiments.

Results are presented in Table 4. A nitrogen source was considered utilized if it supported more growth than the control medium. Inorganic nitrogen in the form of ammonium nitrate supported very poor growth whilst potassium nitrate was not utilized. All simple organic sources apart from urea were utilized, but to varying degrees. Growth on L-asparagine, L-arginine and L-aspartic acid was substantial compared to the others. Glycine, L-alanine and L-phenylalanine were utilized moderately, whereas growth on the remainder was fair. All complex media tested were utilized, and yields were particularly high with mycological peptone and autolysed yeast. Yields on waste yeast and malt extract were moderate and similar to one another.

#### 3.4 Effect of carbon-nitrogen ratio on growth

The effect of carbon-nitrogen (C:N) ratio on growth was studied using basal medium (as in 3.1) supplemented with different amounts of D-glucose and L-asparagine. The C:N ratio in the medium was varied in two ways. Firstly, D-glucose was provided at a fixed initial level (10 g/l) and the concentration of L-asparagine adjusted in accordance with predetermined C:N ratios of 5:1, 9:1, 16:1, 30:1, 60:1, 90:1 and 130:1.

Table 4

Effect of various nitrogen sources on the growth of Pythium oligandrum in a basal liquid medium containing D-glucose as carbon source. Each figure is the mean of four replicates with SEM.

Nitrogen sources	Dry weight biomass*(mg)
Control medium	9.5±0.6
Inorganic	
Potassium nitrate	7.8±0.5
Ammonium nitrate	19.5±1.6
Organic	
1. Simple sources	
L-Asparagine	53.2±3.1
L-Arginine	49.2±2.3
L-Aspartic acid	49.8±1.4
L-Glutamic acid	47.0±1.1
L-Alanine	37.5±1.3
Glycine	32.8±1.4
L-Isoleucine	24.5±1.6
L-Phenylalanine	37.8±1.3
L-Proline	29.8±1.2
L-Serine	26.8±1.0
L-Valine	26.0±1.3
Urea	10.2±0.9
2. Complex sources	
Waste Yeast	25.0±1.7
Autolysed Yeast	44.2±1.9
Mycological Peptone	47.5±1.6
Malt Extract	22.8±0.8
LSD (P=0.05)	4.2

\* Dry weight biomass after 12 d incubation at 25°

Secondly, L-asparagine was provided at a fixed level (1.5 g/l) and the D-glucose concentration varied independently to produce the same range of C:N ratios. In calculating the C:N ratios, allowance for the carbon content of L-asparagine was made. Batches of sterile basal medium and appropriate concentrations of sterile D-glucose and L-asparagine were combined to give the required C:N ratios. General procedures and incubation time were the same as those in the previous experiment.

Biomass yields are shown in Table 5. With the L-asparagine constant yield increased significantly with increasing C:N ratios (or carbon content) up to 60:1. Thereafter, yields declined with further ratio increases, but growth was not inhibited. A different response occurred when D-glucose was constant. Low ratios supported greatest growth, whilst an increase in the ratio (or decline in nitrogen content) significantly decreased yields. Yields in both circumstances showed a direct relationship between nitrogen and carbon content. With an increase in either source, yields increased until conditions for biomass production were optimum.

### 3.5 The effect of pH on growth

Growth, as well as other metabolic activities, is affected by the pH of the medium employed. The pH range and optimum pH for growth on liquid and solid media were therefore determined.

#### 1. Liquid medium

The effect of pH on biomass production was investigated using glucose-asparagine medium (as in 3.1). Double-strength medium was buffered over the range of pH 4.0 to 7.0 and pH 7.5 to 9.5 with the two

Table 5

Effect of variation of the carbon-nitrogen ratio in glucose-asparagine liquid medium on the growth of Pythium oligandrum.

Each figure is the mean of four replicates with SEM.

C:N ratio	L- Asparagine constant		D- Glucose constant	
	Content of C (g/l)	Dry weight biomass (mg)*	Content of N (g/l)	Dry weight biomass (mg)*
5:1	1.40	19.8±1.8	1.21	58.6±0.8
9:1	2.52	29.8±1.5	0.54	57.5±1.7
16:1	4.48	51.8±2.4	0.28	47.0±1.8
30:1	8.40	69.9±3.1	0.14	40.3±1.7
60:1	16.80	87.8±2.6	0.07	33.4±2.0
90:1	25.20	62.4±1.5	0.05	25.1±1.8
130:1	36.40	47.2±1.8	0.03	19.3±1.0
LSD (P=0.05)			5.5	

\* Dry weight biomass after 12 d incubation at 25°

buffers citrate-phosphate (0.05 M citric acid, 0.1 M  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$  and tris(hydroxymethyl)aminoethane (0.1 M Tris, 0.1 M HCl) respectively. There was a 0.5 unit increment between each level. Buffer solutions were prepared to the required pH by varying the proportions of the two components according to Gomori (1955). Batches of medium were adjusted with N-NaOH or N-HCl to the appropriate pH and an equal volume of buffer added to give the correct concentration of medium. Medium pH was then determined with a Whatman PHA 250 pH meter. Individual batches of buffered medium were filter-sterilized and 20 ml aliquots dispensed aseptically into four replicate sterile 100 ml Erlenmeyer flasks. A set of flasks with unbuffered medium of pH 5.1 was included as controls. General procedures were the same as those in previous experiments with the final pH values of media being determined on harvest.

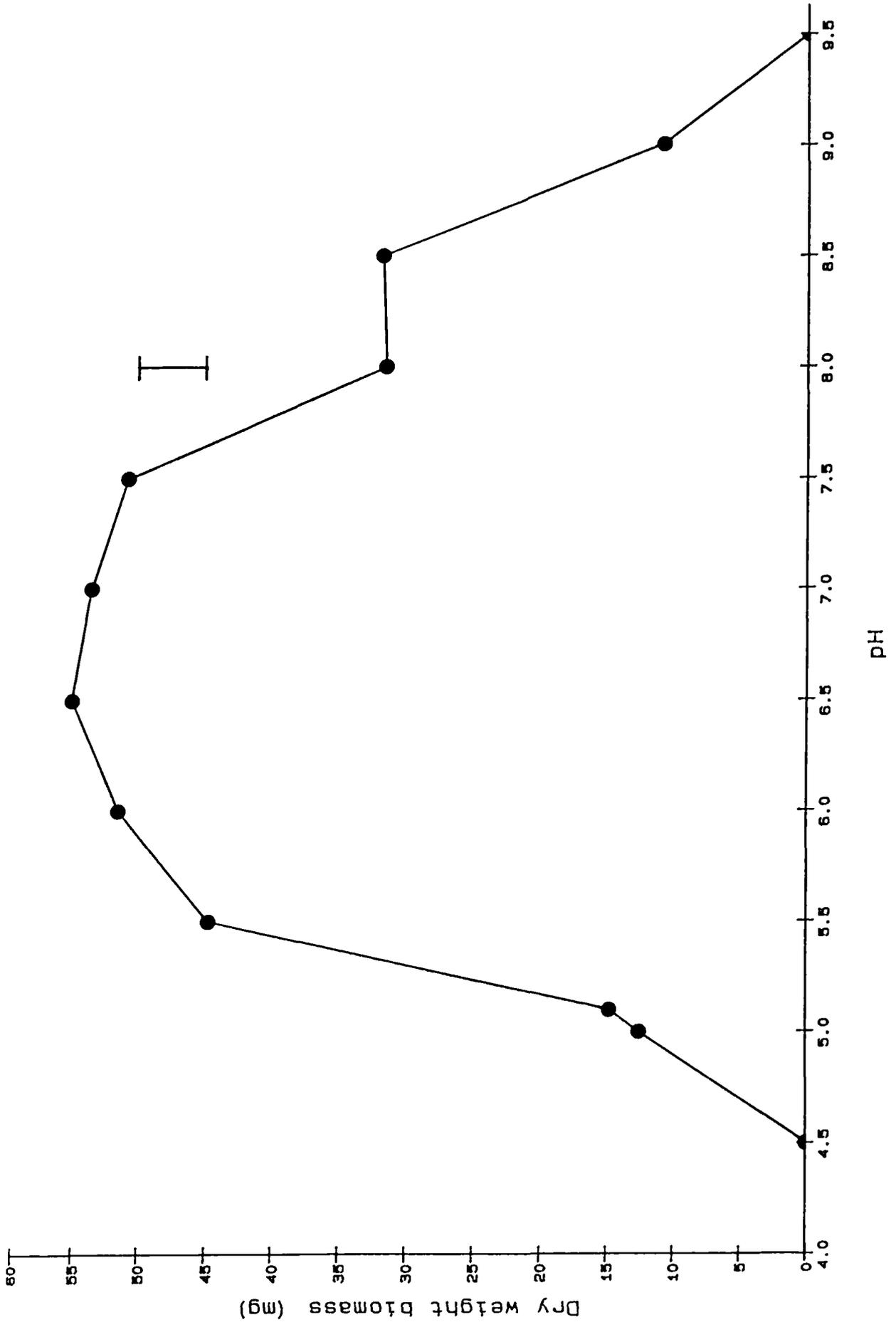
Biomass dry weights are shown in Fig. 2 and Appendix 1.2. Growth occurred over the range of pH 5.0 to 9.0, whilst the optimum range for biomass production was between pH 6.0 and 7.5. Biomass production was adversely affected by either extreme acidic or alkaline conditions and growth was inhibited at pH 4.5 and 9.5. The pH of buffered media was generally maintained during the growth period and only varied by 0.2 to 0.4 units. The pH of unbuffered control medium rose by almost one unit.

## 2. Solid media

The effect of pH on radial growth rates of colonies was investigated using Oxoid CMA and tap water agar (TWA; 15 g no. 3 Oxoid agar in 1-l tap water). Both media were prepared at twice their normal strength and autoclaved. Batches of sterile medium were buffered at different

Figure 2

Effect of pH on the biomass yield (mg) of Pythium oligandrum in glucose-asparagine liquid medium after 12 d incubation at 25°. Points are mean values of four replicates. For SEM, see Appendix 1.2. The bar indicates the least significant difference at P=0.05.



pH levels (pH 4.0 to 9.0) with citrate-phosphate and tris(hydroxymethyl)aminoethane buffers as described before. Buffers were autoclaved separately and added at 60° to an equal volume of molten medium. Batches of medium were then dispensed in 15 ml aliquots into four replicate Petri dishes (9 cm diam). Dishes were each inoculated centrally with a 3 mm diam disc cut from the periphery of a 2-d-old colony. Controls consisted of unbuffered CMA (pH 5.8) and TWA (pH 7.2). Radial growth rates were determined by measuring colony diameters along two axes at right angles at 12 h intervals over a 36 h period. No measurements were made of the final pH of the media.

Colony radial growth rates (mm/d) are shown in Fig. 3 and Appendix 1.3. Growth on CMA and TWA occurred over the range pH 4.4 to 7.9 and pH 4.9 to 8.1 respectively. The optimum pH for growth on both media was between pH 6.0 and 7.0, but growth rates over this range were slightly greater on CMA than TWA.

### 3.6 Effect of temperature on growth

The effect of temperature on growth was assessed at 5, 10, 15, 20, 25, 30, 35 and 40° using liquid and solid media. Growth of five isolates was also compared in a non-defined liquid medium.

#### 1. Liquid medium

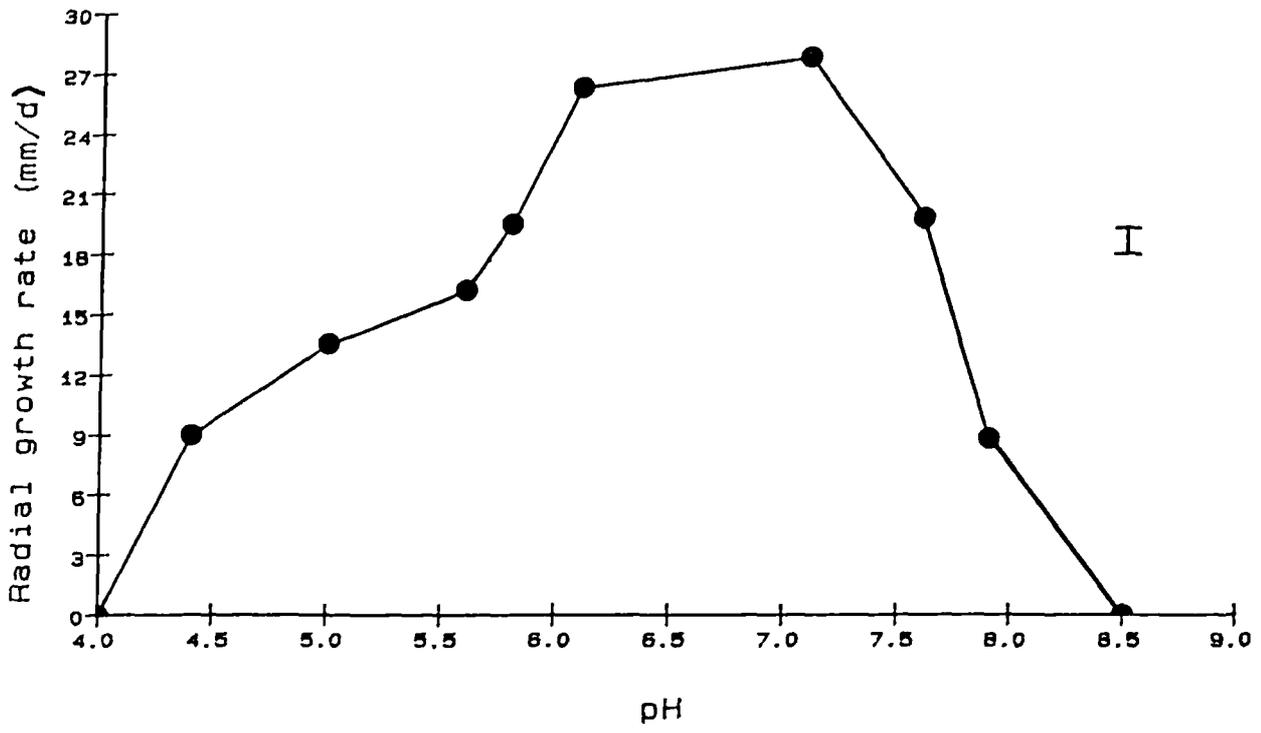
Aliquots (20 ml) of glucose-asparagine medium, pH 6.5, (as in 3.1) were dispensed aseptically into sterile 100 ml Erlenmeyer flasks. Flasks were inoculated and four replicates incubated at each temperature. Biomass dry weights were determined at 12 d as described for previous experiments.

The results are shown in Fig. 4 and Appendix 1.4. The optimum

Figure 3

Colony radial growth rates (mm/d) of Pythium oligandrum on solid media of different pH at 25°. Points are mean values of four replicates. For SEM, see Appendix 1.3. The bars indicate the least significant differences at P=0.05.

CMA



TWA

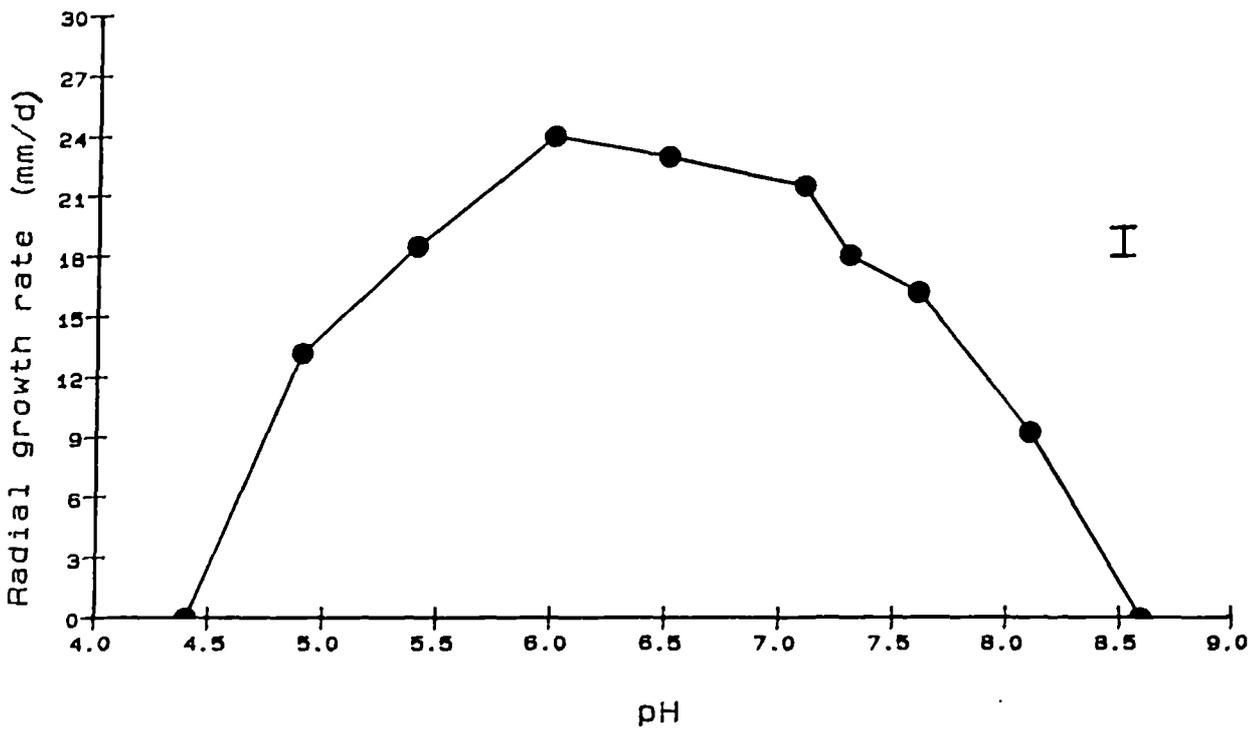
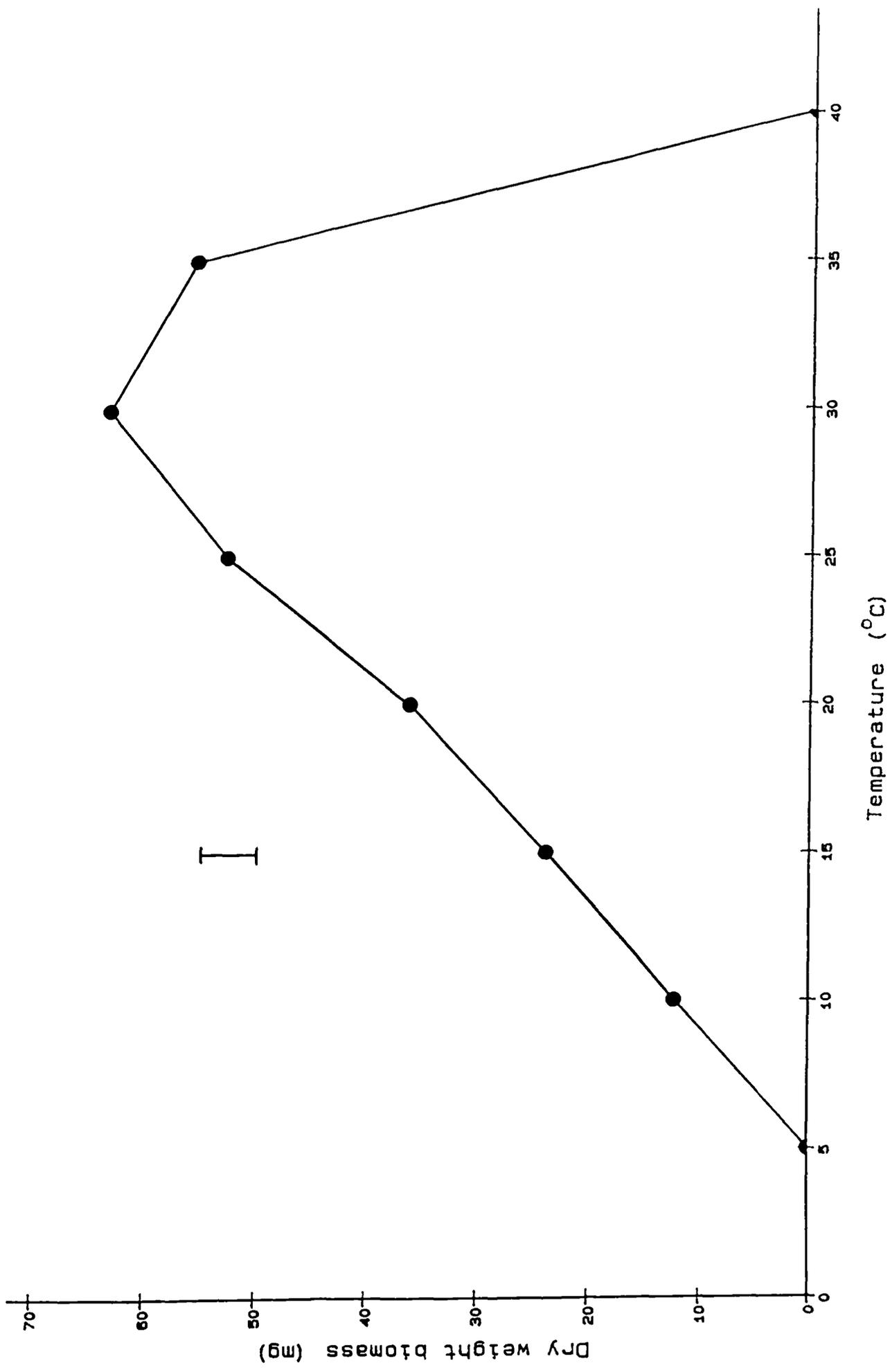


Figure 4

Effect of temperature on the biomass yield (mg) of Pythium oligandrum in glucose-asparagine liquid medium after 12 d incubation. Points are mean values of four replicates. For SEM, see Appendix 1.4 The bar indicates the least significant difference at P=0.05.



temperature for biomass production was 30°. P. oligandrum grew well at 25 and 35°, but biomass yields were less than at 30°. The maximum and minimum temperatures for growth were 35 and 10° respectively.

Growth of five isolates over a range of temperatures was compared using soil extract broth. Details of the isolates used are stated in Table 1 (Chapter II). Soil extract was prepared by modification of the method reported by Ayers & Lumsden (1975). A 100 g sample of Brickearth soil (silt loam type, Hamble series) was added to 1-l distilled water and left to stand at room temperature (c. 22°) for 3 d. The material was then centrifuged at 1200 g for 30 min and the supernatant removed and used to prepare soil extract broth. Aliquots (250 ml) of extract were made up to 1-l with tap water and supplemented with the following nutrients (per l); 1 g L-asparagine, 1 g D-glucose, 1 g K<sub>2</sub>HPO<sub>4</sub>, 0.5 g MgSO<sub>4</sub>.7H<sub>2</sub>O, 0.1 g NaCl and 1 g yeast extract (Oxoid). Medium pH was adjusted to 6.8 with N-HCl and aliquots (20 ml) dispensed into 100 ml Erlenmeyer flasks prior to autoclaving. Medium pH was 6.9 after autoclaving. Batches of flasks were inoculated using a 5 mm diam agar disc of each isolate cut from a 2-d-old colony on CMA. Four replicate flasks of each isolate were inoculated at each test temperature. Cultures were harvested at 10 d and biomass dry weights determined as described in previous experiments.

The results are shown in Table 6. Individual isolates exhibited similar growth responses over the range of temperatures tested. Growth of all isolates was inhibited at 5 and 40°. The optimum temperature range for most isolates was between 20 and 25°. Isolate MPMP05 had a temperature optimum lower than the other four isolates. At 10° biomass yields of isolate IMI 133857 were significantly lower than those of the

Table 6

Biomass dry weight yield (mg) of five Pythium oligandrum isolates after 10 d incubation at different temperatures in soil extract broth. Each figure is the mean of four replicates with SEM.

Isolate	Temperature (°c)									
	5	10	15	20	25	30	35	40		
IMI 133857	N.G.*	8.8±0.3	12.6±0.4	16.2±0.3	18.7±0.6	15.9±0.6	14.1±0.2	N.G.		
MPMP02	N.G.	12.9±0.3	16.6±0.3	18.0±0.4	20.6±0.4	17.3±0.9	12.6±0.6	N.G.		
MPMP03	N.G.	12.4±0.4	14.3±0.6	17.2±0.2	18.5±0.4	15.1±0.5	13.0±0.4	N.G.		
MPMP04	N.G.	10.5±0.5	16.1±0.6	18.8±0.2	18.5±0.2	15.1±0.6	10.8±0.8	N.G.		
MPMP05	N.G.	10.0±1.0	18.9±0.5	19.8±0.4	17.1±1.4	15.4±1.0	10.6±0.3	N.G.		
LSD (P=0.05)									1.5	

\* N.G. - No growth.

other isolates.

## 2. Solid media

Radial growth rates at each temperature were determined on Oxoid CMA (pH 5.8), Oxoid PDA (pH 5.7), TWA (pH 7.2) and soil extract agar (SEA) in 9 cm diam Petri dishes as described in section 3.5. SEA was prepared by dissolving 15 g agar (no. 3 Oxoid) in 1-l of soil extract broth whilst all other media were prepared as before. The initial pH of SEA was 6.9.

Results are shown in Table 7. Growth was inhibited at 5 and 40° on most media tested, but on PDA there was no growth below 15°. Maximum growth rates were between 25 and 30° on all media. Growth rates generally increased with nutrient status of the media, but were comparatively slow on PDA.

## 3.7 Effect of water potential on growth of *Pythium oligandrum* and some target pathogens

The importance of substratum water potential on fungal growth is clearly recognized (Griffin, 1981b). Although there have been in vitro studies on the effect of water potential on the growth and interactions of some major fungal antagonists (Whipps & Magan, 1987; Magan & Whipps, 1988) there is still no information on the effect of water potential on growth of *P. oligandrum*. Since water potential has important implications for biocontrol (Cook & Baker, 1983) a greater understanding of the effects of water potential on the growth of *P. oligandrum*, and some target pathogens, would allow a more rational approach to be taken for the exploitation of this antagonist. The

Table 7

Effect of temperature on colony radial growth rates (mm/d) of Pythium oligandrum on different solid agar media.

Each figure is the mean of four replicates with SEM.

---

Temperature(°C)	Medium			
	TWA	SEA	CMA	PDA
5	N.G.*	N.G.	N.G.	N.G.
10	3.0±0.4	7.0±0.4	4.8±0.5	N.G.
15	8.5±0.3	11.5±1.0	6.5±0.6	3.0±0.4
20	11.2±0.5	17.5±0.6	14.5±0.3	6.5±0.3
25	18.8±0.8	24.0±0.4	20.0±0.4	9.5±0.5
30	22.2±0.5	21.8±0.6	25.0±0.4	13.5±0.3
35	10.5±0.6	18.2±0.5	17.8±0.5	6.0±0.4
40	N.G.	N.G.	N.G.	N.G.
LSD (P=0.05)			1.4	

---

\* N.G. - No growth.

effects of osmotic and matric potential on the growth of P. oligandrum were therefore determined by assessing growth rates and biomass production on media with modified water potential. Growth of P. oligandrum and two target pathogens, P. aphanidermatum and P. ultimum, was also compared at different water potentials.

### 1. Osmotic potential

Solid and liquid media of different osmotic potentials were prepared by the addition of appropriate amounts of solutes: NaCl (Lang, 1967) KCl (Campbell & Gardner, 1971) and glycerol (Dallyn & Fox, 1980). The water potentials of unmodified media were determined psychrometrically (Appendix 1.5) and the potential of modified media calculated as the sum of media plus solute osmotic potentials. Representative samples of the media were checked with a 100 channel automated thermocouple psychrometer (Stevens & Alcock, 1976).

#### i. Liquid media

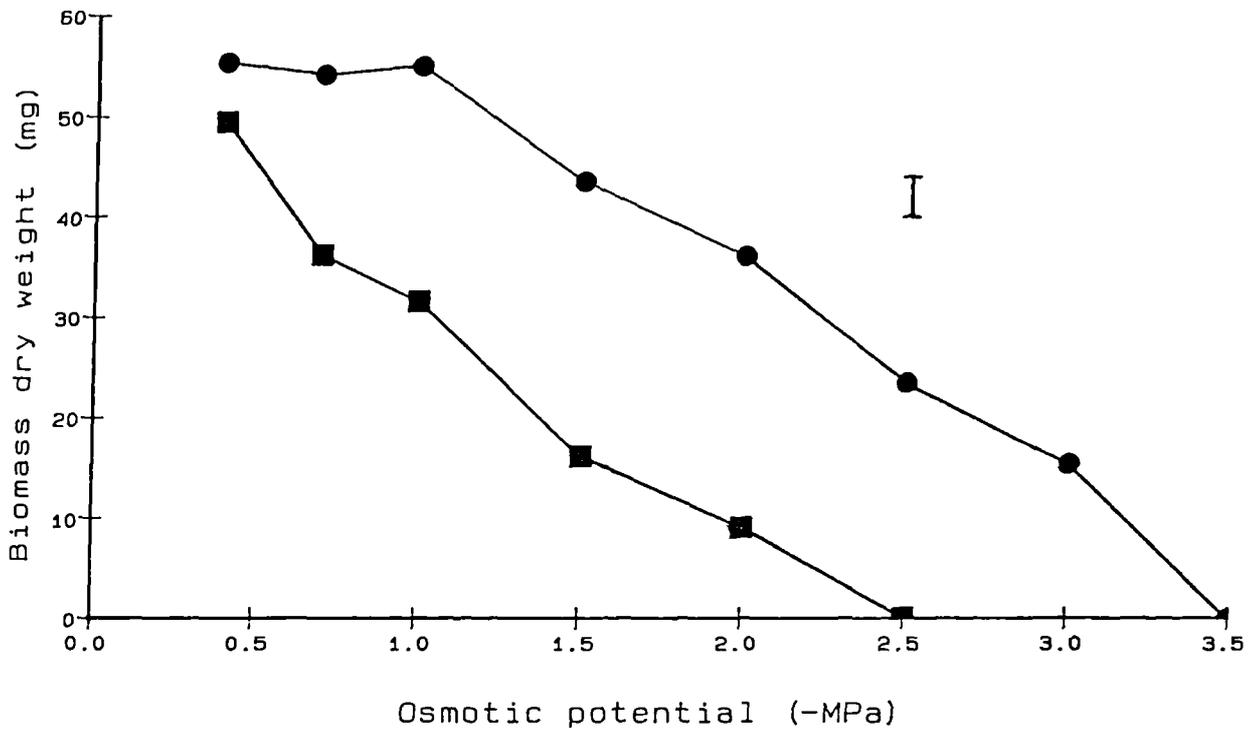
V8 juice broth (20 ml V8 juice, Campbells Soups Ltd., 80 ml water) and glucose-asparagine liquid medium were osmotically maintained over the range -0.2 to -3.5 MPa with the electrolyte NaCl or non-electrolyte glycerol (see Appendix 1.6a). Liquid media adjusted to each osmotic potential were dispensed in 20 ml aliquots into four replicate 100 ml Erlenmeyer flasks. Flasks were inoculated with a standard 5 mm diam agar disc cut from the periphery of a 2-d-old colony of P. oligandrum and incubated at 25° for 12 d after which time biomass dry weight yields were determined.

For results, see Fig. 5 and Appendix 1.6b. Growth in both liquid media generally decreased with decreasing osmotic potential, biomass

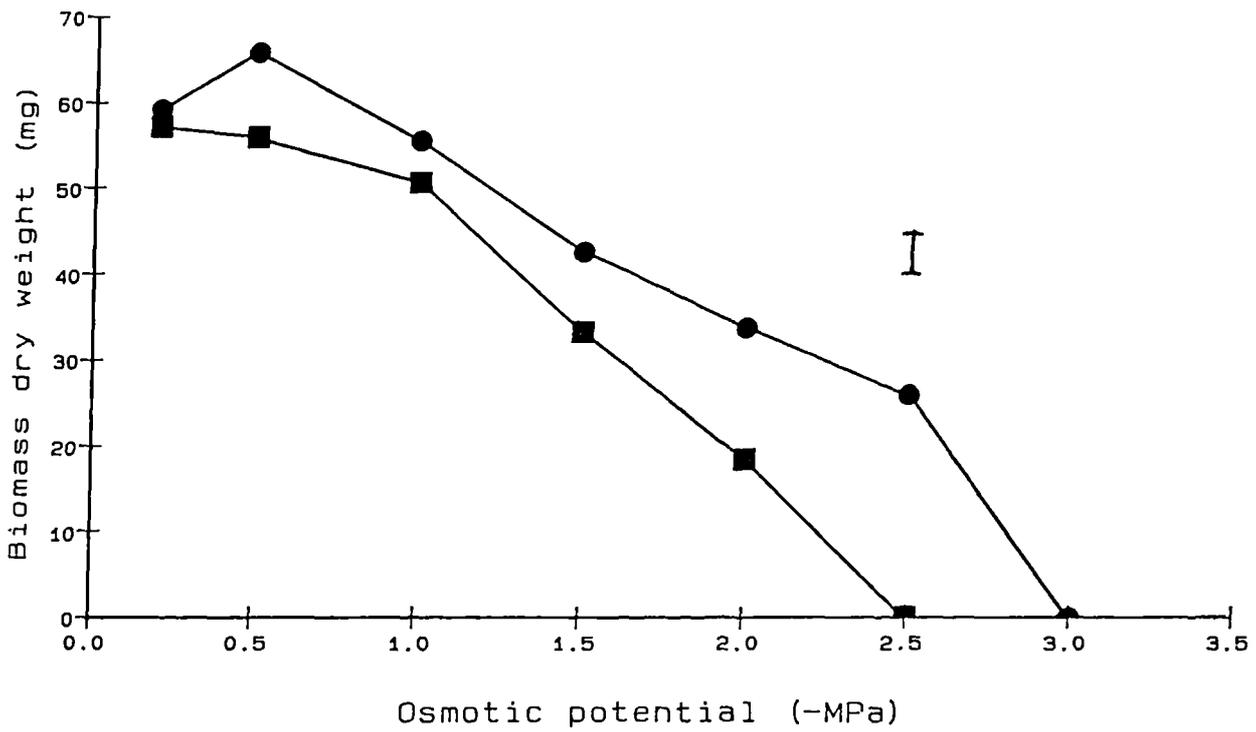
Figure 5

Effect of osmotic potential, modified with NaCl (●) and glycerol (■) on the biomass yield (mg) of Pythium oligandrum in liquid media after 12 d incubation at 25°. Points are mean values of four replicates. For SEM, see Appendix 1.6b. The bars indicate the least significant differences at  $P=0.05$ .

Glucose - asparagine



V8 juice broth



production being reduced markedly below -1.0 to -1.5 MPa, and ceasing altogether at -2.5 to -3.5 MPa, depending upon the media and osmoticum used. However, in V8 juice modified with NaCl there was a small stimulation of growth as the osmotic potential was decreased slightly, but reduction and final cessation of growth occurred with a further decrease in osmotic potential. At most osmotic potentials biomass production was greater on NaCl than the glycerol-modified media. Furthermore, media modified with glycerol also inhibited growth at higher osmotic potentials than media modified with NaCl. For example, growth on glucose-asparagine modified with NaCl was moderate at -2.5 MPa whilst with glycerol it was inhibited.

ii. Solid media

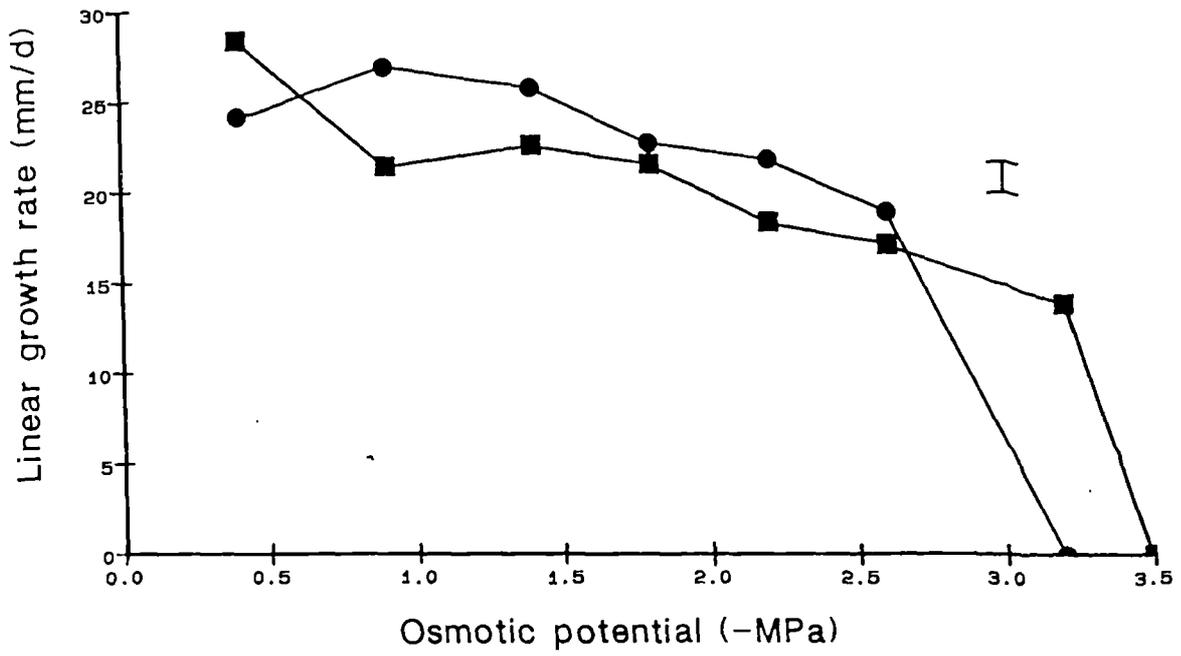
Glucose-asparagine agar (GAA) (1-1 glucose-asparagine (as in 3.1)), 15 g agar, no. 3 Oxoid) and CMA were osmotically maintained with the electrolytes NaCl and KCl over the range -0.4 to -3.5 MPa (see Appendix 1.7a). Sterile media were dispensed in 50 ml aliquots to a series of growth tubes, replication being four-fold. These were inoculated at one end with a 3 mm diam agar disc of P. oligandrum and incubated in the dark at 25°. Each tube was plugged at both ends with cotton wool which were then covered with foil. Water loss was prevented by pouring cooled agar and wrapping growth tube ends in catering grade 'Cling-film'. Mycelial growth on the surface of the agar in each tube was measured at 24 h intervals for 5 d from the margin of the inoculum disc using self-adhesive Scalafix scales (Philip Harris Ltd.). Linear growth rates were then calculated.

Results are shown in Fig. 6 and Appendix 1.7b. Linear growth rates on both solid media decreased with decreasing substratum osmotic

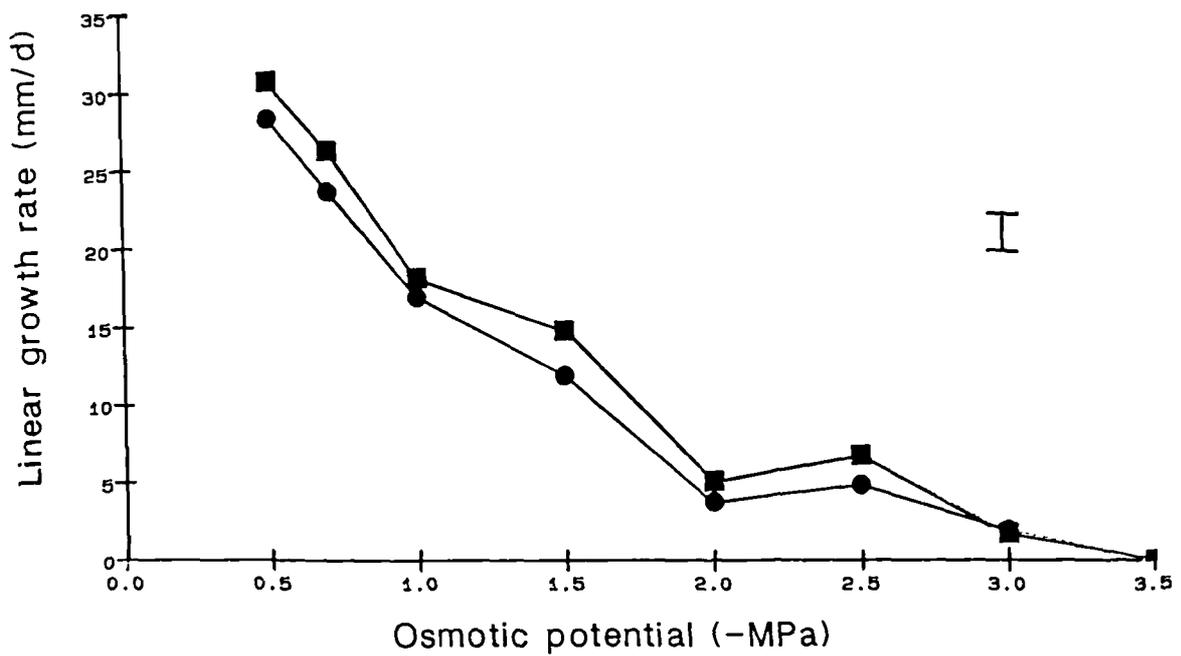
Figure 6

Effect of osmotic potential, modified with NaCl (●) and KCl (■), on the linear growth rate (mm/d) of Pythium oligandrum on solid media at 25°. Points are mean values of four replicates. For SEM, see Appendix 1.7b. The bars indicate the least significant differences at P=0.05.

### GAA



### CMA



potential. The minimum osmotic potentials for growth were again dependent upon the medium and osmoticum used. Growth was inhibited at -3.2 MPa on GAA modified with NaCl, whilst with KCl growth was inhibited at a lower osmotic potential of -3.5 MPa. With CMA growth was inhibited at -3.0 MPa on both electrolyte amended agar. For GAA, linear growth rates on media modified with NaCl were faster than on media modified to the same osmotic potentials with KCl. However, on CMA, KCl tended to support faster growth rates than NaCl at osmotic potentials above -2.6 MPa.

SEA (as in 3.6) was osmotically maintained with NaCl, KCl or glycerol over the range -0.5 to -3.5 MPa (see Appendix 1.7c) and used to compare the growth of P. oligandrum, P. aphanidermatum and P. ultimum. Sterile media were dispensed in 15 ml aliquots to a series of 9 cm diam Petri dishes, replication being four-fold. These were inoculated centrally with standard 3 mm diam agar discs cut from the periphery of 2-d-old colonies and incubated at 20°. Water loss was prevented by pouring cooled agar, wrapping individual dishes in catering grade 'Cling-film' and placing groups of dishes in sealed plastic bags. Colony diameters were measured every 12 h for 36 h as described in section 3.5 and radial growth rates determined.

Results are summarized in Table 8. Colony radial growth rates of each fungus decreased with decreasing osmotic potentials. Growth of all fungi was inhibited between -2.5 to -3.5 MPa, but minimum osmotic potentials for growth were higher for P. oligandrum than for P. aphanidermatum or P. ultimum. Growth responses of P. aphanidermatum and P. ultimum over the range of osmotic potentials tested were similar. The minimum osmotic potential for growth of each fungus when

Table 8

Colony radial growth rates (mm/d) of Pythium oligandrum, Pythium aphanidermatum, and Pythium ultimum at 20° on SEA at a range of osmotic potentials modified with NaCl, KCl and glycerol.

Each figure is the mean of four replicates with SEM.

Osmotic potential (-MPa)	<u>Pythium oligandrum</u>			<u>Pythium aphanidermatum</u>			<u>Pythium ultimum</u>		
	NaCl	KCl	Glycerol	NaCl	KCl	Glycerol	NaCl	KCl	Glycerol
0.5*	15.4±0.2	14.9±0.3	16.1±0.1	19.3±0.5	18.4±0.2	19.6±0.3	20.3±0.2	19.4±0.5	20.6±0.3
0.7	9.2±0.3	12.6±0.2	5.6±0.3	20.2±0.6	22.0±0.3	11.0±0.2	19.8±0.4	22.9±0.5	8.5±0.3
1.0	5.6±0.3	10.8±0.2	4.3±0.1	19.1±0.2	18.8±0.4	9.0±0.9	12.7±0.5	22.7±0.5	4.1±0.1
1.5	4.1±0.1	6.2±0.1	2.7±0.3	14.6±0.3	17.1±0.3	8.1±0.6	13.7±0.6	18.7±1.2	2.2±0.1
2.0	3.0±0.1	4.8±0.2	1.9±0.1	7.6±0.3	15.2±0.3	2.4±0.2	7.5±0.6	13.6±0.4	1.3±0.1
2.5	1.0±0.1	1.9±0.1	N.G.	3.1±0.2	4.7±0.1	1.8±0.1	4.0±0.2	9.3±0.1	0.8±0.1
3.0	N.G.**	N.G.	N.G.	1.2±0.2	1.7±0.1	N.G.	2.5±0.4	2.9±0.4	N.G.
3.5	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.
LSD (P=0.05)									0.9

\* Unmodified media

\*\* N.G. - No growth

modified with the non-electrolyte, glycerol, was higher than when the electrolytes, NaCl or KCl, were used. Both P. aphanidermatum and P. ultimum showed growth stimulation as the osmotic potential on KCl-modified media was decreased slightly. Thereafter, reduction and finally cessation of growth occurred with a further decrease in osmotic potential.

## 2. Matric potential

Much of the work on the influence of water potential on fungal growth has been determined using osmotically controlled systems using salts, sugars or glycerol (Scott, 1957; Griffin, 1981b). A few studies with soil or agar media have demonstrated a difference in the effect of osmotic and matric potential on growth of several fungi (Sommers et al., 1970; Adebayo & Harris, 1971; Cook, Papendick & Griffin, 1972; Monandhar & Bruehl, 1973; Magan, 1988). Since matric potential is the major component of the total soil water potential (Griffin, 1981b) it was thought necessary to determine the matric potential range for growth of P. oligandrum and some target pathogens.

The matric potential of SEA (as in 3.6) was altered with polyethylene glycol 6000 (PEG 6000) as recent evidence suggests that matric potential is the major component (90-95%) of the total water potential of polyethylene glycol of high molecular weight (Steuter, Mozafar & Goodin, 1981). Solutions of SEA/PEG 6000 do not solidify completely below -1.5 MPa, so a modified method of Baudoin & Davis (1987) was used. PEG 6000 (g/kg liquid) of different concentrations (equivalent to -0.6, -0.8, -1.0, -1.5, -2.0 and -2.5 MPa) for incubation temperatures of 25° were determined from a formula derived

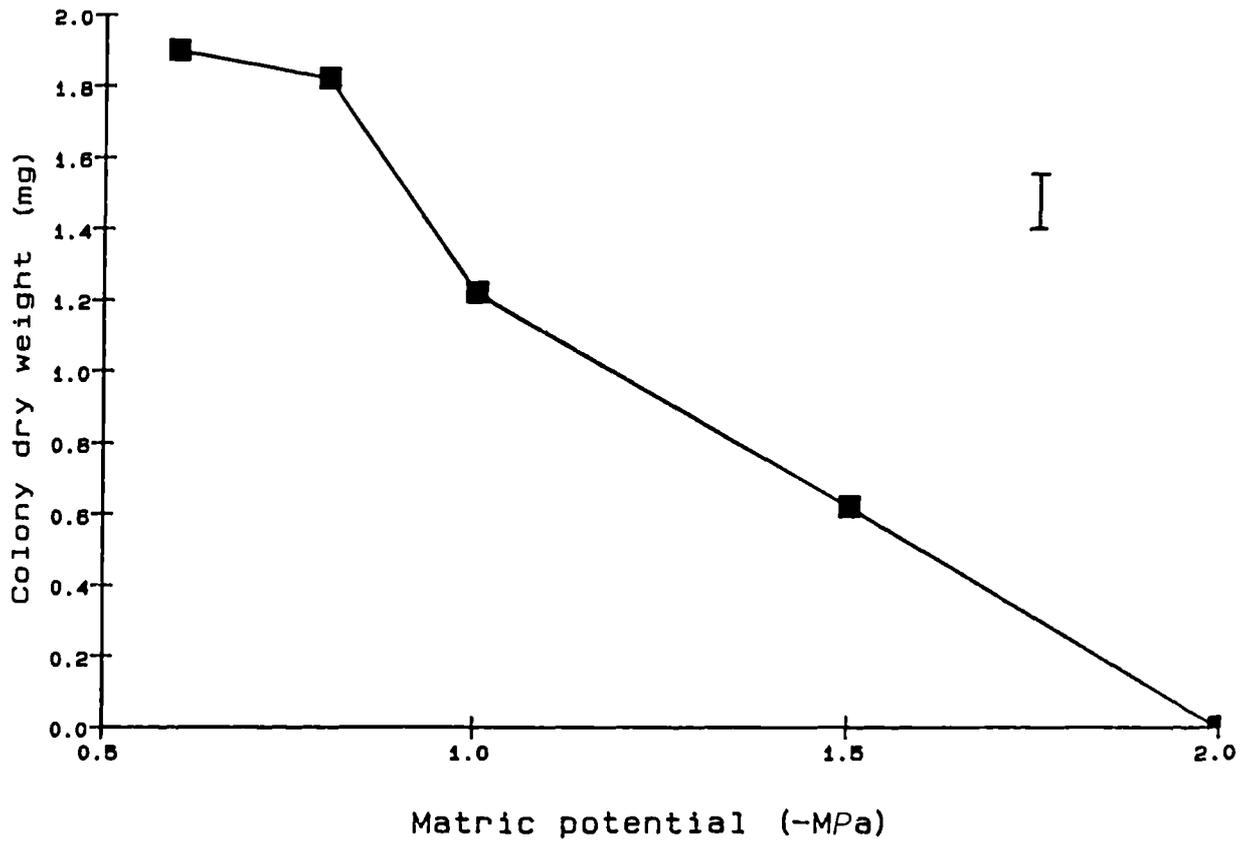
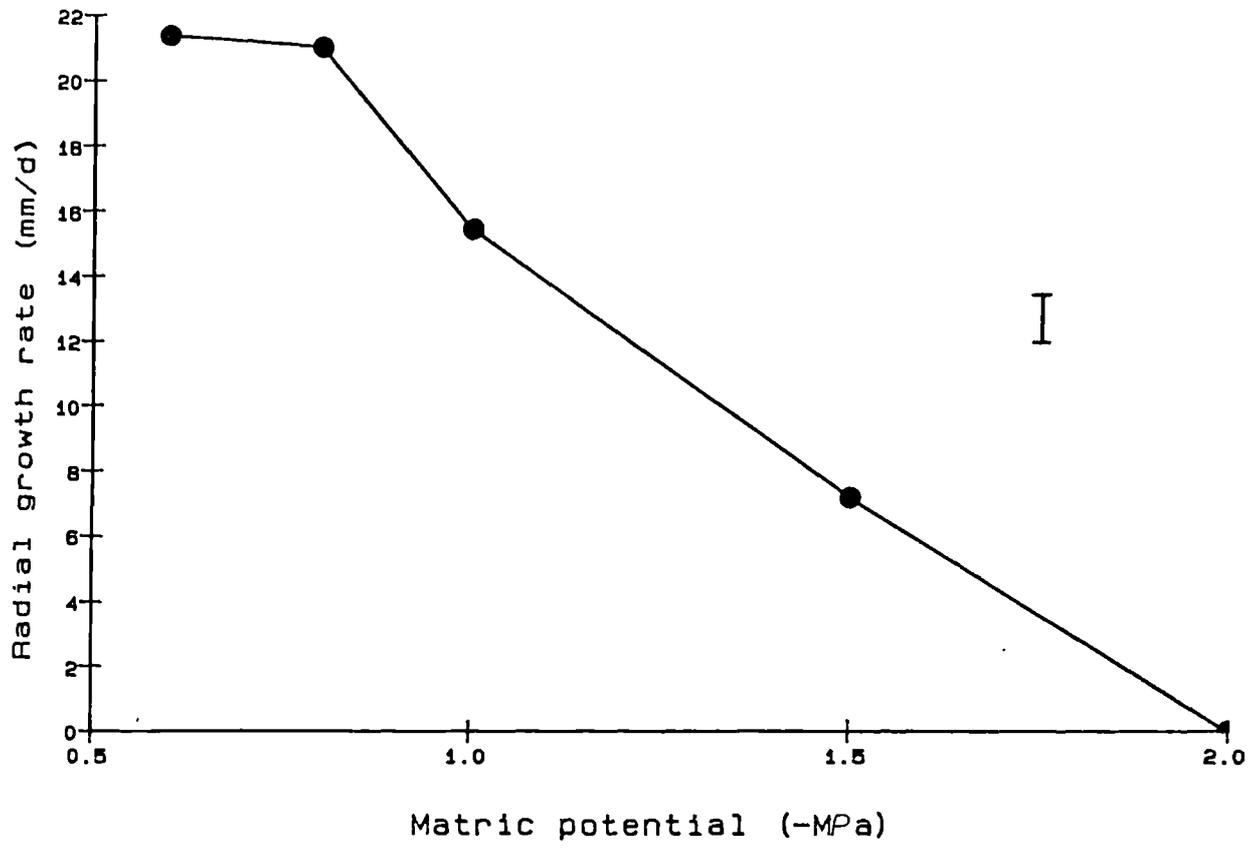
by Michel & Kaufmann (1973), (see Appendix 1.8a). The matric potential of modified media was calculated as the sum of media plus PEG 6000 matric potentials. SEA, pH 6.5, and concentrations of PEG 6000 were autoclaved separately, cooled to 50° and combined to give the required matric potentials. For each matric potential, 50 ml of medium was dispensed into four replicate 14 x 2.5 cm sterile glass Petri dishes. Boiled, autoclaved 13 mm diam cellophane discs (British Cellophane Co. PT600) were placed on the matrically modified media to provide a suitable platform for growth. Petri dishes were inoculated centrally with a 5 mm diam agar disc of P. oligandrum cut from the periphery of a 2-d-old colony. Individual dishes were sealed with Parafilm and groups of replicates stored in sealed plastic bags in an incubator at 25°. Radial growth rates were determined by measuring colonies along two diameters at right angles at 12 h intervals for 72 h. For measuring, dishes were placed on black paper and colonies viewed through the lids under a bright lamp. At 72 h colonies were harvested from cellophane discs by gently scraping the mycelium away with a spatula, dried at 60° for 36 h and weighed.

Results are shown in Fig. 7 and Appendix 1.8b. Radial growth rates of P. oligandrum decreased with decreasing matric potential. Below -1.0 MPa growth rates decreased markedly and growth was inhibited at -2.0 MPa. The dry weight of P. oligandrum colonies on cellophane discs also decreased with decreasing matric potential.

To compare the effect of matric potential on the growth of P. oligandrum, P. aphanidermatum and P. ultimum, soil extract broth (as in 3.6) was modified with PEG 6000 over the range -0.6 to -3.5 MPa as described previously. Media of different matric potentials were

Figure 7

Radial growth rates (mm/d) (●) and dry weights (mg) (■) of 72 h-old colonies of Pythium oligandrum at different matric potentials on cellophane discs 'floating' on polyethylene glycol (PEG) 6000 - amended SEA media at 25°. Points are mean values of four replicates. For SEM, see Appendix 1.8b. The bars indicate the least significant differences at P=0.05.



dispensed in 20 ml aliquots into 100 ml Erlenmeyer flasks, replication being four-fold. These were inoculated with 5 mm diam agar discs cut from the periphery of 2-d-old colonies and incubated at 25° for 8 d. Biomass was filtered off under vacuum on pre-weighed filter paper (Whatman GF/A, 5 cm diam) and washed thoroughly with distilled water at 45° for complete removal of PEG. Filter papers with biomass were oven-dried at 60° for 36 h, cooled in a desiccator for 24 h and weighed.

For results, see Table 9. Biomass production by all fungi was stimulated very slightly as matric potential decreased from -0.6 to -0.8 MPa, but growth was reduced and finally ceased with further decreases in matric potential. Growth of P. oligandrum was inhibited at -2.0 MPa, whilst that of P. ultimum and P. aphanidermatum was inhibited at -2.5 and -3.0 MPa respectively. The greatest biomass yields were produced by P. aphanidermatum, whilst yields of P. ultimum were greater than those of P. oligandrum

P. oligandrum and the two pathogens, P. aphanidermatum and P. ultimum, were generally more affected by low matric than by low osmotic potentials. The matric potential range for growth of these fungi was also shown to be markedly less than the osmotic range.

Table 9

Biomass dry weights (mg) after 8 d at 25° of Pythium oligandrum, Pythium aphanidermatum and Pythium ultimum in soil extract broth at a range of matric potentials modified with polyethylene glycol (PEG) 6000.

Each figure is the mean of four replicates with SEM.

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<u>Matric potential (-MPa)</u>	<u>Pythium oligandrum</u>	<u>Pythium aphanidermatum</u>	<u>Pythium ultimum</u>
0.6	20.2±0.8	28.2±0.5	25.0±0.6
0.8	21.8±0.7	28.6±0.8	26.2±0.8
1.0	15.2±0.7	27.2±0.6	22.0±0.9
1.5	7.7±0.6	16.4±0.9	14.4±0.8
2.0	N.G.*	8.5±0.4	6.3±0.8
2.5	N.G.	3.6±0.7	N.G.
3.0	N.G.	N.G.	N.G.

LSD (P=0.05) 1.9

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\* N.G. - No growth

## CHAPTER IV

### FACTORS AFFECTING OOSPORE PRODUCTION AND GERMINATION

## CHAPTER IV

### FACTORS AFFECTING OOSPORE PRODUCTION AND GERMINATION

It has been demonstrated conclusively that oospores of P. oligandrum are the main propagules upon which survival and consequently biocontrol properties depend (Vesely, 1977, 1979; Al-Hamdani, Lutchmeah & Cooke, 1983; Starling & Butler, 1983; Lutchmeah & Cooke, 1985; Martin & Hancock, 1987; Walther & Gindrat, 1987a). In order to produce large quantities of oospores, utilizing novel techniques, it is essential to have a basic understanding of some of the nutritional factors required for and affecting their production. Various nutritional factors affecting oospore production in a defined liquid medium were therefore studied.

Little is known about the effects of environmental factors on the germination of P. oligandrum oospores. The importance of environmental factors affecting the activity of biocontrol agents has already been outlined in Chapter III. An in vitro study was therefore carried out to determine the effects of temperature, pH and water potential on oospore germination.

#### 4.1 Effect of cholesterol, thiamine and calcium on biomass and oospore production

Pythiaceae fungi are unable to synthesize sterols (Hendrix, 1970; Elliot, 1977), and require exogenous sources of them for sexual and asexual reproduction (Elliot et al., 1964; Haskins, Tulloch & Micetich,

1964; Hendrix, 1964, 1965). Sterols have also been shown to stimulate vegetative growth (Lenney & Klemmer, 1966; Schlösser & Gottlieb, 1968; Child, Defago & Haskins, 1969; Langcake, 1974; Elliot, 1977). In a number of Pythium spp. calcium has been shown to be an important factor in the induction and development of oospores (Yang & Mitchell, 1965; Lenney & Klemmer, 1966). In addition, it is reported that P. oligandrum requires exogenous thiamine or its pyrimidine moiety for growth (Leonian & Lilly, 1938; Ridings, Gallegly & Lilly, 1969; Foley & Deacon, 1986b). The response of P. oligandrum to calcium, cholesterol and thiamine added singly and in all possible combinations to a basal medium was studied by assessing biomass and oospore production.

A basal medium containing (per l) 1.5 g L-asparagine, 1 mg FeCl<sub>3</sub>. 6H<sub>2</sub>O, 10 g D-glucose, 1.23 g KH<sub>2</sub>PO<sub>4</sub>, 170 mg K<sub>2</sub>HPO<sub>4</sub> and 0.4 mg MnSO<sub>4</sub>. 4H<sub>2</sub>O was used. L-asparagine and D-glucose were autoclaved separately and added to the sterile mineral salts solution. Batches of basal media were then further supplemented (per l) with either 50 mg CaCl<sub>2</sub> . 2H<sub>2</sub>O, 30 mg cholesterol (1.5% v/v solution in 95% v/v ethanol) or 2 mg thiamine hydrochloride and in all possible combinations to give the following final media:-

1. Basal medium (B)
2. B + CaCl<sub>2</sub>.2H<sub>2</sub>O (Ca)
3. B + cholesterol (ch)
4. B + thiamine HCl (th)
5. B + Ca + ch
6. B + Ca + th
7. B + ch + th
8. B + Ca + ch + th

Cholesterol and thiamine hydrochloride were added after filter-sterilization. Media were adjusted to pH 6.5 with sterile N-NaOH and each was dispensed in 20 ml aliquots into ten 9 cm diam Petri dishes. Each dish was then inoculated with a 5 mm diam mycelial disc cut from a 2-d-old colony on TWA and incubated in the dark at 25°. After 10 d incubation, five dishes of each test medium were removed. Biomass was harvested and dry weights determined as described in section 3.1. Estimations of oospore number were made by microscopic observation of cultures grown for 28 d in the remaining five Petri dishes. Oospores were counted in a single plane of focus in a field area of 1.13 mm<sup>2</sup> (x 150 magnification) at ten random points, beginning at the colony margin and at c. 0.7 mm intervals along a diameter. Oospore counts were logarithmically transformed and an analysis of variance performed on transformed data.

For results, see Table 10. Growth in basal medium alone was very poor. A single addition of calcium to the basal medium had no stimulatory effect on growth. Supplements of cholesterol alone stimulated growth, and biomass yields were significantly greater than those with basal medium. Biomass yield was markedly increased by the addition of thiamine HCl; yields being almost five times greater than those with the basal medium. Biomass yields with basal medium + cholesterol were similar to those with basal medium + cholesterol + calcium. Yields with basal medium + thiamine HCl were also similar to those with basal medium + thiamine HCl + calcium, suggesting that calcium has little effect on growth. The greatest yields were obtained when combinations of both thiamine and cholesterol were added to the basal medium.

Table 10

Effects of the addition to a basal medium of one multiple and three single additives in all possible combinations on biomass and oospore production of Pythium oligandrum at 25°.

Figures for dry weight biomass are the means of five replicates with SEM.

Figures for oospore numbers are the means from 50 counts, 10 in each of 5 dishes.

---

Medium	Dry weight biomass(mg) (10 d)	No. of oospores (range) per field of view	
		(28 d)	
Basal (B)	7.9±0.5	-*	
B+ CaCl <sub>2</sub> .2H <sub>2</sub> O(Ca)	9.4±0.3	-	
B + cholesterol (ch)	14.2±0.2	10 (3-14) 【0.97】**	
B + thiamine HCl (th)	33.9±1.1	-	
B + Ca + ch	15.3±0.7	208 (179-274) 【2.32】	
B + Ca + th	34.5±1.2	-	
B + ch + th	53.1±1.3	10 (3-18) 【0.98】	
B + Ca + ch + th	53.4±1.4	230 (193-261) 【2.36】	
LSD (P=0.05)	2.7	【0.12】	

---

\* Oospores not detected

\*\* Values in square brackets are means calculated after logarithmic (log.) transformation of replicate counts.

Oospores were only formed in media supplemented with cholesterol, indicating that an exogenous supply is essential for their production under these conditions. Few oospores were formed with basal medium + cholesterol and basal medium + cholesterol + thiamine HCl. Significantly more oospores were formed in basal medium + calcium + cholesterol and basal medium + calcium + cholesterol + thiamine HCl than in the other two media.

#### 4.2 Effect of carbon-nitrogen ratio on oospore production

The importance of the carbon-nitrogen (C:N) ratio on the formation of mature oospores by a Pythium spp. in laboratory media has already been demonstrated (Child et al., 1969). It was therefore considered important to investigate the effect of the C:N ratio on oospore production on glucose-asparagine agar (as in 3.7) by P. oligandrum. The C:N ratio was varied in two ways as described in section 3.4, but one further ratio of 200:1 was included. In calculating C:N ratios, allowance for the carbon and nitrogen content of the agar was not made. Their effects have been considered to be uniform and relatively minor (Leal, Gallegly & Lilly, 1967). Batches of D-glucose and L-asparagine were autoclaved separately and added to sterile mineral solutions to give the required C:N ratios. Media were adjusted to pH 6.5 with sterile N-NaOH and each was dispensed in 15 ml aliquots into five replicate 9 cm diam Petri dishes. Dishes were each inoculated centrally with a 3 mm diam disc cut from the periphery of a 2-d-old colony on TWA and incubated in the dark at 25°. Estimations of oospore number were made by microscopic observation of the cultures grown for 28 d as described in the previous experiment. Oospore counts were

again logarithmically transformed before statistical analysis.

The results are summarized in Table 11. Oospore production was generally favoured at low C:N ratios. With L-asparagine constant, oospore production was greater at ratios of 5-30:1. Ranges of oospore numbers tended to be wider at the lower optimum ratios, but mean oospore counts were not significantly different. There was a significant decline in oospore production above a ratio of 30:1. Similar results were obtained with D-glucose constant, but maximum oospore production was at ratios of 5 and 9:1. Oospore numbers were moderate at ratios of 16 and 30:1, but were not as high as those produced at the same ratios on medium with L-asparagine constant. Beyond a ratio of 30:1 oospore production declined with further ratio increases. On both types of adjusted media oospore production was inhibited at a ratio of 200:1.

#### 4.3 Utilization of D-glucose and L-asparagine in relation to biomass and oospore production

The importance of exogenous cholesterol, calcium and the C:N ratio on oospore production of P. oligandrum has already been shown in previous experiments. For fungi, nutrient exhaustion has been recognized as a stimulus to reproduction at least since the time of Klebs (1899, 1900). Furthermore, oosporogenesis in a few Oomycetes is induced by the exhaustion of a particular nutrient (Klebs, 1899; Elliot, 1989). The amounts of nitrogen and carbon source in culture filtrates and the numbers of oospores present were therefore measured with a view to elucidating the nutrient status of the medium during oospore production by P. oligandrum.

Table 11

Mean number and range of Pythium oligandrum oospores per field of view counted on glucose-asparagine agar medium at various carbon-nitrogen ratios after 28 d at 25°.

Means are calculated from 50 counts, 10 in each of 5 dishes.

C:N ratio	L-asparagine constant		D-glucose constant	
	Content of C(g/l)	No. of oospores (range)	Content of N(g/l)	No. of oospores (range)
5:1	1.4	177 (139-257) <b>【2.25】*</b>	1.21	179 (149-210) <b>【2.25】</b>
9:1	2.52	174 (162-185) <b>【2.24】</b>	0.54	176 (146-210) <b>【2.25】</b>
16:1	4.48	168 (143-194) <b>【2.23】</b>	0.28	118 (88-144) <b>【2.07】</b>
30:1	8.40	158 (136-179) <b>【2.20】</b>	0.14	108 (86-129) <b>【2.04】</b>
60:1	16.80	36 (12-51) <b>【1.55】</b>	0.07	86 (78-101) <b>【1.94】</b>
90:1	25.20	13 (8-25) <b>【1.14】</b>	0.05	29 (14-42) <b>【1.47】</b>
130:1	36.40	4 (0-9) <b>【0.47】</b>	0.03	13 (8-18) <b>【1.13】</b>
200:1	56.00	-**	0.02	-
LSD (P=0.05)		<b>【0.11】</b>		

\* Values in square brackets are means calculated after logarithmic (log.(x+1)) transformation of replicate counts.

\*\* Oospores not detected.

Autoclaved glucose-asparagine (as in 3.1) was adjusted to pH 6.5 with sterile N-NaOH and was dispensed in 15 ml aliquots into sterile 100 ml Erlenmeyer flasks. Each flask was then inoculated with a 5 mm diam mycelial disc cut from a 2-d-old colony on CMA and incubated at 25°. At 2, 4, 6, 8, 12 and 16 d, biomass in four flasks was harvested and dry weights determined as described in section 3.1. Biomass was then suspended in 20 ml distilled water, comminuted in an MSE homogenizer and oospore numbers determined using a haemocytometer. The culture filtrates from replicate flasks were combined for assay of residual D-glucose and L-asparagine.

i. Estimation of residual D-glucose

Residual D-glucose was determined enzymatically using glucose oxidase according to the method of Lloyd & Wheelan (1969). Principles of the assay and the details of the reagents used are outlined in Appendix 2.1. Four 1 ml aliquots of test sample were each added to 2 ml glucose oxidase reagent in replicate test tubes. The contents of each tube were mixed thoroughly and then incubated at 37° for 2 h after which time 5N-HCl (4 ml) was added. Contents were again mixed thoroughly and the colour which developed was read as absorbance on a Philips PV8720 uv/vis Scanning Spectrophotometer at 530 nm against a blank (distilled water, 1 ml; glucose oxidase reagent, 2 ml; 5N-HCl, 4 ml). A glucose standard curve was obtained by plotting absorbance against standard concentrations of D-glucose ranging from 0 to 75 µg/l. The relationship between absorbance and D-glucose was estimated by the above procedure in four replicate 1 ml samples from each culture filtrate. Amounts of D-glucose were calculated from the standard curve

(Appendix 2.1) and multiplied by the appropriate dilution factor to obtain the actual amount of residual D-glucose.

ii. Estimation of residual L-asparagine

Residual L-asparagine was determined enzymatically using asparaginase according to Möllering (1985). Principles of the assay and details of the reagents used are outlined in Appendix 2.2. The following were mixed in a 1 cm pathlength quartz cuvette : phosphate buffer, 1 ml; NADH, 0.1 ml; 2-oxoglutarate, 0.1 ml; MDH, 0.02 ml; sample, 1 ml. The cuvette was placed in the spectrophotometer, as used previously, at 25° and the absorbance measured . GOT (0.02 ml) was then added and the cuvette was incubated for 30 min in the spectrophotometer to allow the endogenous oxidation of NADH. Absorbance at 340 nm was measured, 0.1 ml of asparaginase was added, and the absorbance followed until no further changes occurred (c. 20 min). A blank without a sample added was measured in a similar manner. After correction for the blank, the L-asparagine content was calculated as outlined in Appendix 2.2. L-asparagine was estimated by the above procedure in four replicate 1 ml samples from each culture filtrate. Appropriate dilutions of samples were made to allow for the sensitivity of the assay.

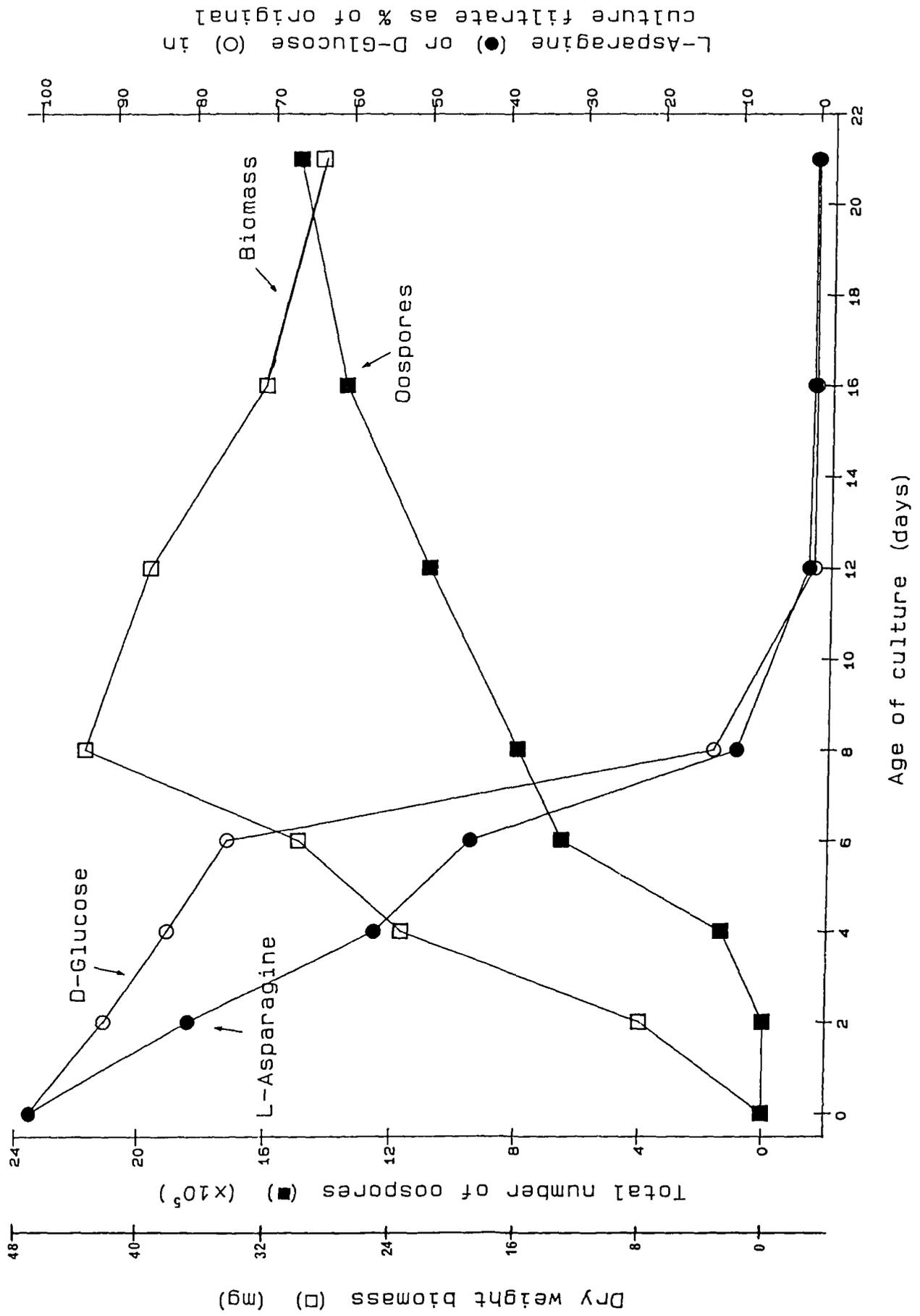
Results are shown in Fig. 8 and Appendix 2.3. Biomass production increased over the incubation period to reach a maximum at 8 d, after which time there was a decline in yield. Oospores were present within biomass after 4 d, and production increased with further increases in incubation period to reach a maximum at 21 d. Levels of L-asparagine and D-glucose in cultures decreased as biomass production increased.

Figure 8

Utilization of D-glucose and L-asparagine by Pythium oligandrum in relation to biomass and oospore production. Media had initially 10 g D-glucose and 1.5 g L-asparagine per l.

●, Amount of L-asparagine in culture filtrate; ○, D-glucose in filtrate; ■, number of oospores; □, dry weight biomass.

Points are mean values of four replicates. For SEM, see Appendix 2.3.



Few oospores were formed while there was more than 0.67 g/l L-asparagine and 7.51 g/l D-glucose left in the medium. Both L-asparagine and D-glucose became exhausted at 12 d but there was continued production of oospores.

#### 4.4 Effect of temperature on oospore germination

Temperature is one of the most important external factors which influence spore germination (Lilly & Barnett, 1951; Cochrane, 1958; Griffin, 1981a). Whilst temperature requirements for germination may be in the same range as for growth, they are not necessarily the same (Sussman, 1965). Temperatures that favour oospore germination are known for certain Pythium spp. (Adams, 1971; Stanghellini & Russell, 1973) but are unknown for P. oligandrum.

Oospores for use in germination studies subsequently described in this Chapter were produced on GAA (as in 3.7) in 9 cm diam Petri dishes. Dishes were each inoculated centrally with a 3 mm diam disc cut from the periphery of a 2-d-old colony and incubated for 21 d at 25°. Cultures were then flooded with c. 10 ml sterile distilled water and oospores were dislodged by gently scraping the colony surface with a sterile spatula. Oospore suspensions were passed through two layers of muslin to remove hyphal fragments, concentrated by centrifugation at 1200 g for 30 min and resuspended.

To assess the effect of temperature on oospore germination at 5,10,15,20,25,30,35 and 40°, stock oospore suspension was diluted with sterile distilled water to produce a spore density of c.  $2 \times 10^3$  per ml, and aliquots (0.5 ml) were spread over the surface of SEA (as in 3.5 ; 10 ml in 9 cm diam Petri dishes) with a glass spreader. Dishes were

Table 12

Effect of temperature on the germination of Pythium oligandrum oospores on SEA after 12h incubation.

Figures in parentheses are means with SEM from five replicates calculated after angular transformation of percentage data.

---

Temperature (°C)	Germination (%)
5	—*
10	—
15	14 (22.1±0.8)
20	22 (27.7±0.5)
25	27 (31.3±0.3)
30	28 (31.7±0.5)
35	—
40	—
LSD (P=0.05)	(1.7)

---

\* Germination not detected after 12 h incubation.

placed in sealed plastic bags and five replicates incubated at each temperature for 12 h, after which time oospores were stained with cotton blue/lactophenol and examined microscopically. Germination of 250 oospores on each plate was scored, oospores with germ-tubes of a length greater than spore diameter being considered to have germinated. Assessment of germination was not possible after this time because of extensive germling growth at most temperatures. To determine the temperature limits for germination a further three dishes were incubated at the appropriate temperatures for 14 d and observed daily. Percentage germination data was angular transformed and analysis of variance performed on transformed data. Similar transformations of germination data from subsequent experiments were also made before analyses.

Results are shown in Table 12. After 12 h, oospore germination occurred over the range 15-30°, whilst the optimum temperatures were 25 and 30°. Germination was reduced below 25°, but it was significantly greater at 20° than at 15°. Oospores incubated at 10 and 35° germinated after 6 d, whilst those incubated at 5 and 40° failed to germinate after 14 d incubation. Oospores incubated at 5 and 40° germinated within 12 h when reincubated at 25°.

#### 4.5 Effect of pH on oospore germination

Spore germination usually has a narrower pH range than growth, presumably because there is little time for metabolic products to modify an unfavourable substrate (Cochrane, 1958). The effect of pH on growth has already been evaluated in section 3.5. The pH range and optimum pH for oospore germination was therefore determined on a solid

medium.

The effect of pH on oospore germination was investigated using SEA (as in 3.6). Sterile media were buffered at different pH levels (pH 4.0 to 9.0) with citrate-phosphate and tris(hydroxymethyl)aminoethane buffers as described in section 3.5. Batches of buffered medium were then dispensed in 10 ml aliquots into four replicate Petri dishes (9 cm diam). Control medium consisted of unbuffered SEA (pH 7.3). Oospore germination on SEA at different pH levels was determined at 25° as described before. To determine the pH limits for germination, a further three dishes per treatment were incubated for 14 d and observed daily.

For results, see Table 13. Germination occurred over a pH range of 4.5 to 9.0, with an optimum between 6.0 and 7.0. Oospore germination was totally inhibited at pH 4.0 and 9.0 after 14 d.

#### 4.6 Effect of water potential on oospore germination

Water potential is recognized as an important factor affecting the initiation of hyphal growth from dormant propagules (Griffin, 1981b). However, little information is available on the possible effects of and differences between osmotic and matric potential effects on spore germination and germ-tube extension (Domsch et al ., 1980). Further, there is no information in the literature on the effect of water potential on oospore germination in P. oligandrum. The effects of osmotic and matric potential on oospore germination were determined using SEA (as in 3.6) and soil extract broth (as in 3.6) respectively.

##### 1. Osmotic potential

SEA was osmotically maintained over the range -0.5 to -3.5 MPa using

Table 13

Effect of pH on the germination of Pythium oligandrum oospores on SEA after 12 h incubation at 25°.

Figures in parentheses are means with SEM from five replicates calculated after angular transformation of percentage data.

---

pH	Germination (%)
4.0	—*
4.5	6 (14.0±1.1)
5.0	10 (18.0±1.2)
5.5	19 (25.5±1.0)
6.0	24 (31.9±2.7)
6.4	27 (31.4±0.7)
7.1	25 (30.2±0.9)
7.3**	16 (23.9±0.6)
7.5	20 (26.3±1.1)
8.0	15 (23.0±1.0)
8.4	5 (13.0±1.0)
9.0	—
LSD (P=0.05)	(2.7)

---

\* Germination not detected after 12 h incubation

\*\* Unbuffered medium

NaCl or KCl as described in section 3.7. Sterile media were dispensed in 15 ml aliquots to a series of 9 cm diam Petri dishes, replication being four-fold. Stock oospore suspension (1 ml) was added to 19 ml sterile distilled water in 25 ml Universal bottles previously modified to the required osmotic potential with NaCl or KCl. The final oospore density was in the range  $1-4 \times 10^3$  per ml. Oospore suspensions were therefore maintained at appropriate osmotic potentials before use. Oospore germination on SEA maintained at different osmotic potentials was determined at 25° as described in section 4.4. To determine the osmotic potential limits for germination, three dishes per treatment were incubated for 14 d and observed daily.

For results, see Fig. 9 and Appendix 2.4. Germination was maximum and fairly uniform over the range -0.5 to -1.5 MPa. On both amended media germination decreased below -1.5 MPa with decreasing osmotic potential and was totally inhibited at -3.5 MPa even after 14 d. For each osmotic potential, germination on both amended media was similar.

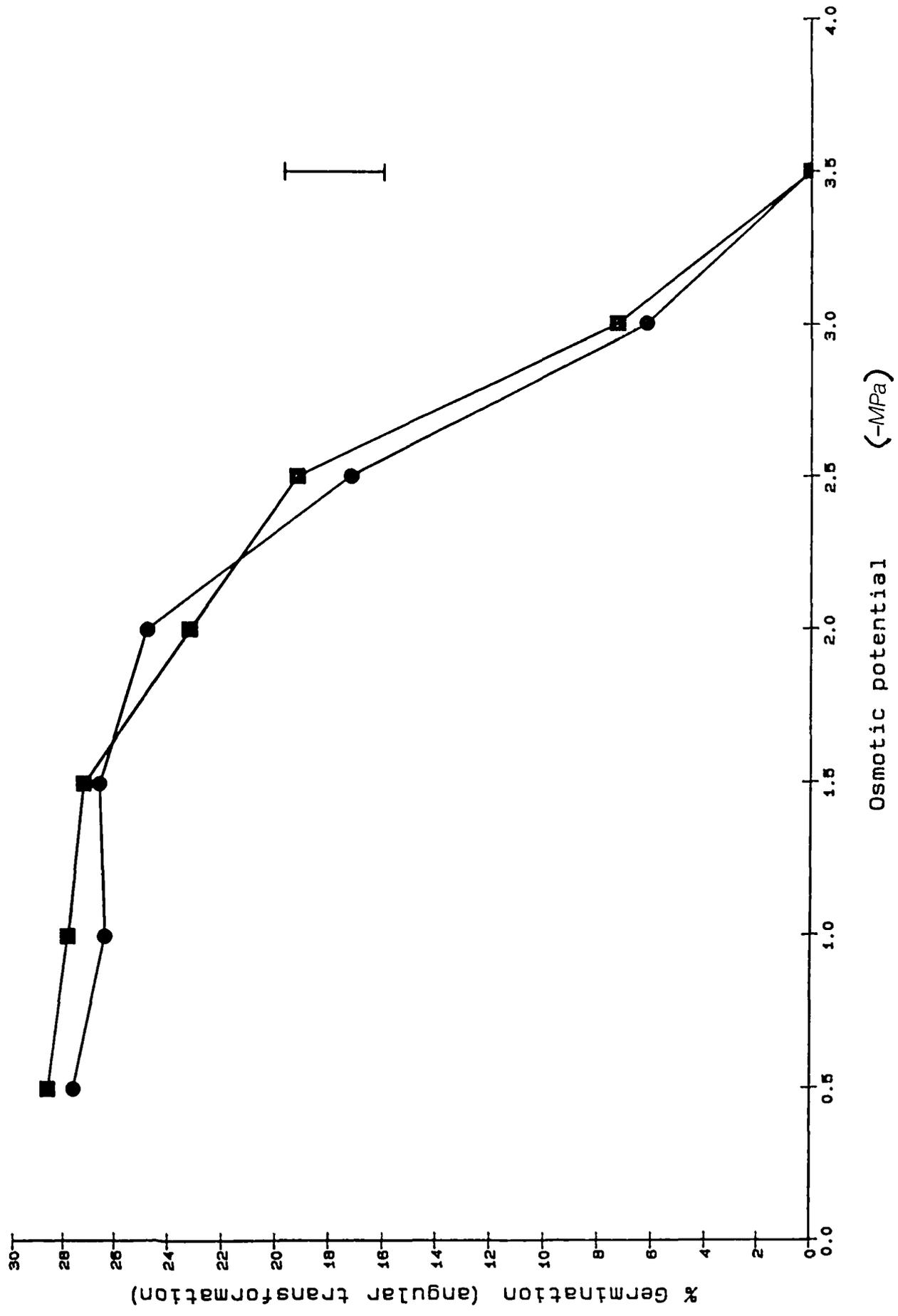
## 2. Matric potential

The matric potential of soil extract broth was altered over the range -0.6 to -3.5 MPa with PEG 6000 as described in section 3.8. Aliquots (5 ml) of oospore suspension (c.  $1-4 \times 10^3$  oospores per ml) in appropriate soil extract broth/PEG 6000 solutions were dispensed into four replicate 5 cm diam glass Petri dishes. Dishes were sealed individually with Parafilm, placed in sealed polyethylene bags and incubated at 25° for 12 h, after which time oospores were fixed by the addition of 0.5 ml formalin/ethanol (1:1 v/v). Germination of 150 oospores in each dish was then scored. The matric potential limits for germination were also determined by observing additional dishes daily

Figure 9

Effect of osmotic potential, modified using NaCl (●) or KCl (■), on germination of Pythium oligandrum oospores on SEA after 12 h incubation at 25°. Points are mean values of four replicates.

For SEM, see Appendix 2.4. The bars indicate the least significant difference at  $P=0.05$ .

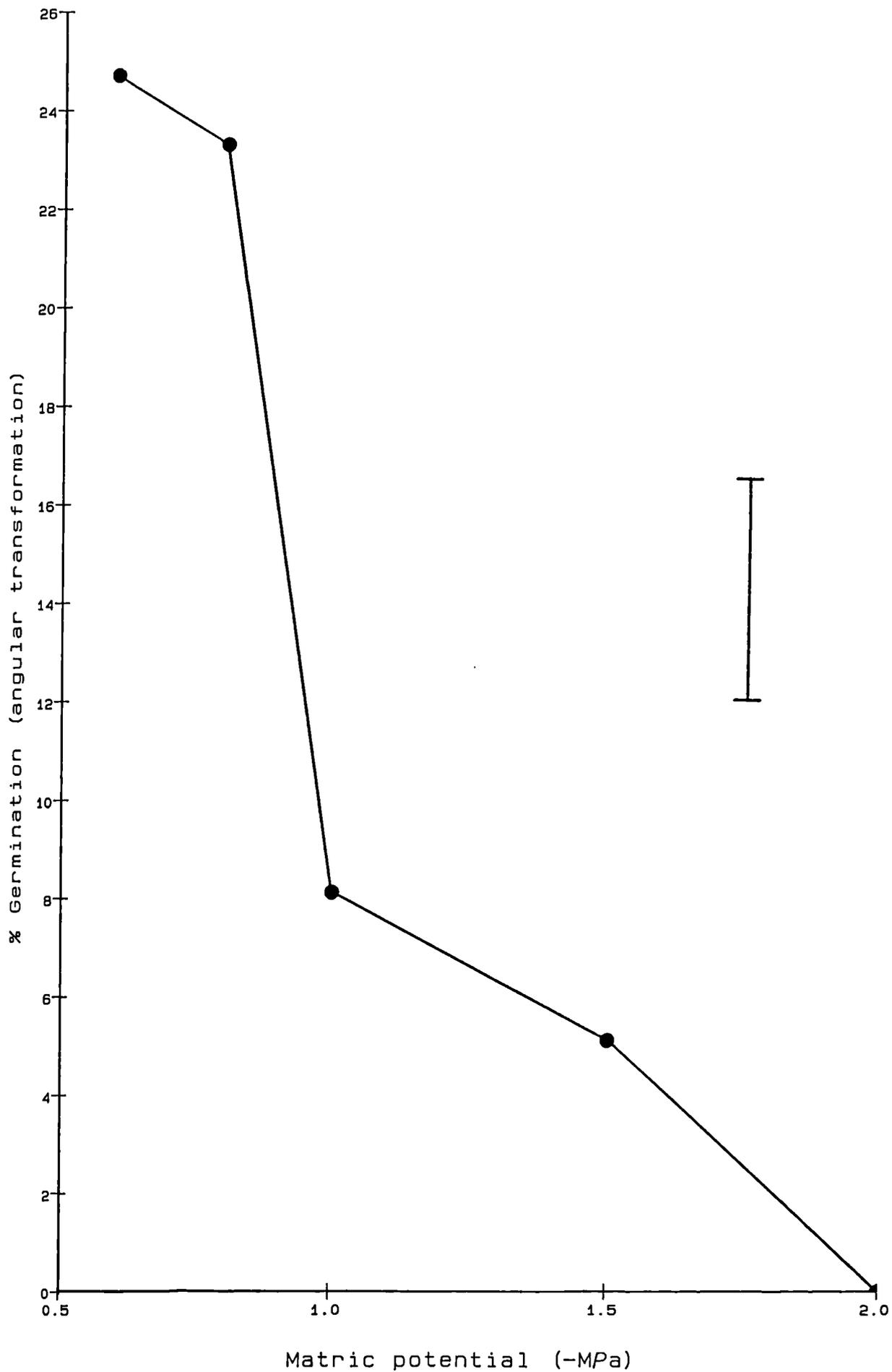


for 10 d.

For results, see Fig. 10 and Appendix 2.5. Germination was maximum and similar at -0.6 and -0.8 MPa. Below -0.8 MPa germination was reduced markedly and was totally inhibited at -2.0 MPa even after 8 d incubation. Oospore germination was therefore more affected by low matric than by low osmotic potentials, and the matric potential range for germination was also narrower than the osmotic range.

Figure 10

Effect of matric potential, modified with PEG 6000, on the germination of Pythium oligandrum oospores in soil extract broth after 12 h incubation at 25°. Points are mean values of four replicates. For SEM, see Appendix 2.5. The bar indicates the least significant difference at P=0.05.



CHAPTER V

USE OF INEXPENSIVE SOLID AND LIQUID MEDIA  
FOR OOSPORE PRODUCTION

## CHAPTER V

### USE OF INEXPENSIVE SOLID AND LIQUID MEDIA FOR OOSPORE PRODUCTION

The importance of P. oligandrum oospores for biocontrol has already been mentioned in Chapter IV. Advancement in research on the biocontrol potential of this mycoparasite has been limited due to the lack of reliable systems for producing viable oospores (Lewis, Whipps & Cooke, 1989). To produce large quantities of oospores for both small and large-scale biocontrol trials, inexpensive, commercially-compatible procedures are required. The goal of mass production of fungal biocontrol agents at an economical cost can be met by using submerged (or deep tank) and semi-solid culture techniques utilizing crude agricultural products and food processing wastes that are readily available in unlimited quantities (Churchill, 1982; Lewis & Papavizas, 1984; Papavizas et al., 1984; Lisansky, 1985; Tabachnik, 1988; Lumsden & Lewis, 1989). It has already been shown that P. oligandrum requires an exogenous supply of sterols for oospore production (Chapter IV). Crude agricultural products and food processing wastes are ideal substrata for oospore production as sterols and related compounds are known to occur in various groups of plants (Bergmann, 1962; Heftmann, 1963, 1969). This Chapter describes studies to develop simple solid and liquid culture techniques for oospore production utilizing a range

of such substrata.

### 5.1 Oospore production on various semi-solid media

Semi-solid media previously used for oospore production include millet saturated with a mineral nutrient solution (Vesely & Hejdanek, 1981, 1982) maize meal-sand (Starling & Butler, 1983) and cornmeal-vermiculite (Lutchmeah & Cooke, 1985). A wide range of inexpensive semi-solid media, including surplus grains supplemented with different nutrient liquids, were screened for their ability to support oospore production.

Each solid was mixed with the inert material perlite (P) (Silvaperl Products Ltd., Harrogate) to provide a high surface area to volume ratio and maintain aeration. The liquids (L) used were either tap water (W), cane molasses (M; 30 g/l) (United Molasses, Hull) or glucose-asparagine (GA; as in section 3.1). Each liquid was adjusted to pH 6.5 with either N-NaOH or N-HCl and was added to individual solid substrata at the volumes stated below. Additional media were included in which rapeseed oil was also added at a rate of 3% (v/v) to each solid supplemented with liquid. Rapeseed oil is a cheap source of sterols and has been shown to be effective in stimulating sexual reproduction of a Pythium sp., (Haskins et al., 1964). The following semi-solid culture media were prepared as follows:

1. Rolled oats : P : L (2:4:1 v/v)
2. Micronised maize meal (2-5mm) : P : L (1:2:1 v/v)
3. Wheat bran : P : L (2:4:1 v/v)
4. Millet : P : L (2:3:1 v/v)

1-4 were supplied by Middletons, Sheffield

5. Spent barley : P : L (1:2:1 v/v)

6. Spent hops : P : L (1:2:1 v/v)

5 & 6 were supplied by Wards' Brewery, Sheffield

7. Cracked barley : P : L (2:2:1 v/v)

8. Cracked wheat : P : L (1:2:1 v/v)

7 & 8 were supplied by Muddle & Sons Ltd, Ashington, W. Sussex

9. Spent mushroom compost (IHR, Littlehampton) : P : L (1:2:1 v/v)

10. Wheat straw : P : L (1:1:1 v/v)

11. Cocoa shells (British Cocoa Mills (Hull) Ltd.): P :L (1:2:1  
v/v)

Components of each medium were well combined and 60 g portions added to four replicate 250 ml wide-necked Erlenmeyer flasks. Flasks were autoclaved on two consecutive days for 1 h. Each flask was inoculated with a 2 ml oospore suspension containing c.  $2 \times 10^4$  oospores per ml which was previously produced by macerating washed biomass grown in GA at 25° for 21 d. Cultures were incubated in the dark at 25° and shaken manually after 3 d. Contents of individual flasks were removed after 21 d, placed in open glass Petri dishes (9 cm diam) and air-dried in a laminar flow cabinet for 5-7 d. To assess oospore production, four 1 g samples of air-dried preparation from each replicate flask were each suspended in 20 ml Triton X100 (0.05% v/v) and blended in an MSE homogenizer for 2 min. Aliquots of suspension were passed through one layer of muslin to remove solid debris and oospores were counted in a haemocytometer.

Oospore production on a range of semi-solid media is shown in Table 14. Production was particularly high on cracked barley and wheat with all liquid supplements, but the greatest number of oospores was

Table 14

Oospore production ( $\times 10^5$ , per g preparation) by Pythium oligandrum after 21 d incubation at 25° on semi-solid media supplemented with either tap water (w), cane molasses (M, 30 g/l); or glucose-asparagine (GA) and rapeseed oil (O). Each figure is the mean from 16 counts with SEM; four for each of four flasks.

Liquid supplement

Solid substrate	W	M	GA	W+O	M+O	GA+O
Cracked barley	209.2±3.1	241.7±7.9	221.1±7.5	228.0±3.7	320.2±4.5	238.0±0.7
Cracked wheat	126.8±1.4	228.5±9.3	198.5±5.3	179.2±7.7	255.0±5.0	200.0±1.9
Millet	96.4±2.3	172.7±8.7	113.9±6.5	94.5±2.6	180.0±2.1	113.2±2.8
Maizemeal	3.2±0.3	97.1±4.5	104.2±4.3	20.2±1.4	110.2±2.6	123.0±1.2
Wheat bran	75.0±1.2	86.2±5.2	100.5±5.3	105.0±1.2	93.8±2.2	102.8±1.9
Rolled oats	17.2±0.8	72.9±1.1	95.7±4.1	29.2±1.4	71.2±2.2	114.8±1.4
Spent mushroom compost	-*	2.7±0.4	2.9±0.1	2.0±0.3	4.4±1.1	3.7±0.2
Spent barley	-	68.9±0.8	73.6±2.6	-	72.8±0.8	121.5±1.9
Wheat straw	-	0.6±0.1	5.5±0.4	0.6±0.1	6.5±0.5	4.1±0.6

LSD (P=0.05)

9.7

Oospores were not produced on cocoa shells or spent hops.

\* Oospore production absent.

produced on cracked barley supplemented with cane molasses and rapeseed oil. Moderate numbers were produced on millet, maize meal, wheat bran and rolled oats, but numbers on spent mushroom compost and wheat straw were very low. No oospores were detected on cocoa shells or spent hops with any of the supplements. For most solids, oospore production was increased with an increase in the nutrient status of the liquid supplement. In general, most solids supported greater oospore numbers with cane molasses or glucose-asparagine than with water alone. Oospore production was inhibited on spent barley, spent mushroom compost and wheat straw supplemented with water, whilst with supplements of molasses or glucose-asparagine oospore production was supported. Oospore numbers produced on solids supplemented with both liquid and rapeseed oil were generally greater compared with those produced on solids with liquid alone.

## 5.2 Oospore production on semi-solid media within autoclavable bags

Large quantities of Coniothyrium minitans Campbell for use in glasshouse biocontrol trials have been grown successfully on a semi-solid maize meal perlite substrate in autoclavable bags (Whipps, Budge & Ebben, 1989). It was therefore considered possible to utilize autoclavable bags containing those semi-solid media which had supported high oospore production in the previous experiment for large-scale oospore production.

Four solids (maize meal, millet, cracked barley and cracked wheat) were mixed with perlite and supplemented with a solution of cane molasses (30 g/l) and rapeseed oil as described in the previous experiment. Components of each medium (c. 1-1) were added to three

replicate Sterilin autoclavable bags (62 x 33 cm). The opening of each bag was wrapped round a metal ring (3.5 cm diam) providing an artificial neck which was stopped with cotton wool. Following autoclaving on two consecutive days, bags were each inoculated with a 50 ml oospore suspension containing c.  $2 \times 10^4$  oospores per ml which was prepared as described before. Bags were incubated at 25° for 21 d after which time contents were removed and oospores counted in four 1 g samples as described in the previous experiment. Quantitative assessments of the number of colony-forming units (cfu) per g of air-dried preparation were also made. Four 1g samples of air-dried preparation from each replicate bag were each suspended in 10 ml 0.1% (w/v) sterile distilled water agar (no. 3 Oxoid) and macerated in an MSE homogenizer for 2 min. After standing for 10 min, the suspension was mixed for 60 s with a Rotamixer and 0.5 ml aliquots were spread over the surface of a selective medium of Martin & Hancock (1986), modified to contain only 50 µg/l rose bengal (see Appendix 3.1), in three replicate 9 cm diam Petri dishes with a glass spreader. Ten fold dilutions were prepared and three replicate Petri dishes used for each dilution. Petri dishes were incubated for 48 h at 25°, after which solid residue was washed from the agar surface under a gentle stream of water. Colonies on the agar surface were then counted. Final counts were made after 72 h incubation.

For results, see Table 15. Oospore numbers were high on barley and wheat, but greater numbers were produced on barley. Millet supported greater numbers than maize meal. Oospore production on each solid medium was also less in autoclavable bags than in 250 ml Erlenmeyer flasks. Suspensions of all solid preparations produced characteristic

Table 15

Oospore production by Pythium oligandrum after 21 d at 25° on semi-solid media supplemented with 3% (w/v) cane molasses and rapeseed oil in Sterilin autoclavable bags. Each figure is the mean with SEM. For oospores per g preparation, means were calculated from 12 counts, four for each of three bags.

Colony-forming units were determined for four replicate 1g samples from each of three bags.

---

Solid substratum	Oospores per g preparation (x10 <sup>5</sup> )	Colony-forming units (log. per g preparation)
Cracked barley	176.0±4.7	3.06±0.04
Cracked wheat	129.2±4.7	3.18±0.03
Millet	87.8±3.7	2.87±0.03
Maizemeal	26.5±2.6	2.57±0.01
LSD(P=0.05)	11.0	0.22

---

colonies of P. oligandrum on the agar medium. Colony counts were highest with barley and wheat, and lowest with maize meal.

### 5.3 Oospore production in a range of liquid media

Oospores for small-scale biocontrol studies have previously been produced utilizing expensive complex media such as V8 juice supplemented with cholesterol (Al-Hamdani et al., 1983). In view of expensive liquid media being commercially incompatible, oospore production in a wide range of inexpensive media was compared with production in V8 juice.

The following liquid media were prepared:

1. Cane molasses (CM) containing 30 g/l cane molasses (United Molasses, Hull).
2. Cane molasses-yeast (CM-Y) containing 30 g/l cane molasses and 5 g/l brewers' yeast (The Distillers Co., Surrey).
3. Cane molasses-corn steep liquor (CM-CSL) containing 30 g/l cane molasses and 5 g/l corn steep liquor (Sigma Chemical Co.).
4. Maize meal broth (MB). Maize meal (Middletons, Sheffield) was ground to pass through a 500  $\mu$ m screen in a Cyclotec sample mill/laboratory grinder (Tecator, Bristol). Thirty gram maize meal was boiled with 1-l distilled water for 1 h, filtered through muslin, and made up to 1-l with distilled water.
5. Wheat bran broth (WBB); 30 g wheat bran (Middletons, Sheffield) was boiled with 1-l distilled water for 30 min, filtered through muslin, made up to 1-l with distilled water.
6. Wheat bran broth-yeast (WBB-Y); 5 g brewers' yeast was added to 1-l wheat bran broth.

7. Wheat bran broth-corn steep liquor (WBB-CSL); 5 g corn steep liquor was added to 1-l wheat bran broth.
8. Spent barley broth (SBB); 20 g spent barley (Wards' Brewery, Sheffield) was boiled in 1-l distilled water for 30 min, filtered through muslin, and made up to 1-l with distilled water.
9. Spent barley broth-yeast (SBB-Y); 5 g brewers' yeast was added to 1-l spent barley broth.
10. Spent barley broth-corn steep liquor (SBB-CSL); 5 g corn steep liquor was added to 1-l spent barley broth.
11. Beet molasses (BM) containing 40 g/l beet molasses (United Molasses, Hull).
12. Beet molasses-yeast (BM-Y) containing 40 g/l beet molasses and 5 g/l brewers' yeast.
13. Beet molasses-corn steep liquor (BM-CSL) containing 40 g/l beet molasses and 5 g/l corn steep liquor.
14. V8 juice broth (V8); 200 ml V8 juice (Campbells Soups Ltd.) was added to 800 ml distilled water.
15. V8 juice broth-cholesterol (V8-ch); 200 ml V8 juice, 30 mg cholesterol as a 1.5% solution in 95% ethanol and 2.5 g CaCO<sub>3</sub> solution (clarified by centrifugation at 13200 g) were added to 800 ml distilled water.

After autoclaving, each medium was adjusted to pH 6.5 with sterile N-NaOH or N-HCl and each was dispensed in 45 ml aliquots into five replicate 250 ml Erlenmeyer flasks. Each flask was then inoculated with a 8 mm diam disc cut from a 2-d-old colony on CMA. Flasks were incubated at 25° for 21 d and biomass harvested by filtering through Whatman No. 1 filter paper. Spent medium from replicate flasks was

combined and the pH determined with a Whatman PHA 250 pH probe. Biomass dry weights from four of the five replicate flasks were determined after oven-drying at 60° for 36 h. Biomass was then suspended in 20 ml distilled water, comminuted in an MSE homogenizer and oospore numbers determined using a haemocytometer. The biomass in the remaining flask of each medium was washed in three changes of sterile distilled water and air-dried in a laminar flow cabinet at 18-21°. It was not possible to assess the germination of oospores produced in all liquid media. Air-dried biomass produced in only six media was resuspended in sterile distilled water to produce oospore suspensions containing c.  $2 \times 10^3$  oospores per ml. Aliquots (0.5 ml) were spread over the surface of CMA in three replicate 9 cm diam Petri dishes and germination of 150 oospores in each dish scored after 16 h at 25° as described in section 4.4.

Growth and oospore production in liquid media is summarized in Table 16. The pH of each medium increased during the growth period. All the media tested supported growth and oospore production, but to varying degrees. Biomass production was the greatest in MB and high yields were also obtained in CM-Y and V8-ch. Yields in V8, CM and CM-CSL were moderately high, whilst those in all the other media were generally lower. The smallest yield was obtained in BM. On a dry weight basis, maximum oospore production occurred in CM. High numbers were also produced in V8-ch, CM-Y, CM-CSL, SBB, and SBB-CSL. Biomass yields in a few media such as SBB and SBB-CSL were small, whereas numbers of oospores produced per unit biomass were quite high in comparison with the other media. On a medium volume basis, the greatest number of oospores was produced in CM-Y. Numbers produced in V8-ch, CM, CM-CSL

Table 16

Growth and oospore production by Pythium oligandrum in liquid media after 21 d at 25°. Each value is the mean with SEM from four replicate flasks.

Medium*	Final pH**	Dry weight biomass (mg)	Dry weight biomass per vol. medium (mg/ml)	Oospores per mg dry weight (x10 <sup>5</sup> )	Oospores per ml medium (x10 <sup>5</sup> )	Germination (%)
V8-ch	7.5	105.4±2.2	2.34±0.05	24.63±1.06	57.50±1.25	12-19
V8	7.2	81.5±0.7	1.81±0.02	16.35±0.23	29.61±0.51	—***
CM	6.6	82.6±0.4	1.84±0.01	25.46±0.56	46.78±1.19	16-19
CM-Y	7.2	124.4±1.6	2.76±0.04	22.80±0.10	63.06±0.98	13-18
CM-CSL	6.9	89.2±0.7	1.98±0.02	24.39±0.29	48.33±0.38	—
MB	7.4	130.9±2.3	2.91±0.05	16.90±0.53	49.11±1.21	—
WBB	8.5	31.0±0.8	0.69±0.02	0.06±0.003	0.04±0.003	—
WBB-Y	8.8	61.6±0.7	1.37±0.01	1.97±0.034	2.70±0.069	13-19
WBB-CSL	8.9	47.8±1.2	1.06±0.02	0.55±0.036	0.59±0.038	—
SBB	8.3	29.1±0.8	0.65±0.02	23.94±0.21	15.45±0.29	—
SBB-Y	8.9	47.8±1.0	1.06±0.02	17.21±0.33	18.29±0.28	16-17
SBB-CSL	8.7	33.4±1.1	0.75±0.02	23.87±0.51	17.70±0.34	—
BM	7.8	11.4±0.4	0.25±0.01	0.16±0.006	0.04±0.002	—
BM-Y	9.2	60.8±2.6	1.35±0.06	1.77±0.075	2.38±0.100	13-19
BM-CSL	8.9	30.6±1.5	0.68±0.03	2.00±0.085	1.35±0.024	—
LSD (P=0.05)		3.9	0.09	1.12	1.83	

\* Media designations are V8-ch (V8 juice broth-cholesterol), V8 (V8 juice broth), CM (cane molasses), CM-Y (cane molasses-yeast), CM-CSL (cane molasses-corn steep liquor), MB (maizemeal broth), WBB (wheat bran broth), SBB (spent barley broth), BM (beet molasses).

\*\* The pH of each medium was adjusted to pH 6.5 before inoculation.

\*\*\* Oospore germination not assessed.

and MB were very high compared to those in the remaining media. Germination of oospores produced in six of the media was between 13 and 19% after 16 h on CMA.

The results suggest that a number of the inexpensive media tested can be used for producing germinable oospores. Cane molasses medium supported both high biomass and oospore yields, which were similar to those obtained with expensive media such as V8-ch. Coupled with the ease at which the medium is prepared in the laboratory, it was chosen as a suitable medium for producing oospores.

#### 5.4 Effect of incubation period on oospore production in static cane molasses liquid culture

A time-course study in static culture was carried out to investigate the relationship between total biomass yield (mycelium and oospores) and number of oospores produced. Twelve 250 ml Erlenmeyer flasks each containing 45 ml sterile cane molasses liquid medium, (pH 5.2-5.6), (as in 5.3) were inoculated with a 2 ml oospore suspension containing c.  $1 \times 10^4$  oospores per ml. Flasks were incubated at 25°, and at 7 d intervals over a 21 d period four flasks were removed and biomass harvested. Biomass dry weights and oospore numbers were determined as described before.

For results, see Table 17. Total biomass and oospore production increased over the incubation period to reach a maximum at 21 d. Oospores were present within biomass after 7 d and there was a marked increase in their production (140% and 290% on a dry weight and medium volume basis respectively) between 7-21 d. Total biomass increased only 63% during this period. Biomass harvested after 21 d consisted

Table 17

Effect of incubation period on biomass and oospore production by Pythium oligandrum in static cane molasses liquid medium at 25°.

Each value is the mean of four replicates with SEM.

---

Incubation period (days)	Dry weight			
	Dry weight biomass (mg)	biomass per vol. medium* (mg/ml)	Oospores per mg dry weight (x10 <sup>5</sup> )	Oospores per ml medium (x10 <sup>5</sup> )
7	46.6±0.9	1.04±0.02	1.11±0.04	1.14±0.03
14	67.9±0.5	1.51±0.01	1.61±0.05	2.42±0.06
21	76.0±1.4	1.69±0.03	2.67±0.03	4.51±0.10
LSD (P=0.05)	3.1	0.07	0.13	0.22

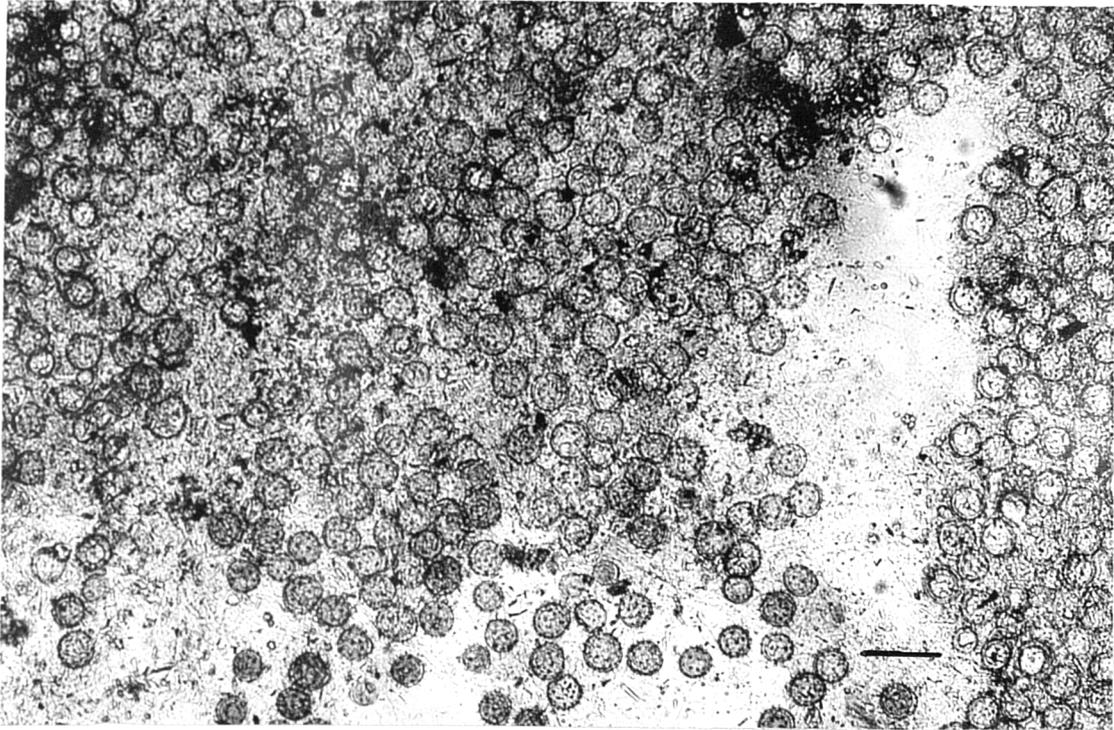
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\* Each flask contained 45 ml medium.

Plate 1

Aggregates of oospores in a biomass preparation of Pythium oligandrum.

Bar = 40  $\mu\text{m}$ .



mainly of oospore aggregates (Plate 1).

#### 5.5 Biomass and oospore production of six isolates in static cane molasses liquid culture

Biomass and oospore production of six different isolates in cane molasses liquid medium was compared. Details of the isolates used are listed in Table 1 (Chapter II). Four replicate 250 ml Erlenmeyer flasks each containing 45 ml medium were inoculated with a 8 mm diam mycelial disc cut from a 2-d-old colony of a single isolate on CMA. Flasks were incubated at 25° for 21 d after which time biomass and oospore production was assessed as described previously.

For results, see Table 18. Biomass production of all isolates varied between 64 and 101 mg. Isolates MPMP01 and MPMP02 produced the greatest yields, whilst the smallest yield was produced by MPMP04. All isolates produced high numbers of oospores. On a medium volume basis, oospore numbers were greatest with IMI 133857. Numbers for MPMP01 and MPMP03 were the lowest in comparison with the other isolates.

#### 5.6 Effect of culture conditions on biomass and oospore production in cane molasses liquid medium

Industrial fermenters are equipped to provide a wide range of culture conditions (Knight, 1988). In order to develop large-scale liquid culture techniques for the production of oospores, it is desirable to have a basic understanding as to how culture conditions affect growth and oospore production. The effects of culture aeration and agitation on biomass and oospore production were determined.

Cultures were either aerated, shaken or kept static, replication

Table 18

Biomass and oospore production of six Pythium oligandrum isolates in cane molasses liquid medium after 21 d at 25°.

Each value is the mean from four replicates with SEM.

---

Isolate	Dry weight			
	Dry weight biomass (mg)	biomass per vol. medium* (mg/ml)	Oospores per mg dry weight ( $\times 10^5$ )	Oospores per ml medium ( $\times 10^5$ )
IMI 133857	76.4 $\pm$ 1.3	1.70 $\pm$ 0.03	2.44 $\pm$ 0.05	4.14 $\pm$ 0.11
MPMP01	98.0 $\pm$ 1.9	2.18 $\pm$ 0.04	0.97 $\pm$ 0.04	2.10 $\pm$ 0.06
MPMP02	85.9 $\pm$ 2.4	1.91 $\pm$ 0.05	1.98 $\pm$ 0.06	3.76 $\pm$ 0.09
MPMP03	100.2 $\pm$ 3.3	2.23 $\pm$ 0.07	0.93 $\pm$ 0.05	2.05 $\pm$ 0.03
MPMP04	64.9 $\pm$ 1.2	1.44 $\pm$ 0.03	2.26 $\pm$ 0.04	3.27 $\pm$ 0.11
MPMP05	78.8 $\pm$ 2.0	1.77 $\pm$ 0.05	1.88 $\pm$ 0.06	3.30 $\pm$ 0.02
LSD (P=0.05)	6.2	0.15	0.15	0.23

---

\* Each flask contained 45 ml medium.

being five-fold. Fifteen 250 ml Erlenmeyer flasks each containing 100 ml cane molasses liquid medium (as in 5.3) (pH 5.2-5.6) were inoculated with a 5 ml oospore suspension containing c.  $1 \times 10^4$  oospores per ml. Aeration and agitation of cultures was provided by passing compressed air at a rate of 450-500 ml/min via 3 cm diam Millipore bacterial vents (3  $\mu$ m pore size) through a glass delivery tube (5 mm diam) 2 cm from the bottom of each flask. Excess air was passed through a Ferris outlet filter. Shake cultures were placed on a Fisons gyratory shaker at 110 rev/min. Flasks were incubated at 25° for 21 d and biomass and oospore production was determined as before.

The results are shown in Table 19. *P. oligandrum* grew as dispersed filaments in static culture. In aerated and shake culture, the fungus produced small, discrete mycelial pellets. Biomass production in aerated and shake cultures was similar, and yields were greater than in static culture. The greatest number of oospores was produced in shake culture and numbers in aerated culture were greater than those in static culture.

#### 5.7 Large-scale oospore production in static and aerated cane molasses liquid culture

Large volume static liquid cultures were produced in 2-l glass mould-culture flasks, 9 cm in depth of 17 cm diam, and fitted with side inoculation and sampling ports. Four flasks each containing 200 ml medium were each inoculated with three 15 mm diam mycelial discs cut from a 2-d-old colony on CMA. Flasks were incubated for 18 d at 25°, being shaken at 3 d to disperse inoculum. Biomass was removed by vacuum filtration as before, washed in sterile distilled water, and

Table 19

Effect of culture conditions on biomass and oospore production of Pythium oligandrum in cane molasses liquid medium after 21 d at 25°.

Each value is the mean from five replicates with SEM.

---

Culture	Dry weight biomass (mg)	Dry weight		
		biomass per vol. medium* (mg/ml)	Oospores per mg dry weight (x10 <sup>5</sup> )	Oospores per ml medium (x10 <sup>5</sup> )
Static	112.9±1.4	1.13±0.01	2.20±0.06	2.39±0.04
Shake	162.9±1.0	1.63±0.01	3.24±0.02	5.28±0.03
Aerated	157.6±3.3	1.57±0.03	2.58±0.03	4.07±0.09
LSD (P=0.05)	6.6	0.07	0.13	0.19

---

\* Each flask contained 100 ml medium.

dried overnight in a laminar flow cabinet at 18-21° prior to weighing.

Aerated liquid cultures were grown in 2-l autoclavable aspirators (Plate 2). Aeration and agitation was provided as before by passing compressed air at a rate of 600-800 ml/min via 3 cm diam Millipore bacterial vents (3 µm pore size) through a ring glass sparger 2.5 cm from the bottom of each vessel. Excess air was passed through two Ferris outlet filters. Autoclaved medium was adjusted to pH 6.5 with sterile N-NaOH, and 1-l aliquots were added to each of eight vessels together with 3 ml sterile Antifoam A (Sigma Chemical Co.). Each vessel was inoculated with 100 ml oospore suspension containing c.  $1 \times 10^4$  oospores per ml. Vessels were incubated at 25° and after 7 and 14 d, biomass from four replicates was harvested. Biomass and spent medium were separated by centrifugation at 1200 g for 30 min followed by washing in sterile distilled water, air-drying overnight and weighing.

To quantify oospore production in both culture systems, dried biomass was ground to a fine powder. A 50 mg sample was then suspended in 40 ml distilled water, blended in an MSE homogenizer for 2 min and oospores counted in a haemocytometer. To assess oospore germination, biomass suspensions were diluted with sterile distilled water to produce a spore density of c.  $2 \times 10^3$  per ml. Aliquots (0.5 ml) were spread over the surface of CMA in five replicate 9 cm diam Petri dishes and germination of 250 oospores in each dish scored after 16 h at 25° as described in section 4.4.

Growth morphologies in static and aerated cultures were identical to those in similar culture conditions in the previous experiment. For results, see Table 20. In aerated culture, total fungal biomass and oospore production increased slightly between 7 and 14 d incubation.

Plate 2

Aspirators fitted with inlet (A), outlet (B) filters and glass sparger (C).

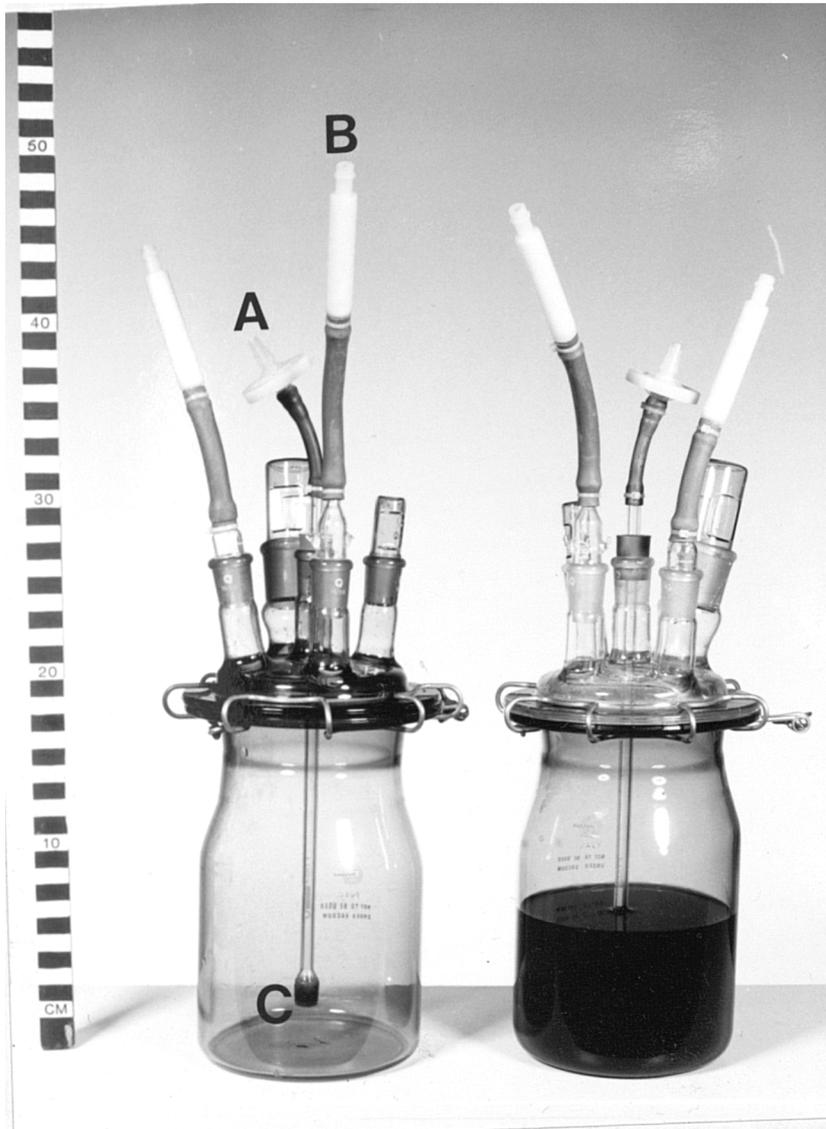


Table 20

Growth and large-scale oospore production by Pythium oligandrum in static and aerated cane molasses liquid medium at 25°.

Each value is the mean from four replicates with SEM.

Culture	Incubation period (days)	Dry weight biomass (mg)	Dry weight per vol. medium (mg/ml)	Oospores per ml medium		Germination range (%)
				dry weight ( $\times 10^5$ )	( $\times 10^5$ )	
Static	18	217.7± 9.7	1.09±0.05	1.40±0.07	1.53±0.11	19-23
Aerated	7	1781±19.7	1.78±0.02	2.06±0.11	3.68±0.23	16-19
	14	1971±32.0	1.97±0.03	2.14±0.05	4.22±0.04	16-20

Biomass and oospore production was greater in aerated compared with static culture. Oospores produced in the aerated culture system had a similar germination (16-20% after 16 h on CMA at 25°) as those produced in static culture (19-23%).

## CHAPTER VI

### PREPARATION, STORAGE AND SURVIVAL OF OOSPORE FORMULATIONS

## CHAPTER VI

### PREPARATION, STORAGE AND SURVIVAL OF OOSPORE FORMULATIONS

The lack of formulation and delivery systems for antagonistic micro-organisms is an obstacle to the advancement of biocontrol research. It has already been shown in the previous Chapter that viable oospores can be mass-produced on inexpensive semi-solid and liquid media. In order to attempt widespread biocontrol of soil-borne diseases with P. oligandrum, suitable formulations for delivering oospores to the soil microbiome are required. Previously, this has been done by incorporation of solid substrate preparations directly to the sowing sites or manual seed-coating using mycelium and oospores produced on expensive complex liquid laboratory media (Vesely, 1977, 1979; Al-Hamdani et al., 1983; Starling & Butler, 1983; Vesely & Hejdanek, 1981, 1982, 1984; Martin & Hancock, 1987; Walther & Gindrat, 1987a; Vesely, 1989). Here, studies were carried out to develop a range of suitable formulations mainly using oospore biomass produced in static cane molasses liquid culture (as in 5.7). Moreover, the behaviour and survival of the oospore formulations in soil, in a soilless mix, and in laboratory storage at a range of temperatures was also monitored.

#### 6.1 Effect of storage temperature on the germination of oospores produced in liquid culture

Oospore biomass produced from 18-21 d static cultures was air-dried overnight in a laminar flow cabinet at 18-21°. Biomass was then ground

to a fine powder in a Wiley mill. To monitor oospore survival, dried powdered biomass was divided into fifteen 400 mg samples, each being placed in an 18 ml vial. Vials were covered loosely and stored at 5, 15, 20, 25, and 30°, with three replicates for each temperature. After 0, 3, 6, 9 and 16 wk samples of c. 50 mg were removed from each vial and oospore suspensions were prepared as described in section 5.3. To assess oospore germination, aliquots (0.5 ml) of oospore suspension were spread over the surface of CMA in three replicate 9 cm diam Petri dishes and germination of 250 oospores in each dish was scored after 16 h at 25° as described in section 4.4.

Results are shown in Fig. 11 and Appendix 4.1. Germination of freshly-produced oospores on CMA was 19-23% after 16 h, but storage at 5-30° reduced germination. This reduction was more pronounced at 20, 25 and 30° than at 5 and 15° over the 16 wk storage period. Storage at 20, 25 and 30° resulted in a significant reduction in germination after only 3 wk, but germination was still above 1% even after 16 wk.

## 6.2 Preparation of formulations from oospore biomass

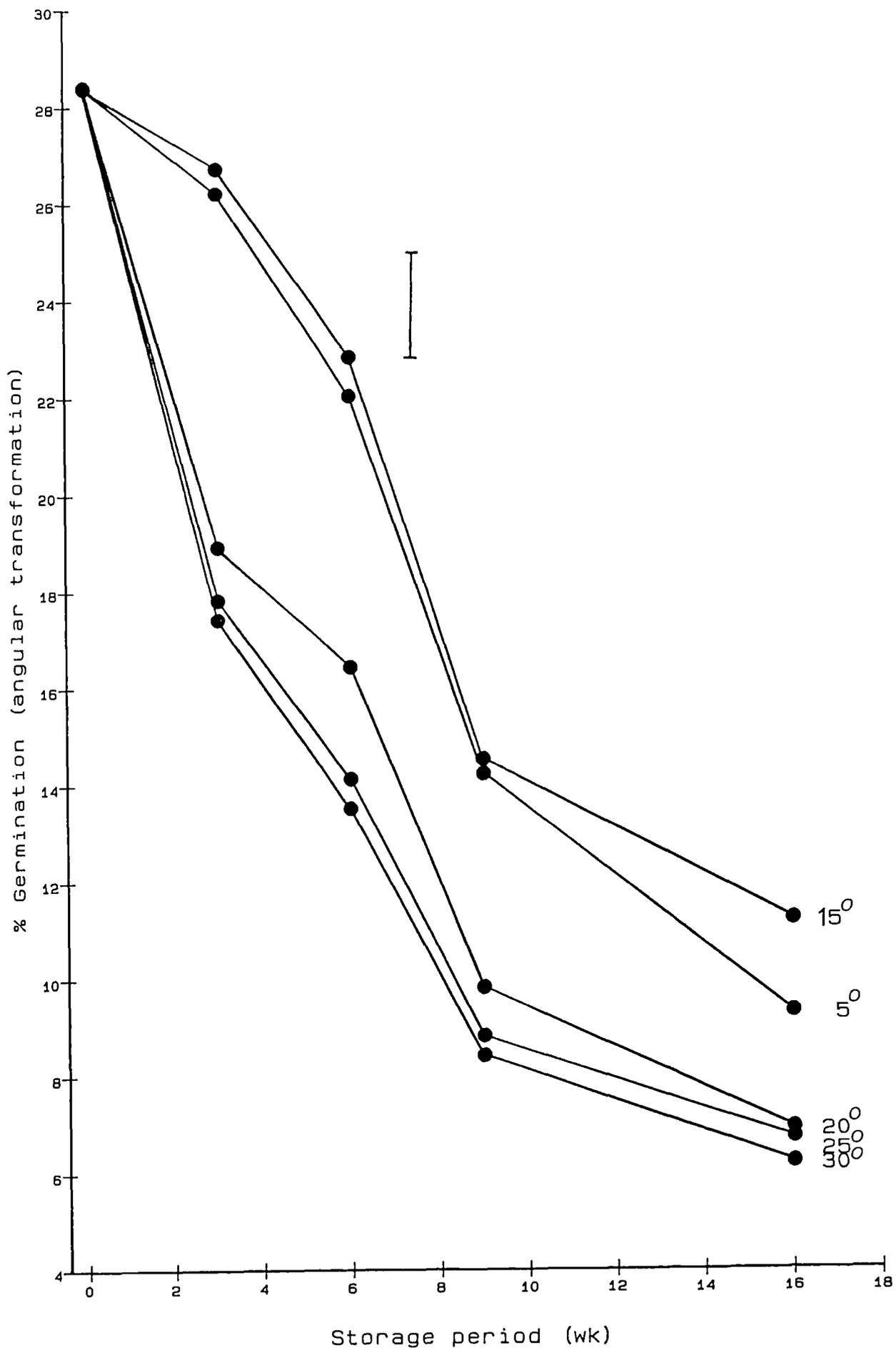
Four different formulations were prepared from oospore biomass produced from 18-21 d static cultures. After harvest, biomass was first washed in three changes of distilled water and air-dried in a laminar flow cabinet at 18-21°.

### 1. Oospore coated seed

Cress seed (Lepidium sativum L., cv. 'Extra Curled'; W. J. Unwin Ltd., Histon, Cambs.) was coated with oospores by a modification of a method of Al-Hamdani et al., (1983). Oospore biomass (c. 300 mg) was suspended in 40 ml sterile distilled water and blended in an MSE

Figure 11

Effect of storage temperature on germinability of oospores from biomass of Pythium oligandrum. Each point is the mean germination of oospores stored in three vials. For SEM, see Appendix 4.1. The bar indicates the least significant difference at  $P=0.05$ .



homogenizer for 2 min. Suspensions were centrifuged at 5000 g for 10 min, resuspended, and washed in three changes of sterile distilled water. Equal amounts 3% (v/v) carboxymethyl cellulose (CMC) and oospore suspension (5 ml) were mixed thoroughly for 15 min on a mechanical shaker. Cress seeds were surface-sterilized in 5% sodium hypochlorite for 10 min followed by rinsing in 3-4 changes of sterile distilled water, and air-drying overnight in a laminar flow cabinet. Approximately 250 seeds were mixed with the CMC suspension for 15 min. Seeds were then spread sparsely in sterile open Petri dishes and allowed to dry overnight in a laminar flow cabinet. Batches of seed were also prepared using oospore-free CMC.

To determine the number of oospores applied to each seed, 15 seeds were selected and five placed in each of three McCartney bottles containing 5 ml water. The bottles were then vigorously agitated on a mechanical shaker for 3-4 min which removed and suspended the oospores. Oospore numbers were determined using a haemocytometer. The ability of oospores to generate a mycelium from seed was assessed immediately after seed coating and at 3-monthly intervals during storage for 1 yr at 15°. Twenty seeds were incubated on CMA for 48 h at 25° after which time seeds giving rise to mycelium of P. oligandrum were counted.

Between  $2-6 \times 10^4$  oospores adhered to each seed. When placed on CMA mycelium of P. oligandrum grew from all seeds within 24 h throughout their 12 month storage period.

## 2. Alginate pellets

It has recently been shown that use of alginate pellet formulations is an innovative and feasible approach to applying some micro-organisms to soil for biocontrol of soil-borne diseases (Fravel et al., 1985,

1986a, 1986b; Lewis & Papavizas, 1985, 1987, 1988; Lumsden & Locke, 1989; Papavizas & Lewis, 1989; Lumsden & Lewis, 1989). Previously, spores of various biocontrol fungi have been encapsulated in alginate pellets with a carrier, which may be an inert clay, a nutrient base or a combination of both. It was therefore considered possible to encapsulate oospores of P. oligandrum in alginate pellets containing combinations of kaolin clay and two nutrients.

Alginate pellets containing oospores were prepared by a modified method of Walker & Connick (1983). Oospore biomass suspensions were mixed with sodium alginate containing either a clay kaolin carrier, or D-glucose and myo-inositol, or all three in combination, to give the following oospore mixtures:

1. Sodium alginate + kaolin + nutrients
2. Sodium alginate + kaolin
3. Sodium alginate + nutrients
4. Sodium alginate

myo-Inositol was used as germinability and survival of oospores has previously been increased by oospore treatments such as washing with a myo-inositol solution (Walther & Gindrat, 1987a). Sodium alginate (20 g) was dissolved in 500 ml aliquots of distilled water at 40° on a stirring hot plate and autoclaved. Depending upon the contents of the final oospore mixture, batches of cool sodium alginate solution (500 ml) were either made upto 1-l with sterile distilled water or sterile kaolin light solution (BDH Ltd.: 100 g/500 ml) and then blended together for 30 s at high speed. One litre aliquots of alginate solution and alginate-kaolin mixture were each further supplemented with D-glucose (10g) and myo-inositol (100g). Aliquots (180 ml) of each

mixture were then amended with 20 ml of oospore suspension which was prepared by homogenizing c. 250 mg oospore biomass in 40 ml sterile distilled water. Final mixtures contained  $2.0 \times 10^5$  oospores per ml and had pH's between 5.5 and 7.0.

Individual alginate-oospore mixtures were dispensed into a glass dish, 6 cm deep and of 9 cm diam fitted with an outlet coupling at its base. The mixture was stirred continuously on a magnetic stirrer while it was dripped through plastic tubing (4 mm, internal diam) and a Pasteur pipette with a 1 mm diam orifice into a solution of 0.25 M  $\text{CaCl}_2$  (pH 5.4). As it entered the  $\text{CaCl}_2$  solution, each droplet gelled and a distinct, spherical pellet formed (Plate 3). After 20 min in the gellant, pellets were separated from the solution by gentle filtration, washed, and dried overnight in a stream of air at 19-23° in a laminar flow cabinet. The initially formed spongy, gelatinous alginate pellets (3-4 mm diam) dried to hard, spherical granules (1-2 mm diam) with a rough surface (Plate 4). Pellets were generally used in biocontrol and storage experiments within 2 d of preparation. Control pellets without oospore biomass were also prepared.

### 3. Oospore dust

Air-dried oospore biomass was ground in a Wiley mill to produce a fine dust. The resulting powder was mixed (1:5 v/v) with kaolin clay, pH 5-6, as a diluent to increase the volume for distribution purposes.

### 4. Perlite preparation

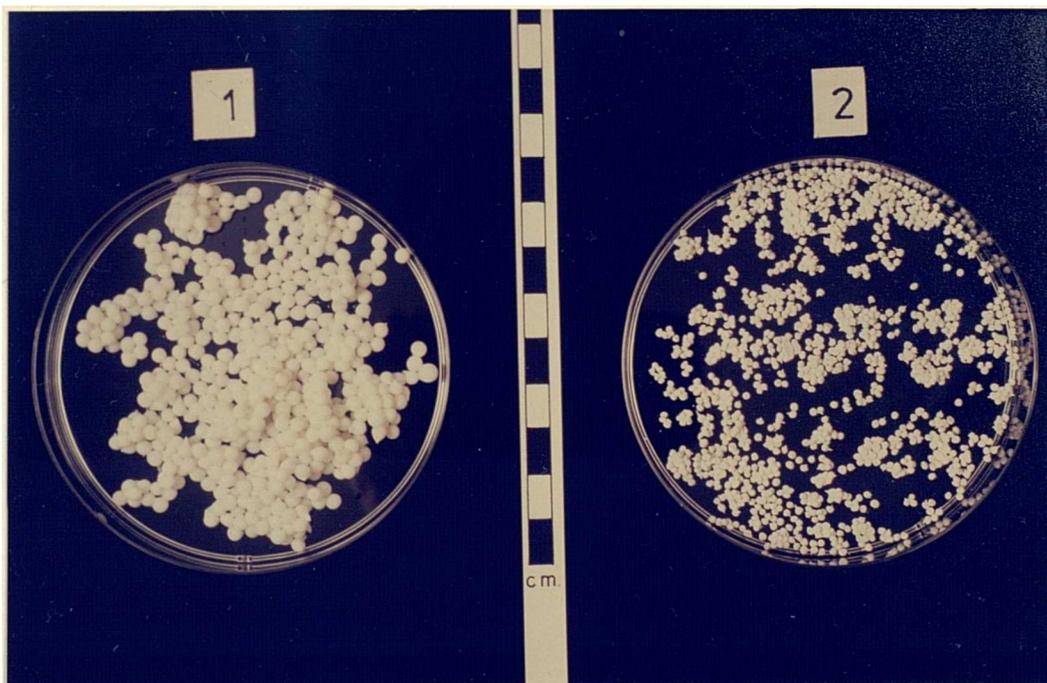
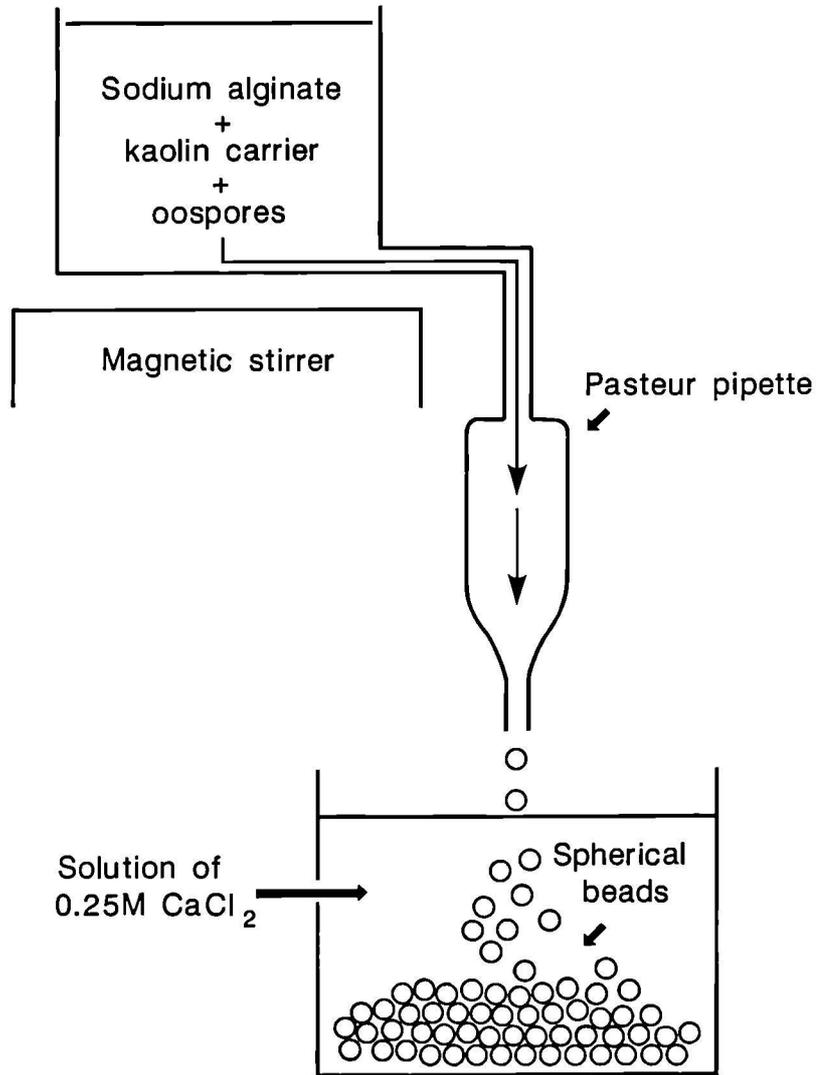
Perlite (Silvaperl Products Ltd., Harrogate) was impregnated with an aqueous solution containing 3% (v/v) CMC as an adhesive. The perlite, while still wet, was coated with powdered air-dried oospore biomass (perlite : oospore biomass, 8:1, w/w) by mixing the mixture and

### Plate 3

Diagram of the apparatus used to form sodium alginate pellets containing oospores of Pythium oligandrum. An aqueous suspension of oospores, sodium alginate, nutrients and a kaolin clay carrier is dripped into a solution of calcium chloride. The calcium displaces the sodium, causing formation of a solidified shell around the drop.

### Plate 4

Alginate-clay pellets containing oospores of Pythium oligandrum.  
Immediately produced pellets (1) and air-dried (2).



air-drying it overnight in a laminar flow cabinet. The impregnated perlite was light, easy to use and contained  $2-6 \times 10^6$  oospores per g.

### 6.3 Growth from alginate pellets in soil

The ability of P. oligandrum to grow actively from the oospore formulated pellet in soil was tested using two methods. In both a medium loam soil (pH 6.5) collected from the University of Sheffield Experimental Garden was used. The water potential of the soil was adjusted to c.  $-0.05$  MPa by adding known amounts of water to air-dried soil; these being determined from a water adsorption curve (see Appendices 4.2 & 4.3). The first method involved the use of a soil-sandwich technique previously described by Grose et al., (1984). Two nucleopore membranes (Nucleopore, 25 mm diam,  $0.2 \mu\text{m}$ ) were carefully placed on a layer of soil in a 9 cm diam glass Petri dish, replication being five-fold. In a second method, cellophane discs were used instead of nucleopore membranes. Single boiled, autoclaved, 11 mm diam cellophane discs (British Cellophane Co. PT600) were placed on soil in six replicate 14 cm diam glass Petri dishes. Individual alginate pellets containing oospores of P. oligandrum, kaolin, D-glucose and myo-inositol were placed in the centre of a nucleopore membrane or cellophane disc and covered with soil. Lids of individual Petri dishes were sealed in 'Cling-film' and placed in sealed polyethylene bags together with dishes of KCl-modified TWA of the same water potential to help maintain the required water status. Dishes were incubated at  $18^\circ$  for 10 d after which time membranes or discs were carefully removed from the soil using forceps, and soil particles removed by gentle shaking. Discs and membranes were stained with a few

Plate 5

Mycelial growth of Pythium oligandrum (A) from an alginate pellet (B) on a cellophane disc (C) carefully placed on a layer of soil (D) in a 14 cm diam glass Petri dish. The alginate pellet was previously covered with soil. Surface soil was removed carefully and the colony of P. oligandrum (A) stained blue with lactophenol in cotton blue.



drops of cotton blue. Mycelial growth was examined using a microscope and colony diameters measured where possible.

When pellets were buried in soil on nucleopore membranes, no mycelial growth of P. oligandrum was observed after 10 d incubation. In contrast, mycelial growth occurred from pellets placed on cellophane discs with the clear formation of colonies (Plate 5). Mycelial colonies were  $41.2 \pm 1.05$  mm in diam after 10 d.

#### 6.4 Effect of storage temperature on the survival of oospores in oospore biomass formulations

Storage and stability are important criteria for an effective biocontrol formulation. The effect of storage temperature on the survival of oospores in alginate pellets and the perlite preparation was studied.

##### 1. Alginate pellets

Survival of oospores incorporated into four different alginate pellet formulations (as in 6.2) was monitored at three storage temperatures. Each air-dried alginate pellet formulation was divided into nine 6 g samples. Individual samples were placed in a 50 ml screw-top glass container, and stored at 5, 15 and 25°, with three replicates for each temperature. After 0, 2, 4, 8, 12 and 24 wk, quantitative assessment of the number of colony-forming units (cfu) per g of air-dried pellets was determined. A 0.5 g sample of alginate pellets was removed from each container and ground with a surface-sterilized pestle and mortar. The powder was brushed into a test-tube containing 10 ml 0.1% (w/v) sterile distilled water agar (no. 3 Oxoid) and the suspension was mixed for 60 s with a Rotamixer. A

dilution series was prepared down to  $10^3$  using sterile disposable pipettes. Each dilution was mixed for 60 s and 0.2 ml aliquots were spread over the surface of the modified selective medium (see Appendix 3.1) of Martin & Hancock (1986), in three replicate 9 cm diam Petri dishes. Petri dishes were incubated for 48 h at 25° after which time solid residue was washed from the agar surface under a gentle stream of water. Dishes were assessed by counting those which had between 4 and 12 colonies of P. oligandrum.

Table 21 shows the changes in cfu recovered per g of air-dried pellets over the 24 wk storage period at 5, 15 and 25°. Oospores survived the rigours of the pellet formation process and similar colony counts were observed using all freshly-produced formulations. Colony counts from all pellet formulations were reduced with lengths of storage at each temperature, but oospores in pellets still produced colonies on agar media after 24 wk. This reduction in colony count was generally more pronounced at 15° and 25° than at 5° over the storage period. During the storage period, colony counts from oospore pellets of sodium alginate alone were similar to those from pellets of sodium alginate plus kaolin stored at the same temperature. In addition, pellets formulated with nutrients generally produced greater colony counts than those containing nutrients plus kaolin; suggesting that kaolin may have an adverse effect on oospore survival.

## 2. Perlite preparation

Air-dried oospore-coated perlite (as in 6.2) was divided into nine 8 g samples, each being placed in a 50 ml screw-top glass container. Containers were stored at 5, 15 and 25° with three replicates for each temperature. After 0, 4, 8, 12 and 24 wk, samples were removed.

Table 21

The effect of storage temperature on the numbers of colony-forming units (log. cfu/g) from oospores of Pythium oligandrum in four different formulations of air-dried alginate pellets. Clay kaolin (K) and, D-glucose plus myo-inositol (Glu + Inos), were added singly and in combination to sodium alginate (NaA). Colony-forming units were determined for three replicate 1 g samples, one from each of three glass containers.

Storage period (wk)		Formulation												
		NaA			NaA + K			NaA + (Glu + Inos)			NaA + K + (Glu + Inos)			
5°	15°	25°	5°	15°	25°	5°	15°	25°	5°	15°	25°	5°	15°	25°
0	4.84	4.87	4.89	4.85	4.86	4.83	4.89	4.85	4.89	4.85	4.89	4.82	4.84	4.82
2	3.92	3.93	3.49	4.07	4.07	3.90	4.11	4.10	3.84	4.05	4.04	4.07	3.86	3.86
4	2.93	2.83	2.44	3.20	2.80	2.66	4.01	3.92	3.79	4.07	3.86	4.07	3.86	2.92
8	2.90	2.59	2.20	2.99	2.75	2.28	4.15	3.00	3.90	3.96	3.00	3.96	3.00	2.48
12	2.98	2.83	2.41	3.05	2.68	2.43	3.74	3.88	3.83	3.96	2.91	3.96	2.91	2.34
24	2.64	1.63	1.62	2.50	1.52	1.45	3.65	3.20	2.65	3.82	2.88	3.82	2.88	1.72
LSD (P=0.05)														0.22

Twenty-five oospore-perlite granules (c. 2-4 mm diam) from each storage container were placed on CMA in 9 cm diam Petri dishes, at five per dish. Petri dishes were incubated at 25° and the number of perlite granules (out of 25) showing growth after 48 h was determined.

Results are shown in Table 22. The number of oospore-coated perlite granules giving rise to P. oligandrum mycelium declined at 25° and 15° over the 24 wk duration of the experiment. This decline was more pronounced at 25° than at 20°. However, at 5° the proportion of perlite granules from which growth occurred remained high. Even after 24 wk storage at 5°, 100% of the granules tested gave rise to a luxuriant mycelium.

#### 6.5 Survival of oospore dust in soil and a soilless potting compost

Survival of the oospore dust preparation (as in 6.2) was monitored in a medium loam soil (as in 6.3) and potting compost, both moistened to c. -0.05 MPa. The compost, pH 6.8, consisted of 50% Irish sphagnum peat and 50% sand amended with the following: chalk, (0.62 g/l); macronutrients, (KNO<sub>3</sub>, 0.38 g/l and superphosphate 0.77 g/l); and micronutrients, (Frit WM255, FENO Chemical Division, 400 mg/l). A preliminary experiment showed that no Pythium spp. were present in the compost. The soil carried a natural population of P. ultimum, but P. oligandrum was absent. Samples (6 g) of oospore dust were mixed with three replicate 100 g (dry weight equivalent) of each medium and placed in glass containers, 10 x 7.5 cm, covered with Parafilm punctured to permit gas exchange. After 0, 1, 4, 8, 12 and 16 wk incubation at 15°, 10 g (equivalent dry weight) samples were removed from each container. Each sample was added to 100 ml 0.1% (w/v) distilled water agar (no. 3

Table 22

The effect of storage temperature on the ability of Pythium oligandrum to grow from perlite impregnated with oospores plated on CMA after 48 h at 25°C.

Values in parentheses are means with SEM from three replicates calculated after angular transformation.

---

% of perlite pieces giving rise to mycelium\*

---

Temperature (°c)

---

Storage (wk)	5	15	25
0	100 (90.0)	100 (90.0)	100 (90.0)
4	88 (69.9±2.1)	100 (90.0)	65 (53.9±1.6)
8	97 (84.5±5.5)	92 (73.9±2.5)	49 (44.6±2.8)
12	100 (90.0)	69 (56.4±1.7)	55 (47.7±0.8)
24	100 (90.0)	45 (42.3±1.6)	39 (38.4±2.1)
LSD (P=0.05)		(5.8)	

---

\* Twenty-five pieces of oospore-coated perlite (c. 2-5 mm diam) were removed from each of three glass jars and plated on CMA.

Oxoid) and macerated in an MSE homogenizer for 2 min. Dilutions were prepared and colony-forming units (cfu/g dry weight) were determined on media as described in section 6.4, but by plating 0.5 ml aliquots of each dilution on agar.

On soil plates, colonies of P. oligandrum could be distinguished morphologically from those of P. ultimum. After 48 h at 25°, colonies of P. oligandrum were c. 12 mm in diam with diffuse, regularly branched hyphae radiating from the centre. Colonies of P. ultimum differed in that they were c. 25 mm in diam after 48 h incubation and grew from propagules as several thick hyphae covered with short, densely branched hyphae in the centre which gave way to longer branches at the periphery.

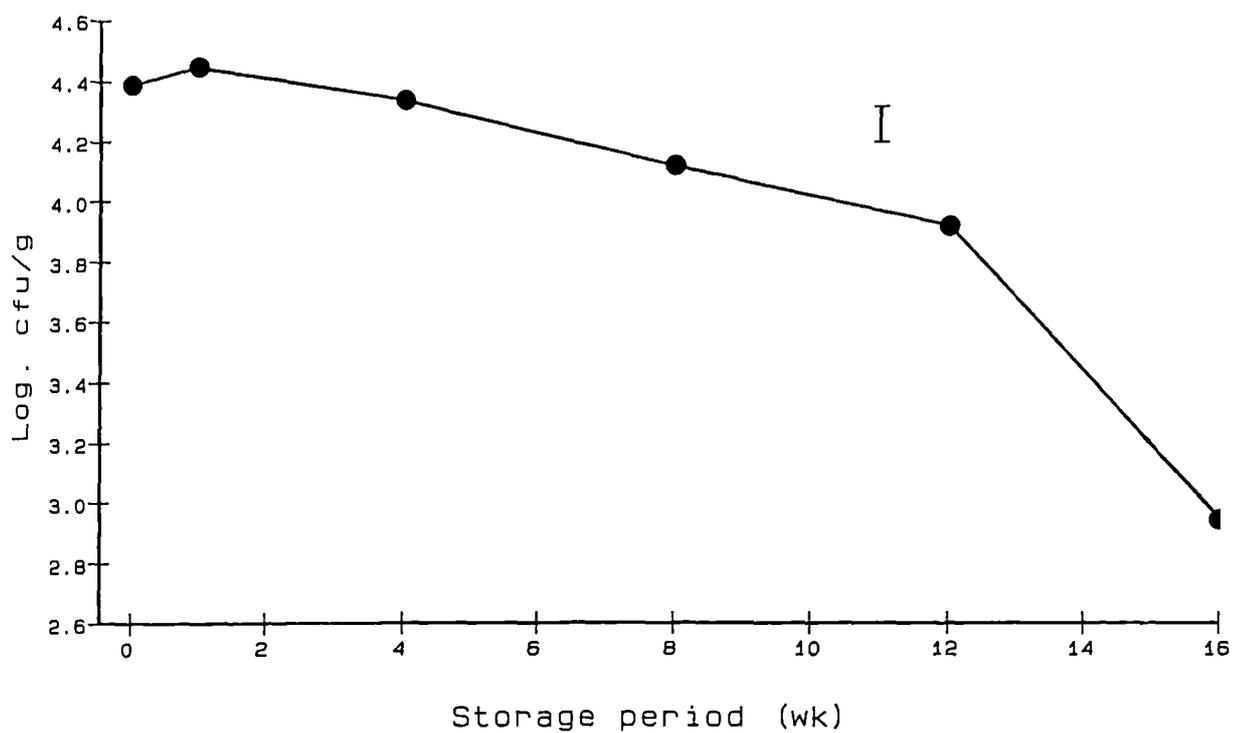
Results are shown in Fig. 12 and Appendix 4.4. One week after addition of oospore dust to soil and potting compost, survival of P. oligandrum was relatively high. In fact, there was a marginal increase in the number of colony-forming units recovered compared to those recovered after the initial incorporation of the oospore preparation. However, with each successive 4 wk period of storage there was a decrease in the number of colony-forming units recovered. This decrease was generally greater for soil than for compost.

Figure 12

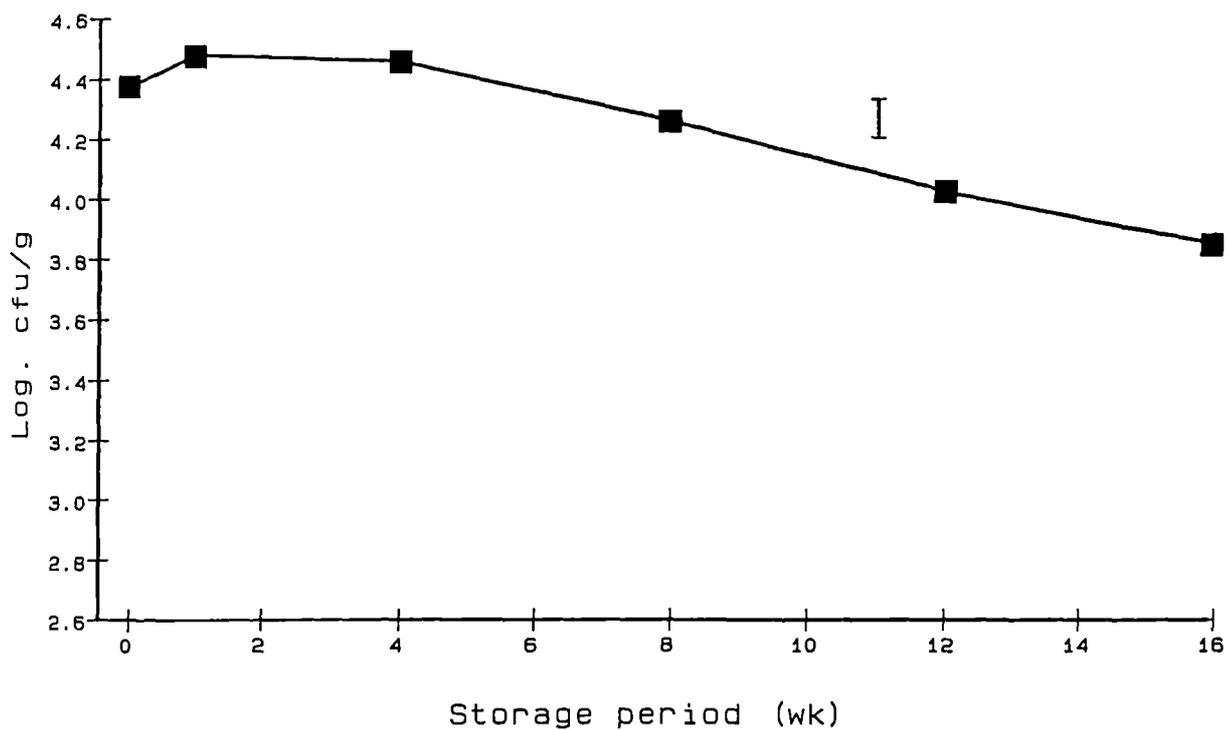
Effect of storage on the numbers of colony-forming units of Pythium oligandrum from oospore biomass dust in a medium loam soil (●) and potting compost (■). Each point is the mean log. cfu/g dry weight soil or compost in three replicate glass containers stored at 15°C.

For SEM, see Appendix 4.4. The bars indicate the least significant differences at P=0.05.

### Soil



### Compost



CHAPTER VII

USE OF OOSPORE FORMULATIONS FOR BIOLOGICAL  
CONTROL OF DAMPING-OFF

## CHAPTER VII

### USE OF OOSPORE FORMULATIONS FOR BIOLOGICAL CONTROL OF DAMPING-OFF

It was shown in Chapter VI that oospores of P. oligandrum could be incorporated successfully into a number of suitable formulations. The next stage towards using these on a commercial scale was to evaluate their effectiveness in controlling disease under defined experimental conditions. Here, experiments in artificially infested sand were conducted to evaluate the effectiveness of formulations in reducing damping-off of cress (Lepidium sativum L.) caused by P. ultimum and Rhizoctonia solani. Cress, cv. 'Extra Curled', obtained from W. J. Unwin Ltd., Histon, Cambridge was chosen as a test host because of its high susceptibility to damping-off fungi. The efficacy of some of the formulations was also determined following storage in the laboratory. Furthermore, biocontrol activity of formulations was tested in a soil which carried a natural population of P. ultimum sufficient to induce severe damping-off.

#### 7.1 Pathogenicity tests

Preliminary experiments were carried out to determine suitable inoculum densities for artificially infesting sand with P. ultimum and R. solani. Specific inoculum densities were then used in experiments for evaluating oospore formulations.

### 1. Inoculum production

To infest sand, sporangia of P. ultimum (MPM02) were produced by modification of a procedure of Ayers & Lumsden (1975). Three day-old cultures on CMA were flooded with sterile 10% soil extract (as in 3.6) and incubated at 25° for 1 wk. Mycelia containing sporangia were then scraped from the agar surface and blended in a laboratory mixer at full speed for 1 min. Sporangia were counted in a haemocytometer and diluted in tap water to provide appropriate numbers per g dry weight equivalent of sand.

Millet seed inoculum was used to infest sand with R. solani (RS21) (Lewis and Papavizas, 1985). Portions (60 g) of panicum millet (Pennisetum glaucum L.) were placed in 250 ml Erlenmeyer flasks containing 20 ml distilled water and were autoclaved. Individual flasks were inoculated with two 5 mm diam PDA discs cut from the periphery of 4-d-old culture of R. solani. Preparations were incubated at 25° for 14 d and then air-dried. Inoculum was used to infest sand within 1 wk of drying.

### 2. Preliminary experiments in artificially infested sand

Pathogenicity tests and all subsequent controlled experiments described in this Chapter were carried out by modification of the method reported by Al-Hamdani et al. (1983) using clear plastic boxes (16 x 28 x 5 cm) each containing 1500 g sand infested with the appropriate pathogen. Sterile washed sand (moistened with sterile water to a moisture level of c. 60% on a dry weight basis) was infested with P. ultimum (MPM02) sporangia (10, 20, 30, 50, and 100 sporangia per g dry weight equivalent of sand) and added to boxes. After

smoothing the sand surface, each box was sown with 55 seeds, arranged in a 5 x 11 grid pattern, each being 2 cm from its neighbour and c. 1 cm deep. Three replicates were used for each treatment, and controls consisted of seed sown in pathogen-free sand. Boxes were arranged in a randomised complete block design within a controlled environment room at 20°, with alternating periods of 12 h light and dark, where the radiant flux at bench level during the light period was 38 W/m<sup>2</sup>. Boxes were watered as required to maintain a moist sand surface. Sand was also individually infested with R. solani millet inoculum (0.5, 1.0, 2.0, 4.0 and 8.0 g/3kg dry weight equivalent of sand) and inoculum densities tested as for P. ultimum. Numbers of dead seedlings were scored at 2 d intervals and measurements of final seedling stand made after 16 d for experiments in P. ultimum-infested sand and 21 d for those in R. solani-infested sand.

All percentage stand data were angularly transformed and two-way analysis of variance was performed on transformed data. Treatment means were compared with the least significant difference (LSD) at a probability of 5%.

Results are shown in Tables 23 and 24. Germination of cress was 93-98% in pathogen-free sand. Damping-off occurred with all inoculum densities of P. ultimum. As inoculum density increased the final seedling stand declined concomitantly, and at the highest densities almost all seedlings were killed after 16 d. An inoculum density of 10 sporangia per g sand gave a final seedling stand of 36%. This inoculum density was used in subsequent experiments described in this Chapter to evaluate the effectiveness of oospore formulations against damping-off induced by P. ultimum (MPM02). In sand infested with R. solani, all

Table 23

Pathogenicity test. Percentage stand of cress seedlings grown in sand artificially infested with various amounts of inoculum of Pythium ultimum (MPM02) for 16 d.

Figures in parentheses are means from three replicates with SEM calculated after angular transformation of percentage data.

---

Inoculum of <u>P. ultimum</u> (MPM02) (sporangia per g dry wt sand)	Seedling stand (%)
0*	93 (75.2±1.8)
10	36 (37.1±1.0)
20	21 (27.5±0.5)
30	17 (24.1±2.6)
50	4 (11.6±2.1)
100	1 (2.7±2.7)
LSD (P=0.05)	(6.6)

---

\* Pathogen-free sand.

Table 24

Pathogenicity test. Percentage stand of cress seedlings grown in sand artificicically infested with various amounts of inoculum of Rhizoctonia solani (RS21) for 21 d.

Figures in parentheses are means from three replicates with SEM calculated after angular transformation of percentage data.

---

<u>R. solani</u> millet inoculum (g/3kg dry wt sand)	Seedling stand (%)
0*	95 (77.6±0.9)
0.5	64 (53.3±0.9)
1.0	28 (32.1±0.7)
2.0	2 ( 5.7±3.3)
4.0	0
8.0	0
LSD (P=0.05)	(4.9)

---

\* Pathogen-free sand.

inoculum densities gave damping-off. Increases in density were again accompanied by a decrease in seedling stand. All seedlings were killed after 21 d with densities above 2 g/3 kg sand. The inoculum density of 1 g/3kg sand, which gave a final seedling stand of 28%, was used in further experiments to evaluate the effectiveness of oospore formulations against R. solani-induced damping-off.

## 7.2 Effect of solid substrate oospore preparations on Pythium ultimum-induced damping-off

The ability of three different solid substrate oospore preparations to reduce damping-off was evaluated in sand artificially infested with P. ultimum. Oospore preparations were produced in autoclavable bags containing either barley grain, wheat grain or maize meal semi-solid media as described in section 5.2. Each freshly-harvested oospore preparation was divided into two equal samples. One sample was air-dried in a laminar flow cabinet for 48 h at 18-21°, whilst the other was autoclaved on two consecutive days to kill oospores. Portions (40 g) of each preparation were then mixed thoroughly with 5 kg (dry weight equivalent) of P. ultimum-infested sand in large plastic bags. Bags were incubated for 3 d at 20° after which time 1500 g quantities of each sand-solid substrate mix were added to three replicate plastic boxes (16 x 28 x 5 cm). The sand surface was made smooth and each box was then sown with 55 cress seeds as described before. Controls consisted of seed sown in pathogen-infested and pathogen-free sand, both of which contained no substrate preparation. Boxes were incubated in a controlled environment room and general procedures were the same as those described previously in section 7.1.

Results are shown in Table 25. The solid substrate oospore

Table 25

Effect of solid substrate oospore preparations of Pythium oligandrum added to sand artificially infested with Pythium ultimum (MPMO2) on seedling stand of cress 16 d after sowing.

Values in parentheses are means with SEM from three replicates calculated after angular transformation of percentage data.

---

Solid substrate oospore preparation	Seedling stand (%)
Control (Uninfested)	96 (79.2±1.4)
Control (Infested)	39 (38.6±0.7)
Barley	34 (35.5±1.3)
Barley (A)*	33 (35.3±1.4)
Maize	33 (35.3±1.4)
Maize (A)	35 (36.3±0.3)
Wheat	40 (39.0±0.8)
Wheat (A)	37 (37.3±0.8)
LSD (P=0.05)	(3.3)

---

\* (A) - Oospore preparations were autoclaved on two consecutive days to kill oospores.

preparations had no significant effect in reducing damping-off. Seedling stand of seed sown in sand treated with oospore preparations was equivalent to or less than those sown in the pathogen-infested control. Oospore preparations gave levels of seedling stand equivalent to their corresponding autoclaved preparations. Fungal contaminants were observed on the sand surface of boxes containing sand treated with solid substrate preparations.

### 7.3 Effect of a CMC-oospore seed-coating on *Pythium ultimum* and *Rhizoctonia solani*-induced damping-off

The ability of *P. oligandrum* CMC-oospore seed-coatings to give control of damping-off was evaluated in three experiments. In the first, the efficacy of various *P. oligandrum* isolates for control of *P. ultimum*-induced damping-off was compared. In the second, the biocontrol activity of oospores incorporated into the CMC-seed-coating was tested against *P. ultimum* following storage in the laboratory. Finally, the effectiveness of the CMC-oospore seed-coating in reducing damping-off induced by *R. solani* was evaluated.

Oospore biomass for use in these experiments was produced in liquid 18-21 d static cultures as described in section 5.7. Batches of cress seed were coated with oospores as outlined in section 6.2.

#### 1. Control of damping-off in *Pythium ultimum*-infested sand

##### i. A comparison of the efficacy of various isolates

Details of the six isolates of *P. oligandrum* used are listed in Table 1 (Chapter II). To ensure that seeds were coated with approximately equal numbers of oospores, the density of each isolate oospore suspension was adjusted prior to coating. Oospore

seed-coatings were also compared with a fungicide drench. Propamocarb hydrochloride (Filex 72.2% SL; Fisons plc.) was applied before sowing as a 90 ml drench (162 mg a.i. per box) and general procedures were the same as those described in section 7.1.

For results, see Table 26 and Plate 6. All seed coatings with isolates of P. oligandrum gave significant increases in seedling stand but none of these were as effective as the propamocarb hydrochloride drench treatment. All isolates were equally effective in reducing damping-off and gave equivalent seedling stands 16 d after sowing.

ii. Effect of storage on biocontrol activity of isolate IMI 133857

Air-dried biomass of isolate IMI 133857 was stored in sealed glass containers at 15°. After 0, 3, 6 and 12 months storage, samples were removed, oospore suspensions prepared and cress seeds coated with the CMC-oospore preparation. To monitor the effect of storage on oospore biocontrol activity, oospore-coated and appropriate control seeds were sown in sand artificially infested with P. ultimum. General procedures were the same as those described in section 7.1, except that fresh sand was infested after each storage period.

Results are shown in Table 27. Length of storage had some effect on the subsequent biocontrol activity of oospores when coated onto seed. Storage for three months significantly reduced effectiveness but there was no significant decline thereafter. After each storage period, the oospore-coated seed still gave significantly greater seedling stands than the CMC-coated and uncoated control seed sown in P. ultimum-infested sand.

2. Control of damping-off in Rhizoctonia solani-infested sand

In this experiment oospores of isolate IMI 133857 were used to coat

Table 26

Effect of CMC-oospore seed-coatings of Pythium oligandrum isolates on seedling stand of cress 21 d after sowing in sand artificially infested with Pythium ultimum (MPM02).

Figures in parentheses are means from three replicates with SEM calculated after angular transformation of percentage data.

---

Treatment	Seedling stand (%)
Uninfested	
Control (uncoated)	98 (81.6±2.4)
CMC*	97 (80.7±2.5)
Infested ( <u>P. ultimum</u> )	
Control (uncoated)	40 (39.0±1.0)
CMC	41 (39.8±1.7)
IMI 133857	63 (52.7±1.4)
MPM01	65 (53.5±1.3)
MPM02	66 (54.5±1.9)
MPM03	62 (52.2±1.4)
MPM04	69 (56.0±1.1)
MPM05	63 (52.5±1.2)
Propamocarb HCl drench (uncoated)	89 (70.7±1.0)
LSD (P=0.05)	(4.5)

---

\* CMC - Carboxymethyl cellulose adhesive.

Plate 6

Surviving cress seedlings 16 d after sowing in sand.

- A. CMC-oospore coated seeds sown in uninfested sand.
- B. CMC coated seeds sown in sand artificially infested with Pythium ultimum.
- C. CMC-oospore coated seeds sown in sand artificially infested with Pythium ultimum.

Seeds were coated with oospores of isolate IMI 133857.

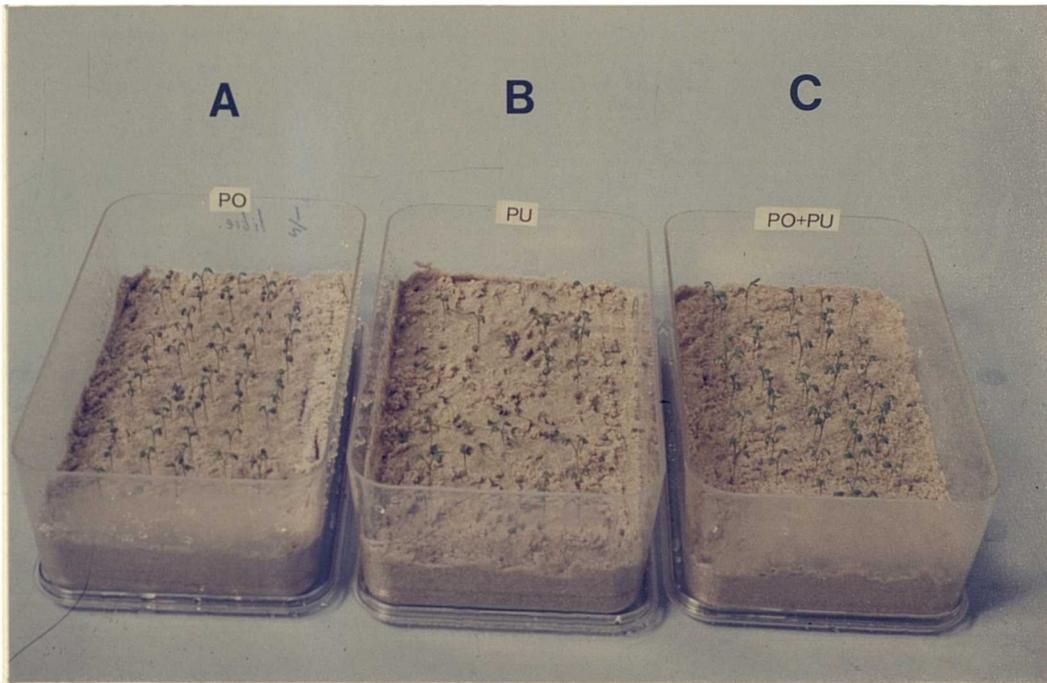


Table 27

Effect of a CMC-oospore seed coating of Pythium oligandrum applied to seed after storage at 15° on seedling stand of cress 16 d after sowing in sand artificially infested with Pythium ultimum (MPMO2). Figures in parentheses are means from three replicates with SEM calculated after angular transformation of percentage data.

Treatment	Seedling stand (%)			
	0	3	6	12
Uninfested				
Control (uncoated)	96 (78.7±1.4)	95 (77.2±1.6)	96 (78.2±2.0)	97 (79.9±2.4)
CMC*	97 (79.6±1.1)	94 (76.3±1.1)	95 (77.0±2.3)	97 (82.4±3.9)
+ Oospores	93 (75.3±1.8)	97 (82.4±3.9)	95 (77.1±0.8)	98 (83.3±3.3)
Infested ( <u>P. ultimum</u> )				
Control (uncoated)	35 (36.5±1.4)	40 (39.2±1.5)	37 (37.2±1.9)	36 (36.9±2.1)
CMC	40 (39.4±1.2)	42 (40.4±0.3)	37 (37.5±1.0)	39 (38.8±1.0)
+ Oospores	61 (51.5±1.0)	53 (45.9±1.8)	51 (45.8±1.5)	51 (45.6±1.3)
LSD (P=0.05)	(5.6)			

\* CMC - Carboxymethyl cellulose adhesive.

cross seed. Appropriate uncoated controls were included and treated seed was sown in sand artificially infested with the millet inoculum of R. solani. General procedures were the same as those described in section 7.1.

Results are shown in Table 28. After 21 d, the oospore-coated seed gave significantly greater seedling stands than the CMC-coated and uncoated control seed sown in sand infested with R. solani. However, the increases in seedling stand were generally not as great as those recorded in previous experiments in which oospore-coated seed was sown in P. ultimum-infested sand.

#### 7.4 Effect of alginate pellets, oospore dust and oospore-coated perlite on Pythium ultimum-induced damping-off

To test biocontrol activity, alginate pellets, oospore dust and oospore-coated perlite were incorporated individually into P. ultimum-infested sand. All three formulations were prepared as described in Chapter VI (section 6.2), and contained oospores of isolate IMI 133857 produced in liquid 18-21 d static cultures as outlined in Chapter V (section 5.7). The experiments were carried out using the plastic box-sand system. Each formulation was mixed thoroughly with P. ultimum-infested sand in large plastic bags, stored for 3 d at 20° and 1500 g quantities of each mix added to three replicate plastic boxes prior to sowing seed.

At the end of experiments, infested sand previously amended with oospore formulations was tested for the presence of P. oligandrum. Samples of sand were removed from plastic boxes and passed through a 1.0 mm mesh screen to separate sand from alginate pellets or perlite.

Table 28

Effect of a CMC-oospore seed-coating of Pythium oligandrum on seedling stand of cress 21 d after sowing in sand artificially infested with Rhizoctonia solani.

Figures in parentheses are means from three replicates with SEM calculated after angular transformation of percentage data.

---

Treatment	Seedling stand (%)
Uninfested	
Control (uncoated)	96 (78.5±2.6)
CMC*	92 (73.9±2.4)
+ Oospores	96 (78.3±1.2)
Infested ( <u>R. solani</u> )	
Control (uncoated)	30 (32.2±0.9)
CMC	26 (30.6±1.4)
+ Oospores	41 (39.6±0.8)
LSD (P=0.05)	(4.4)

---

\* CMC - Carboxymethyl cellulose adhesive.

Sand dilutions were prepared and tested for P. oligandrum by plating aliquots on selective agar media as previously described in section 6.5. Colonies were observed after incubation and confirmation of the presence of P. oligandrum was made based on colony morphology (section 6.5). No quantitative estimation of populations was made.

#### 1. Alginate pellets

Alginate pellets containing oospores, kaolin, and D-glucose and myo-inositol were added to sand at 15 g/kg sand (dry weight equivalent). Oospore-free pellets were also added at the same rate.

Results are shown in Table 29. In sand infested with P. ultimum, oospore pellets gave greater seedling stands than the oospore-free pellets and infested control. However, these increases in seedling stand were not statistically significant ( $P=0.05$ ). In pathogen-free sand, pellets had no adverse effects on seedling stand. At the end of the experiment, P. oligandrum was recovered on agar media from infested sand previously treated with oospore pellets.

#### 2. Oospore dust

Kaolin-oospore dust was added to sand at 4 and 8 g/kg sand (dry weight equivalent). Oospore-free kaolin was also added at a single rate of 8 g/kg sand (dry weight equivalent).

For results, see Table 30. In sand infested with P. ultimum, both application rates of kaolin-oospore dust gave increases in seedling stand compared with the oospore-free kaolin and infested control. With the high application (8 g/kg sand), these increases in seedling stand were statistically significant ( $P=0.05$ ) compared with the oospore-free kaolin treatment, but not with the infested control. The high application rate gave significantly greater seedling stands than the

Table 29

Effect of alginate pellets containing oospores of Pythium oligandrum added to sand artificially infested with Pythium ultimum (MPMO2) on seedling stand of cress 16 d after sowing.

Values in parentheses are means with SEM from three replicates calculated after angular transformation of percentage data.

---

Treatment	Seedling stand (%)
Uninfested	96 (78.4±2.1)
+ Pellets *	95 (76.7±0.9)
+ Oospore pellets	96 (81.1±4.6)
Infested ( <u>P. ultimum</u> )	40 (39.3±1.2)
+ Pellets	38 (38.1±1.4)
+ Oospore pellets	51 (45.6±0.7)
LSD (P=0.05)	(7.5)

---

\* Control alginate pellets contained D-glucose and myo-inositol but no oospore biomass.

Table 30

Effect of a kaolin-oospore biomass dust\* of Pythium oligandrum added to sand artificially infested with Pythium ultimum (MPM02) on seedling stand of cress 16 d after sowing.

Values in parentheses are means with SEM from three replicates calculated after angular transformation of percentage data.

---

Treatment	Seedling stand (%)
Uninfested	88 (69.8±1.8)
+ Kaolin **	93 (75.4±2.4)
Infested ( <u>P. ultimum</u> )	41 (39.8±0.7)
+ Kaolin	38 (37.9±0.7)
+ Kaolin-oospore dust ( 4g/kg)***	45 (42.1±1.0)
+ Kaolin-oospore dust ( 8g/kg)	53 (46.7±1.3)
LSD (P=0.05)	(3.8)

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\* Air-dried oospore biomass was mixed with kaolin clay (1:5 v/v) to increase the volume for distribution purposes.

\*\* Kaolin added to sand (8 g/kg sand (dry weight equivalent)) was oospore-free.

\*\*\* Kaolin-oospore dust was added to sand at 4 and 8 g/kg sand (dry weight equivalent).

low application rate. Kaolin had no adverse effects on seedling stand in pathogen-free sand. P. oligandrum was recovered on agar media from infested sand previously treated with kaolin-oospore dust.

### 3. Perlite preparation

Oospore-coated perlite was added to sand at 2 and 4 g/kg (dry weight equivalent). Oospore-free perlite was also added at a single rate of 4 g/kg sand (dry weight equivalent).

Results are shown in Table 31. Both rates of oospore-coated perlite gave no significant improvements in seedling stand compared with the oospore-free and infested control. Once more, P. oligandrum was recovered on agar media from infested sand treated with oospore-coated perlite.

### 7.5 Effect of formulations on damping-off in naturally infested soil

This experiment was carried out to evaluate the effectiveness of various oospore formulations of isolate IMI 133857 in controlling damping-off of cress in naturally infested soil. Details of the formulations tested and their application rates are shown in Table 32. Formulations were also compared with a fungicide drench. A medium loam soil (as in 6.3), which carried a natural population of P. ultimum sufficient to induce damping-off but from which P. oligandrum was absent, was used. Formulations were mixed with soil, stored for 7 d at 15° and 1500 g (dry weight equivalent) quantities added to three replicate boxes prior to sowing. Propamocarb hydrochloride (Filex 72.2% SL; Fisons plc.) was applied before sowing as a 90 ml drench (162 mg a.i. per box). Water potential of soil was maintained at c. -0.03 MPa and general procedures were as described before, except that final

Table 31

Effect of an oospore-coated perlite preparation of Pythium oligandrum added to sand artificially infested with Pythium ultimum (MPM02) on seedling stand of cress 16 d after sowing.

Values in parentheses are means with SEM from three replicates calculated after angular transformation of percentage data.

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Treatment	Seedling stand (%)
Uninfested	92 (74.4±3.9)
+ Perlite *	94 (76.5±2.0)
Infested ( <u>P. ultimum</u> )	39 (38.5±1.4)
+ Perlite	38 (38.2±2.0)
+ Oospore-coated perlite (2 g/kg)**	45 (42.1±0.7)
+ Oospore-coated perlite (4 g/kg)	49 (44.4±1.0)
LSD (P=0.05)	(7.1)

---

\* Perlite added to sand (4 g/kg sand (dry weight equivalent)) was oospore-free.

\*\* Oospore-coated perlite was added to sand at 2 and 4 g/kg sand (dry weight equivalent).

seedling stands were assessed 14 d after sowing.

All formulations gave significant increases in seedling stand, but none of these were as effective as the propamocarb HCl drench. The CMC-oospore seed treatment gave significantly greater seedling stands than the soil-incorporated formulations. No differences in disease control were apparent between the soil-incorporated formulations.

Table 32

Effect of formulations of Pythium oligandrum on seedling stand of cress 14 d after sowing in soil naturally infested with Pythium ultimum. Values in parentheses are means with SEM from three replicates calculated after angular transformation of percentage data.

---

Treatment	Seedling stand (%)
Autoclaved soil*	92 (74.0±0.7)
Untreated soil	39 (38.8±1.7)
CMC-oospore coated seed	79 (62.5±0.9)
Oospore alginate pellets (15 g/kg)**	60 (51.0±1.9)
Kaolin-oospore dust (8 g/kg)	56 (48.3±1.3)
Oospore-coated perlite (4 g/kg)	57 (49.2±0.8)
Barley grain (10 g/kg)	57 (49.4±1.5)
Propamocarb HCl drench	84 (66.2±1.6)
LSD (P=0.05)	(4.1)

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\* Soil was autoclaved for 1 h at 120° and 103.4 kPa on two consecutive days.

\*\* Application rates are per kg of soil (dry weight equivalent).

CHAPTER VIII

COMMERCIAL SEED-COATING WITH OOSPORES FOR  
BIOLOGICAL CONTROL OF FUNGAL SOIL-BORNE DISEASES

## CHAPTER VIII

### COMMERCIAL SEED-COATING WITH OOSPORES FOR BIOLOGICAL CONTROL OF FUNGAL SOIL-BORNE DISEASES

The only previous commercial seed-coating system tested involved incorporation of a cornmeal-vermiculite-oospore preparation into a clay-based carrier which was then used to form a pellet around seeds of cress, sugar beet and carrot (Lutchmeah, 1985; Lutchmeah & Cooke, 1985). This pelleting procedure has now been superseded commercially by one based on organic filler materials (Dewar et al., 1988). Such a seed pelleting system has been used recently to apply fungal metabolites of Penicillium claviforme Banier to sugar beet seeds for the control of Pythium ultimum (Thompson & Burns, 1989). In addition, the seed-coating industry is tending towards the use of a film-coating technique to deliver fungicides and pesticides to seeds rather than bulky pelleting formulations (Halmer, 1988). It was shown in Chapter V that oospores of P. oligandrum can be produced successfully on a large-scale in liquid culture. This avoids many of the problems associated with solid substrate formulations and allows controlled quantities of oospores to be applied to seeds relatively easily by both seed-coating systems.

Work in this Chapter describes the successful coating of oospores onto cress and sugar beet seeds using two commercial processes. Moreover, trials were also carried out in order to assess the ability

of these treatments to give control of damping-off induced by P. ultimum, R. solani and Aphanomyces cochlioides Drechsler in growth room and glasshouse conditions. Finally, a range of pesticides used as prophylactic seed treatments were tested for their effect on mycelial growth of P. oligandrum under laboratory conditions.

### 8.1 Oospore production and commercial seed-coating of cress and sugar beet

For seed-coating, oospores were produced by the method outlined in Chapter V (section 5.7). This procedure was modified by growing the fungus in 1-l Roux bottles containing 200 ml autoclaved 3% (w/v) cane molasses liquid medium. Bottles were inoculated aseptically with three 20 mm diam CMA discs cut from a 3-day-old culture of P. oligandrum (IMI 133857) and incubated on their sides at 25° for 21 d. Oospore biomass was harvested by filtering cultures through Whatman No. 1 filter paper. After harvest, biomass was washed in three changes of distilled water and air-dried overnight in a laminar flow cabinet at 18-21°. The biomass, which contained 1.4-1.8 x 10<sup>4</sup> oospores per mg, was then used for seed-coating.

Seed-coating of cress (Lepidium sativum L. cv. Curled; Suttons) and sugar beet (Beta vulgaris L. cv. Amethyst) was carried out under the supervision of Dr. Peter Halmer, Germain's (UK) Ltd., Kings Lynn, Norfolk, employing two commercial coating processes. Fungicides and P. oligandrum oospores were incorporated separately into seed pellets using the 'EB3 pellet' process and coated onto seed surfaces using a film-coating binder system. The 'EB3 pellet', which contains a high proportion of 'wood flour', replaced the 'Filcoat' process in 1985

(Dewar et al., 1988). The film-coating binder system is a new process where active ingredients are formulated with liquid based polymeric adhesives and coated onto seeds (Halmer, 1988). For reasons of commercial confidentiality it is not possible to give full technical details of the two seed-coating techniques.

Sugar beet seeds were either steeped (12 h at 25°) in water or a 0.2% suspension of thiram (Agrichem Flowable Thiram 60% FS; Agrichem Ltd.). A further treatment of thiram was applied in the pellet or film-coating to give a final application rate of 4.8 g a.i. per Unit (100,000 seed). Thiram controls seed-borne Phoma betae Frank and all sugar beet sown in Britain is now treated routinely with this fungicide (Dewar et al., 1988). Seed was also treated with two fungicides at the following rates per Unit seed: hymexazol (Tachigaren 70% WP; Sumitomo Chemical Co. Ltd.), 10.50 g a.i., or metalaxyl (Apron 25% WP; Ciba-Geigy Agrichem.), 0.29 g a.i.. Tachigaren controls both Pythium spp. and A. cochlioides, whereas metalaxyl only controls Pythium spp. (Asher & Payne, 1989). Cress seeds were not treated with fungicides. One kilogram of cress seed and a Unit of sugar beet seed were treated with c. 4.0 and 4.5 g of oospore biomass respectively. Seeds were pelleted and film-coated without additives and a water-steeped only treatment was included for sugar beet. All pelleted seed was dried at 30°, apart from some batches of oospore treated seeds which were dried at 45°. Film-coated seed were all dried at 25°. Treated seeds were stored in plastic bags at laboratory temperature (18-22°) until required.

## 8.2 Number and survival of oospores in coating materials

The number of oospores applied to each pelleted seed was determined by the method of Lutchmeah & Cooke (1985). Briefly, five seeds of each type were shaken vigorously in water to suspend the pelleting material. Aliquots (0.05 ml) of suspension were mixed with a few drops of cotton blue in lactophenol on a microscope slide, spread out over a known area and counted under the microscope. Vigorous shaking failed to remove oospores from film-coated seeds and so another technique was used. Five randomly selected seeds were crushed individually with a small pestle and mortar. Crushed material was then suspended in 5 ml dilute water agar (0.01 g agar (no. 3 Oxoid), 1-1 distilled water), blended in an MSE homogenizer for 2 min and the oospores counted as described above. Numbers of oospores carried by each seed were then calculated. In addition, film-coated seeds were examined by scanning electron microscopy in the frozen hydrated state (Atkey & Pegler, 1987). Seeds were attached to specimen stubs with silver paint and frozen on the preparation stage of an Emscope SP2000 sputter cryo apparatus at  $-186^{\circ}$  under a vacuum of 65 Pa. The frozen specimens were then sputter coated with gold and transferred, still frozen and under vacuum to the cooled stage ( $-150^{\circ}$ ) of a Jeol T330 SEM. The specimens were then examined at 25 kv voltage.

Pelleting and film-coating processes both produced similar numbers of oospores per seed achieving 86-94% and 76-86% target rate for cress and sugar beet respectively (Table 33). With the film-coating technique, oospores were clearly visible in high density on the seed surface (Plate 8), whereas in the pellet they were positioned within the thick organic layer surrounding the seed (Halmer, pers. comm.).

Germination of oospores in inoculum used to coat the seeds was

Table 33

Number of oospores of Pythium oligandrum carried per treated seed.

Values are means from five replicates.

---

Seed	Number		Range	
	EB3 pellet	Film-coated	EB3 pellet	Film-coated
Cress	33,060	31,360	31,200-34,900	30,400-32,000
Sugar beet	43,160	41,160	40,200-46,900	38,200-43,700

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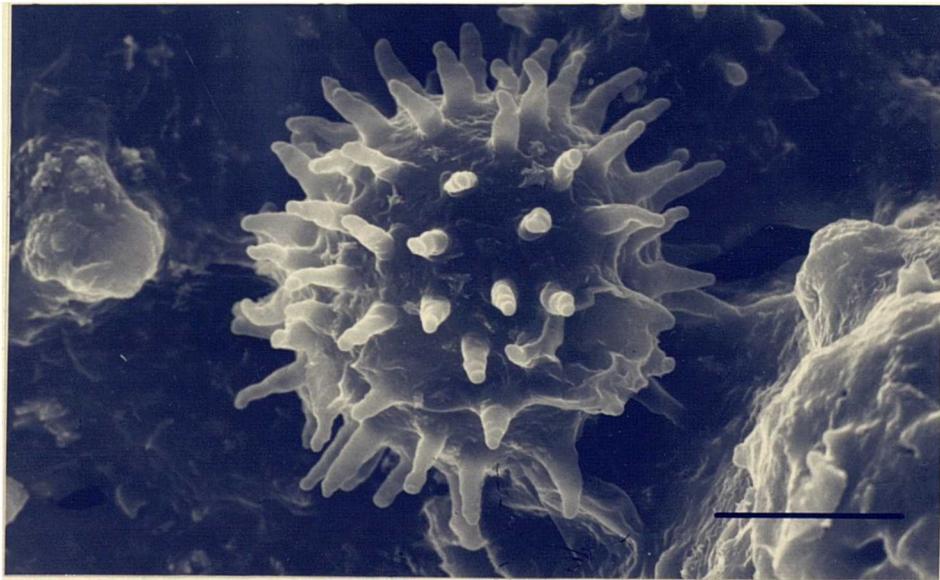
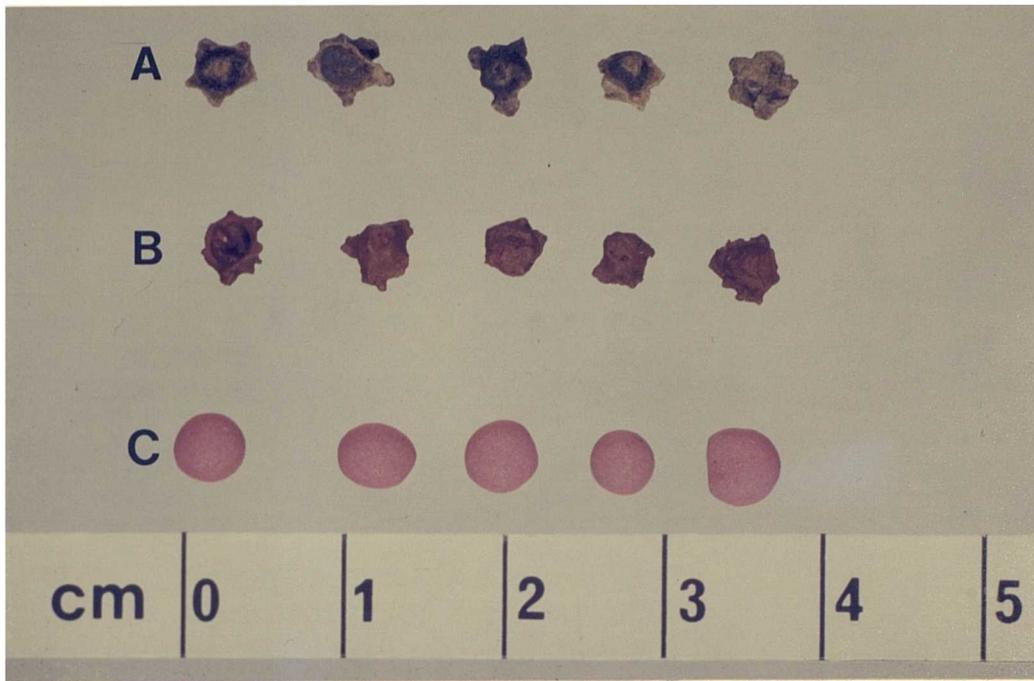
Plate 7

Sugar beet seeds.

- A. Untreated
- B. Film-coated
- C. Pelleted (EB3)

Plate 8

High power view of a single intact oospore of Pythium oligandrum on the surface of a film-coated sugar beet seed (Bar = 10  $\mu$ m).



assessed before seed treatment and compared with those which were pelleted and coated onto seeds. Oospores were removed from seeds as described previously, and suspensions passed through two layers of muslin to remove hyphal fragments and seed-coat debris. Germination was assessed on CMA supplemented with 100 µg/ml of vancomycin HCl using the method described in section 4.4. All antibiotics were supplied by Sigma Chemical Co. The ability of oospores to produce a mycelium from seed was assessed immediately after the coating processes and at fortnightly intervals during seed storage for 16 wk. Twenty seeds of each type were incubated on CMA for 48 h at 25° after which time seeds giving rise to mycelium of P. oligandrum were counted.

Oospores obtained from the treated seeds had a similar germination level (9-19% after 16 h at 25°) as those present in the inoculum used to coat seeds (14-23%). Mycelium of P. oligandrum grew from all seed coatings when seeds were placed on CMA c. 5 d after coating (see Plate 9). Growth of P. oligandrum still took place within 48 h incubation from all seeds tested 16 wk after coating.

### 8.3 Effect of seed treatments on damping-off of cress in sand and potting compost

Experiments in artificially infested sand and potting compost were conducted to evaluate the effectiveness of P. oligandrum oospore seed treatments in reducing damping-off of cress caused by P. ultimum and R. solani. Oospore seed treatments were also compared with fungicide drenches.

#### 1. Sand experiments

Two experiments were carried out using the method described in

Plate 9

Growth of Pythium oligandrum mycelium from oospore pelleted cress seed on CMA (48 h at 25°) c. 5 d after pelleting.



section 7.1 in which cress seeds were sown in clear plastic boxes (16 x 28 x 5 cm) each containing 1500 g sand infested with the appropriate pathogen.

i. Control of damping-off in *Pythium ultimum*-infested sand

Washed sand (moistened with sterile water to a moisture level of c. 60% on a dry weight basis) was infested with *P. ultimum* (MPMO2) sporangia (10 sporangia per g dry weight equivalent of sand) and added to boxes. After smoothing the sand surface, each box was sown with 55 seeds, arranged in a 5 x 11 grid pattern, each being 2 cm from its neighbour and about 1 cm deep. Three replicates were used for each treatment and controls consisted of oospore-free pelleted and film-coated seed. Propamocarb hydrochloride (Filex 72.2% SL; Fisons plc.) was applied before sowing as a 90 ml drench (162 mg a.i. per box). Boxes were arranged in a randomised complete block design within a controlled environment room but under slightly different conditions from those in experiments described in Chapter VII. The temperature was maintained at 20°, with alternating periods of 14 h light and 10 h dark, where the radiant flux at bench level during the light period was 25 W/m<sup>2</sup>. Boxes were watered as required to maintain a moist sand surface. Seedling emergence was noted and numbers of dead seedlings scored at 2 d intervals. Measurements of final seedling stand and shoot dry weight were made after 16 d.

All percentage stand and emergence data were angularly transformed and two-way analyses of variance performed on transformed data. Similar transformations of percentage data from subsequent experiments were also made before analyses. Treatment means were then compared with the least significant difference (LSD) at a probability of 5%.

Results are shown in Table 34. In sand artificially infested with P. ultimum, P. oligandrum pellets gave increases in seedling stand and shoot dry weight equivalent to the fungicide drench. Film-coating with P. oligandrum oospores increased seedling stand and shoot dry weight (only the latter significantly ( $P=0.05$ )) but was not as effective as the fungicide treatment.

ii. Control of damping-off in Rhizoctonia solani-infested sand

Sand was also infested with R. solani millet inoculum (1 g/3kg dry weight equivalent of sand) and seed treatments tested as for P. ultimum. The fungicide treatment included tolclofos-methyl (Basilex 50% WP; Fisons plc.) which was applied before sowing as a 45 ml drench (45 mg a.i. per box). Measurements of final seedling stand and shoot dry weight were made 21 d after sowing.

For results, see Table 35. All oospore seed treatments gave significant increases in seedling stand and shoot dry weight but none of these were effective as the tolclofos-methyl drench treatment.

2. Potting compost experiments

A preliminary pathogenicity test was carried out to determine a suitable inoculum density of P. ultimum (MPM02) for use in evaluating oospore seed treatments against damping-off in compost.

i. Pathogenicity test

To infest potting compost, sporangia of P. ultimum (MPM02) were produced as described in section 7.1. The compost (as in 6.4) was infested with sporangia (100, 200, 400 and 600 sporangia per g dry weight equivalent of compost) and added to plastic pots (7.5 cm diam). Twenty seeds were sown 1 cm deep in pots. Pots in saucers were arranged in a randomised complete block design on a glasshouse bench

Table 34

Effect of seed treatments on seedling stand and shoot dry weight of cress 16 d after sowing in sand artificially infested with Pythium ultimum (MPMO2).

Values for seedling stand in parentheses are means with SEM from three replicates calculated after angular transformation of percentage data.

Values for dry weight shoot are means from three replicates with SEM.

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Treatment	Seedling stand (%)	Dry weight shoot (mg)
EB3 pellet (30°)*	47 (43.1±0.8)	42.6±3.9
+ <u>P. oligandrum</u>	96 (78.3±2.1)	108.8±1.8
+ <u>P. oligandrum</u> (45°)	93 (75.8±4.4)	103.6±3.2
+ Propamocarb HCl drench	94 (76.4±1.2)	113.1±1.2
Film-coated (25°)	38 (38.0±2.3)	39.8±5.3
+ <u>P. oligandrum</u>	51 (45.6±2.8)	56.8±2.6
+ Propamocarb HCl drench	93 (74.5±2.0)	109.0±1.3
LSD (P=0.05)	(7.8)	9.4

---

\* The temperature at which seeds were dried after commercial treatment.

Table 35

Effect of seed treatments on seedling stand and shoot dry weight of cress 21 d after sowing in sand artificially infested with Rhizoctonia solani.

Values for seedling stand in parentheses are means with SEM from three replicates calculated after angular transformation of percentage data.

Values for dry weight shoot are means from three replicates with SEM.

---

Treatment	Seedling stand (%)	Dry weight shoot (mg)
EB3 pellet (30°)*	47 (43.1±0.8)	45.0±2.9
+ <u>P. oligandrum</u>	62 (51.8±1.8)	64.3±2.6
+ <u>P. oligandrum</u> (45°)	68 (55.8±1.8)	71.9±1.8
+ Tolclofos-methyl drench	97 (79.6±1.1)	108.8±1.0
Film-coated (25°)	44 (41.3±2.7)	44.6±1.4
+ <u>P. oligandrum</u>	58 (49.4±1.9)	63.0±1.5
+ Tolclofos-methyl drench	98 (81.9±0.3)	107.9±1.4
LSD (P=0.05)	(5.3)	5.7

---

\* The temperature at which seeds were dried after commercial treatment.

maintained at 15-25° and watered as required. Eight replicates were used for each treatment and controls consisted of seed sown in pathogen-free compost. Seedling emergence was noted and numbers of dead seedling scored at 2 d intervals. Measurements of final seedling stand and shoot dry weight were made 16 d after sowing.

Results are shown in Table 36. Damping-off occurred with all inoculum densities of P. ultimum. As inoculum density increased, the final seedling stand declined concomitantly. An inoculum density of 200 sporangia per g compost gave a seedling stand of 50%. This was used to artificially infest compost in order to evaluate oospore seed treatments against damping-off.

ii. Control of damping-off in Pythium ultimum-infested compost

General procedures were the same as those described in the pathogenicity test. Oospore seed treatments were compared with propamocarb HCl applied before sowing as a 45 ml drench (81 mg a.i. per pot). Controls consisted of oospore-free pelleted and film-coated seed. Disease progress was monitored over a 16 d period as described before.

For results, see Table 37 and Plate 10. All oospore seed treatments gave significant increases in seedling stand and shoot dry weight but none of these were as effective as the propamocarb HCl drench.

Table 36

Pathogenicity test. Percentage stand of cress seedlings grown in potting compost artificially infested with various amounts of inoculum of Pythium ultimum (MPMO2) for 16 d.

Figures in parentheses are means from eight replicates calculated after angular transformation of percentage data.

---

Inoculum of <u>P. ultimum</u> (MPMO2) (sporangia per g dry wt compost)	Seedling stand (%)
0*	99 (87.4±2.6)
100	66 (54.4±1.1)
200	50 (45.0±1.6)
400	26 (30.6±0.6)
600	13 (20.5±2.8)
LSD (P=0.05)	(5.2)

---

\* Pathogen-free compost.

Table 37

Effect of seed treatments on seedling stand and shoot dry weight of cress 16 d after sowing in potting compost artificially infested with Pythium ultimum (MPMO2).

Values for seedling stand in parentheses are means with SEM from eight replicates calculated after angular transformation of percentage data.

Values for dry weight shoot are means from eight replicates with SEM.

---

Treatment	Seedling stand (%)	Dry weight shoot (mg)
EB3 pellet (30°)*	47 (43.2±1.3)	163.2±3.4
+ <u>P. oligandrum</u>	68 (55.3±1.0)	207.8±2.4
+ <u>P. oligandrum</u> (45°)	62 (51.9±0.9)	210.6±2.0
+ Propamocarb HCl drench	89 (72.6±2.0)	314.1±3.9
Film-coated (25°)	36 (37.0±1.3)	145.5±3.5
+ <u>P. oligandrum</u>	50 (45.0±1.1)	162.7±4.6
+ Propamocarb HCl drench	92 (74.9±2.7)	304.8±4.4
LSD (P=0.05)	(4.6)	10.3

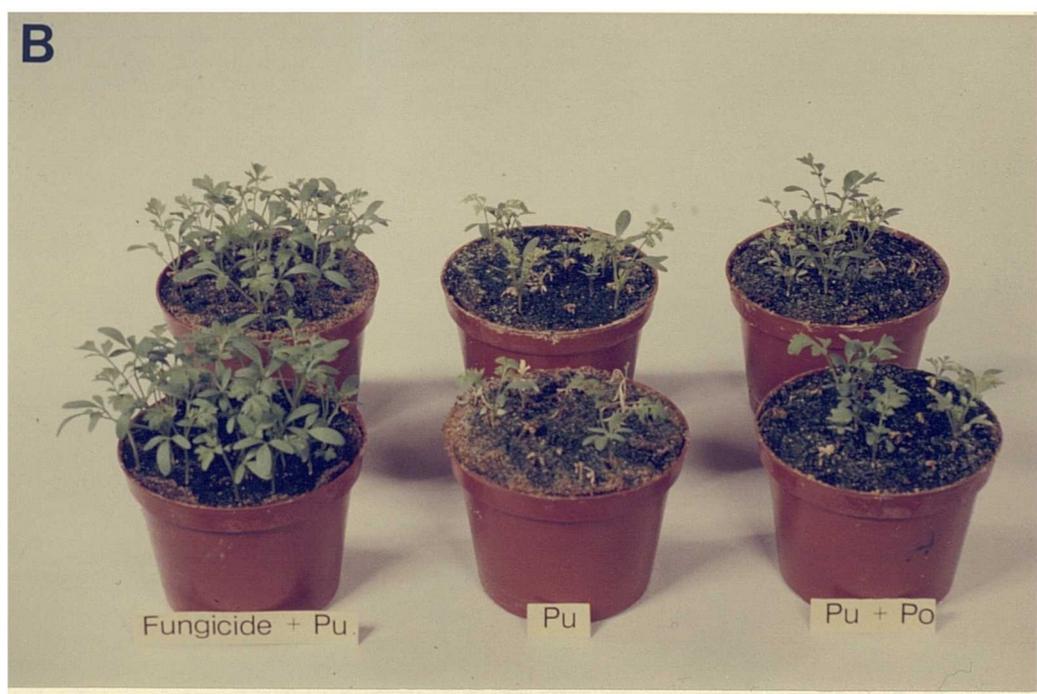
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\* The temperature at which seeds were dried after commercial treatment.

Plate 10

Surviving cress seedlings 16 d after sowing pelleted (A) and film-coated (B) seed in Pythium ultimum (Pu)-infested potting compost. Pythium oligandrum (Po) oospore seed treatments were compared with a fungicide (Propamocarb HCl) drench.

All pelleted seed were dried at 30°, apart from some batches of oospore treated seeds, which were dried at 45°. Film-coated seed was dried at 25°.



#### 8.4 Effect of seed treatments on damping-off of sugar beet in sand and soil

The most important soil-borne fungi causing damage to, and loss of sugar beet seedlings are A. cochlioides and Pythium spp. (Asher & Payne, 1989; Payne & Asher, 1989). Serious losses due to Pythium spp. have been recorded in Finland (Vestberg et al., 1982), northern France and, more recently, in Yugoslavia (Asher & Payne, pers. comm.). In England the fungus has been recorded in about one third of fields where sugar beet is grown (Asher & Payne, 1989) but is generally considered not to be a serious problem. The optimum temperature for infection is about 18° (Vestberg, 1984) though damage can occur at temperatures lower than this under conditions of high soil moisture. Seedling losses can be either pre- or post-emergence (Asher & Payne, 1989). In contrast, death of seedlings caused by A. cochlioides occurs almost entirely post-emergence. This fungus requires relatively high soil temperatures (optimum c. 25°) for maximum infection and is favoured by high soil moisture and low pH (Byford, 1975). Again, it occurs in about one third of fields in England (Asher & Payne, 1989), but because of the high soil temperature requirements, it generally causes problems only in late-drilled crops. Serious losses have been recorded in several regions of France (Bouhot, 1983; Bouhot, Moncorge & Richard-Molard, 1985) and Germany (Asher & Payne, pers. comm.).

The ability of P. oligandrum seed treatments to reduce damping-off was compared with standard fungicide seed treatments in sand artificially infested with P. ultimum and in soil naturally infested with A. cochlioides and Pythium spp..

## 1. Sand experiments

Experiments were carried out using the method described in section 7.1 in which cress seeds were sown in clear plastic boxes (16 x 28 x 5 cm) each containing 1500 g sand infested with sporangia of P. ultimum (MPM01). P. ultimum (MPM01) was isolated from a damped-off sugar beet seedling grown in soil from Broom's Barn Experimental Station, Higham, Suffolk (Table 2). A preliminary pathogenicity test was carried out to determine a suitable inoculum density of P. ultimum (MPM01) for use in evaluating oospore seed treatments against damping-off in sand.

### i. Pathogenicity test

To infest sand, sporangia of P. ultimum (MPM01) were produced as described in section 7.1. Sand was infested with sporangia (5, 10, 50 and 100 sporangia per g dry weight equivalent of sand) and added to boxes. General procedures were the same as those in sand experiments described in section 8.3, except that disease progress was monitored over a 21 d period.

For results, see Table 38. As in previous pathogenicity tests, seedling stand declined with an increase in the inoculum density. Increasing the inoculum density from 5 to 10 sporangia per g sand gave a marked decrease in seedling stand. The inoculum density of 5 sporangia per g sand, which gave a seedling stand of 43%, was used in the subsequent experiment.

### ii. Control of damping-off in Pythium ultimum-infested sand

Sugar beet seeds were sown in plastic boxes containing sand artificially infested with P. ultimum (MPM01) sporangia (5 sporangia per g dry weight equivalent of sand) as described previously and disease progress monitored in the controlled environment room as before.

Table 38

Pathogenicity test. Percentage stand of sugar beet seedlings grown in sand artificially infested with various amounts of inoculum of Pythium ultimum (MPM01) for 21 d.

Figures in parentheses are means from three replicates calculated after angular transformation of percentage data.

---

Inoculum of <u>P. ultimum</u> (MPM01) (sporangia per g dry wt sand)	Seedling stand (%)
0*	97 (82.6±4.1)
5	43 (42.8±1.9)
10	14 (21.3±4.9)
50	7 (14.2±3.4)
100	6 (13.7±3.0)
LSD (P=0.05)	(12.9)

---

\* Pathogen-free sand.

Results are shown in Table 39. Only low levels of pre-emergence damping-off occurred, with over 90% of all seeds emerging. Slight improvements in emergence were found with the P. oligandrum 45° pelleted treatment, the pelleted fungicides, and the P. oligandrum film-coated treatments. After 21 d, the P. oligandrum pelleted treatment gave significantly greater seedling stands than the pelleted control and were equivalent to, or just less than, the two pelleted fungicide treatments. The P. oligandrum film-coated treatment gave significantly greater seedling stand compared with the film-coated control and was equivalent to the film-coated fungicide treatments. Both pelleted and film-coated controls gave significant improvements in seedling stand compared with uncoated controls.

## 2. Soil experiments

A brown sandy loam (Moulton series), pH 7.0, from Broom's Barn Experimental Station was used in all experiments. After collection from the top 10-15 cm of the profile, soil was stored in polyethylene bags at room temperature and was sieved through a 2 mm mesh screen before use. To test the seed treatments at a range of inoculum potentials, soil from a site naturally infested with a high inoculum potential of A. cochlioides was diluted with varying proportions of sand or soil from a site with a low inoculum potential of A. cochlioides. Soils from both sites carried a low natural population of Pythium spp. Information on inoculum potentials of A. cochlioides and Pythium spp. in the soil was provided by Dr. M. J. C. Asher and P. A. Payne of Broom's Barn Experimental Station.

Table 39

Effect of seed treatments on seedling emergence and stand of sugar beet 21 d after sowing in sand artificially infested with Pythium ultimum (MPM01).

Values in parentheses are means with SEM from three replicates calculated after angular transformation of percentage data.

---

Seed treatment	Emergence (%)	Seedling stand (%)
Control (uncoated)	90 (71.8±2.1)	33 (34.7±3.4)
EB3 pellet (30°)*	91 (72.4±2.0)	57 (49.2±0.8)
+ Metalaxyl	97 (79.6±1.1)	93 (75.1±0.4)
+ Hymexazol	98 (83.4±3.4)	97 (80.7±1.1)
+ <u>P. oligandrum</u>	93 (75.0±0.4)	86 (68.1±1.7)
+ <u>P. oligandrum</u> (45°)	97 (81.4±4.4)	89 (71.2±3.7)
Film-coated (25°)	93 (74.6±2.3)	61 (51.4±1.5)
+ Metalaxyl	97 (79.6±1.1)	91 (72.6±1.2)
+ Hymexazol	96 (78.7±1.8)	93 (75.2±1.8)
+ <u>P. oligandrum</u>	97 (82.5±4.1)	92 (73.7±1.6)
LSD (P=0.05)	(7.8)	(6.1)

---

\* The temperature at which seeds were dried after commercial treatment.

i. Control of damping-off in a soil-sand mix naturally infested with *Aphanomyces cochlioides* and *Pythium* spp.

In the first series of experiments, the soil with a high inoculum potential of *A. cochlioides* was mixed thoroughly with an equal volume of sand and adjusted to c. 60% moisture content. Plastic pots (7.5 cm diam) containing c. 200 g of soil-sand mixture were each planted with 20 sugar beet seeds, with five replicates per treatment. The control consisted of uncoated seed. Pots in saucers were arranged in a randomised complete block design in a Fisons 600 H growth chamber with a 14 h light (24°, 40 W/m<sup>2</sup>, 80% r.h.) and 10 h dark (18°, 80% r.h.) regime and were watered daily.

Dead seedlings were removed at regular intervals and placed in sterile distilled water in 9 cm diam Petri dishes at room temperature (c. 20°) for 24-48 h. During this time, growth of fungal structures from dead seedlings occurred, allowing disease diagnosis. Seedlings killed by *A. cochlioides* were identified by the clusters of encysted zoospores which developed at tips of hypha-like zoosporangia (Byford & Stamps, 1975) and those killed by *Pythium* by characteristic sporangium production.

After 28 d, seedling stand and shoot dry weight were measured. The soil-sand mixture from all pots was then thoroughly mixed together and the whole experiment repeated using this mixture. As all seedlings in all treatments were dead 12 d after sowing, the mixture was again mixed and diluted further with additional sand to give a final 1:7 v/v soil-sand mixture, and the whole experiment repeated.

Results are shown in Tables 40 and 41. In the 50:50 v/v soil-sand mixture, there were no significant increases in emergence with fungicide or *P. oligandrum* treatments in comparison with appropriate pelleted or film-coated controls. After 28 d, all *P. oligandrum* and

Table 40

Effect of seed treatments on seedling emergence, stand and shoot dry weight of sugar beet 28 d after sowing in a natural soil-sand mix (50:50 v/v) infested with Aphanomyces cochlioides and Pythium spp.. Values for emergence and seedling stand are means with SEM from five replicates calculated after angular transformation of percentage data. Values for dry weight shoot are means from five replicates with SEM.

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Seed treatment	Emergence (%)	Seedling stand (%)	Dry weight shoot (mg)
Control (uncoated)	77 (65.7±9.2)	21 (27.2±0.7)	80.2±3.9
EB3 pellet (30°)*	87 (69.8±3.5)	20 (26.2±2.9)	73.9±6.4
+ Metalaxyl	97 (83.7±3.9)	30 (33.2±1.0)	123.7±15.2
+ Hymexazol	91 (74.7±4.4)	63 (53.0±4.3)	187.4±9.6
+ <u>P. oligandrum</u>	97 (83.7±3.9)	62 (52.6±5.0)	192.8±10.4
+ <u>P. oligandrum</u> (45°)	85 (69.8±5.5)	45 (42.1±3.8)	139.6±23.6
Film-coated (25°)	70 (60.0±9.5)	28 (30.8±5.1)	107.5±27.7
+ Metalaxyl	78 (62.5±5.2)	24 (28.1±5.2)	82.4±26.0
+ Hymexazol	82 (65.3±2.6)	48 (43.8±1.7)	191.3±9.0
+ <u>P. oligandrum</u>	84 (67.7±4.4)	48 (43.8±2.2)	147.5±15.2
LSD (P=0.05)	(15.0)	(9.7)	47.6

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\* The temperature at which seeds were dried after commercial treatment.

Table 41

Effect of seed treatments on seedling stand of sugar beet 9 and 13 d after sowing in a natural soil-sand mix (1:7 v/v) infested with Aphanomyces cochlioides and Pythium spp..

Values in parentheses are means with SEM from five replicates calculated after angular transformation of percentage data.

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Seed treatment	Seedling stand (%)	
	9d	13d
Control (uncoated)	30 (31.8±6.2)	0 (0.0)
EB3 pellet (30°)*	20 (24.3±7.1)	0 (0.0)
+ Metalaxyl	43 (41.0±4.8)	5 (10.0±4.2)
+ Hymexazol	81 (65.1±4.0)	25 (29.1±4.8)
+ <u>P. oligandrum</u>	44 (41.3±5.3)	25 (29.0±4.7)
+ <u>P. oligandrum</u> (45°)	56 (48.6±3.2)	31 (33.6±2.7)
Film-coated	18 (24.5±4.1)	5 (10.0±4.2)
+ Metalaxyl	22 (27.7±2.3)	4 (8.9±3.7)
+ Hymexazol	87 (71.3±5.2)	39 (37.6±8.1)
+ <u>P. oligandrum</u>	50 (45.0±2.6)	33 (34.7±3.5)
LSD (P=0.05)		(11.9)

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At 21 d, all seedlings were killed in all treatments.

\* The temperature at which seeds were dried after commercial treatment.

hymexazol treatments had significantly greater seedling stands and shoot dry weights compared with controls, although the P. oligandrum film-coated treatment was not significantly greater than the film-coated control. Metalaxyl was significantly less effective in controlling damping-off compared with hymexazol. Microscopic observations showed that 95% of seedlings were killed by A. cochlioides but only 5% by Pythium spp.. In a repeat of this experiment using the same soil-sand mixture, all seedlings in all treatments were killed 12 d after sowing, indicating an increase in inoculum potential.

In the 1:7 v/v soil-sand mixture, a reduction in disease with concomitant increase in seedling stand was obtained 9 d after planting with all P. oligandrum and hymexazol treatments (Table 41). The seedling stands were reduced further in all treatments 13 d after sowing, and only with P. oligandrum and hymexazol treatments did a significant number of seedlings survive. By 21 d after planting all seedlings were dead. Again, 95% of damped-off seedlings were killed by A. cochlioides and only 5% by Pythium spp..

ii. Control of damping-off in soil at a range of inoculum potentials of Aphanomyces cochlioides and Pythium spp.

In the second series of experiments, the soil with high inoculum potential of A. cochlioides was diluted with soil with a low inoculum potential of A. cochlioides to give proportions of 100, 50, 10, 5, 2, 1 and 0% v/v of high inoculum potential soil. Plastic pots (7.5 cm) were then filled with soils at 60% moisture content from this dilution series and each planted with 20 sugar beet seeds, with five replicates per treatment. Pots in saucers were arranged in a randomised complete block design on a bench in the glasshouse maintained between 20-28° and

watered and monitored as before. Because EB3 pellets gave results generally equivalent to those from film-coated oospore treatments in the previous tests only EB3 pelleted treatments were used here.

Results are shown in Tables 42 and 43, and Plate 11. A clear effect of inoculum potential dilution on seedling stand was found (Table 42). With proportions above 10% of heavily infested A. cochlioides soil, neither pelleted treatments of P. oligandrum or hymexazol had any effect on seedling stand. However, at proportions of 10% or below, both these treatments consistently increased seedling stand. When only the low disease incidence soil was used (0% infested soil) the pelleted control gave levels of seedling stand equivalent to the fungicide and P. oligandrum treatments. These effects were mirrored in results expressed as seedlings killed due to A. cochlioides only (Table 43). The only difference observed was that the first significant effect of P. oligandrum occurred at a proportion of 5% rather than 10% high A. cochlioides inoculum potential soil.

Table 42

Effect of seed treatments on seedling stand of sugar beet 28 d after sowing in natural soil infested with Aphanomyces cochlioides and Pythium spp..  
 Values in parentheses are means from five replicates calculated after angular transformation of percentage data.

Seed treatment	Seedling stand (%)						
	100	50	10	5	2	1	0
Control (uncoated)	11 (18.7±2.7)	15 (21.9±3.6)	24 (29.3±1.2)	19 (25.1±3.5)	27 (31.2±1.6)	21 (26.5±3.6)	48 (43.8±4.2)
EB3 pellet (30°)**	16 (23.2±2.3)	16 (21.2±5.4)	9 (14.9±4.8)	11 (19.0±2.2)	33 (35.0±0.8)	29 (32.3±2.5)	78 (63.7±6.5)
+ Hymexazol	29 (31.8±5.3)	24 (28.9±3.3)	80 (65.1±5.5)	71 (61.2±9.8)	84 (71.8±8.2)	89 (75.1±6.5)	77 (61.9±3.3)
+ P. <u>oligandrum</u>	13 (18.4±5.4)	25 (27.1±7.1)	56 (56.2±6.2)	67 (56.2±6.2)	73 (59.5±4.9)	74 (60.7±5.8)	86 (70.4±5.2)
LSD (P=0.05)	(14.4)						

\* Soil infested with a high inoculum of A. cochlioides was diluted with soil with a low inoculum potential of A. cochlioides. Both soils contained a low inoculum potential of Pythium spp..

\*\* The temperature at which seeds were dried after commercial treatment.

Table 43

Effect of seed treatments on post-emergence damping-off of sugar beet caused by Aphanomyces cochlioides 28 d after sowing in natural soil infested with A. cochlioides and Pythium spp.. Values in parentheses are means from five replicates calculated after angular transformation of percentage data.

Seed treatment	Number of seedlings killed (%)						
	100	50	10	5	2	1	
	% <u>A. cochlioides</u> infested soil* (v/v)						
Control (uncoated)	55 (47.9±3.2)	51 (45.5±5.4)	63 (52.6±1.5)	58 (49.7±2.6)	51 (45.5±4.7)	54 (47.4±5.2)	4 ( 7.4±4.5)
EB3 pellet (30°)**	67 (55.3±3.7)	65 (53.9±2.9)	86 (68.7±3.1)	66 (55.9±7.0)	56 (48.5±2.6)	67 (55.1±2.4)	15 (17.4±8.3)
+Hymexazol	67 (55.4±4.6)	75 (60.3±2.7)	10 (11.0±8.2)	28 (27.7±10.1)	15 (17.1±8.2)	10 (14.0±6.3)	6 (9.0±5.7)
+ <u>P. oligandrum</u>	67 (55.7±5.0)	68 (56.7±5.4)	42 (37.2±9.5)	28 (28.3±8.8)	25 (29.4±3.9)	22 (24.0±8.3)	3 (4.6±4.5)
LSD (P=0.05)	(16.2)						

\* Soil infested with a high inoculum potential of A. cochlioides was diluted with soil with a low inoculum potential of A. cochlioides. Both soils contained a low inoculum potential of Pythium spp..

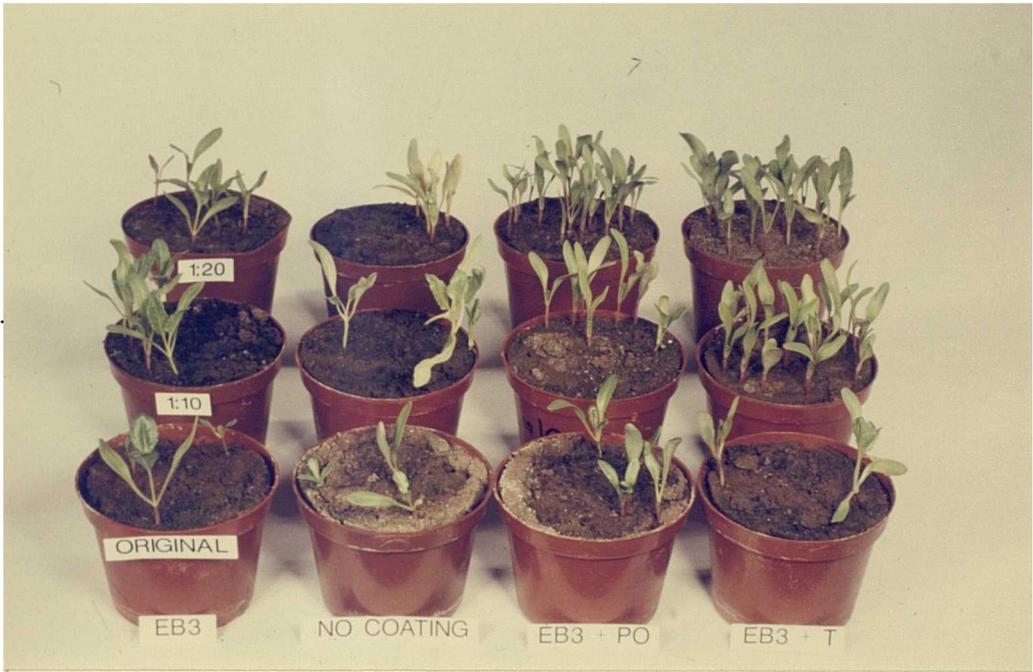
\*\* The temperature at which seeds were dried after commercial treatment.

Plate 11

Surviving sugar beet seedlings 28 d after sowing pelleted (EB3) seed in soil containing Aphanomyces cochlioides and Pythium spp. at a range of inoculum potentials. Pythium oligandrum (Po) oospore seed treatments were compared with a fungicide (hymexazol (T)) seed treatment.

Note:

'Original'	=	100%	(v/v)	infested	soil
'1:10'	=	10%	"	"	"
'1:20'	=	5%	"	"	"



### 8.5 Rhizosphere competence of *Pythium oligandrum* on cress and sugar beet

Rhizosphere competence was measured by sowing 20 seeds of each seed-coating treatment in pots containing either sterile sand or potting compost. Pots were placed in a Fisons 600H growth cabinet for 8 d, after which time seedlings were carefully removed. Ten randomly selected seedlings were then treated as described by Ahmad & Baker (1987a). Hypocotyl and individual root segments (1 cm) were plated on the modified selective medium (see Appendix 3.1) of Martin & Hancock (1986), and incubated at 25° for 4 d. Plates were observed daily for characteristic colony growth of *P. oligandrum* from segments.

After 8 d, *P. oligandrum* was isolated from at least 50% of the root segments 1 cm below the seed from all coated seeds in both sand and potting compost. However, it was only recovered in three root segments each of cress and sugar beet 2 cm below the seed from all those sampled. It was never found below this zone. In contrast, it was recovered from all segments of hypocotyl 1 cm above the seed (data not shown).

### 8.6 The effect of pesticides on mycelial growth of *Pythium oligandrum* in vitro

Seed-coatings commonly contain a mixture of fungicides and insecticides (Halmer, 1988). In the U.K., fungicides such as thiram and hymexazol are routinely incorporated into seed pellets of commercially-grown sugar beet for control of damping-off diseases (Dewar et al., 1988; Halmer, 1988; Asher & Payne, 1989). The carbamate insecticide, methiocarb, is also usually incorporated into seed pellets

along with the fungicides for partial control of soil-inhabiting pests (Durrant et al., 1986). Tefluthrin (pyrethroid) and a range of carbamates including carbofuran, carbosulfan and furathiocarb are currently being tested in field trials with a view to introducing new insecticide seed treatments (Halmer, 1988). Consequently, P. oligandrum must be active in the presence of these pesticides if it is to have any success as a commercial seed treatment in an integrated pest management system. Even if P. oligandrum were sensitive to any of these pesticides, it might be possible to develop tolerant isolates by exposing the fungus to increasing pesticide concentrations in vitro. A benomyl-tolerant isolate of P. oligandrum has already been produced using this technique (Lewis, 1988; Lewis et al., 1989).

In this study, pesticides used as seed treatments were tested on mycelial growth of P. oligandrum in agar media. The pesticides tested consisted of three fungicides and two carbamate insecticides: hymexazol (Tachigaren 70% WP; Sumitomo Chemical Co. Ltd.), metalaxyl (Apron 25% WP; Ciba-Geigy Agrichem.), thiram (Agrichem Flowable thiram 60% FS; Agrichem Ltd.), methiocarb (Mesurool 50% WP; Bayer UK Ltd.) and benfuracarb 40% WP (Farm Protection Ltd.).

Stock suspensions of each pesticide were prepared and appropriate amounts added to autoclaved molten CMA previously cooled to 50° to give a range of pesticide concentrations (Table 44). Batches of amended CMA were then poured aseptically into 12 cm diam plastic Petri dishes, with three replicates for each pesticide concentration. Petri dishes were each inoculated centrally with a 5 mm mycelial disc of P. oligandrum (IMI 133857) cut from the periphery of a 3 d colony on CMA. Dishes were incubated at 25° for 4 d by which time the mycelia had reached the

edge of the control plates. Colony diameters were measured along two predetermined diameters at right angles. Dose responses were obtained as the concentration required to inhibit fungal growth by 50% (IG50) using probit-line analysis according to Bliss (1935).

Results are shown in Table 44. Mycelial growth was most inhibited by metalaxyl followed by thiram, hymexazol and the two carbamate insecticides. The strong effect of metalaxyl on P. oligandrum is due to the specificity of this fungicide for Oomycetes (Schwinn & Urech, 1986). The insecticides had very little effect on mycelial growth at concentrations below 10 mg a.i./l and did not inhibit growth at the highest concentration tested (50 mg a.i./l).

Table 44

Mean colony diameter (mm) of Pythium oligandrum (IMI 133857) on CMA amended with various pesticides after 4 d at 25°. Each value is the mean from three replicates with SEM.

Pesticide	Pesticide concentration (mg a.i./l)										IG50
	0	0.1	0.05	0.1	0.5	1.0	5.0	10.0	50.0	IG50	
Hymexazol	107±2.5	104±3.6	102±1.5	101±1.0	101±1.3	60±2.4	21±1.7	14±2.5	0	1.81	
Metalaxyl	106±1.8	81±2.1	68±2.4	41±2.5	8±0.9	0	0	0	0	0.05	
Thiram	107±2.5	83±1.9	80±2.5	72±2.0	57±1.8	0	0	0	0	0.16	
Benfurabarb	108±1.9	-*	-	107±1.8	104±3.4	104±2.5	103±1.4	73±1.4	22±2.1	11.21	
Methiocarb	109±3.1	-	-	108±2.8	107±1.9	107±2.1	106±0.9	101±0.5	54±1.6	35.12	

\* Growth not assessed.

CHAPTER IX

DISCUSSION

## CHAPTER IX

### DISCUSSION

The excessive and irrational use of fungicides to control plant pathogens has resulted in the incidence of high levels of toxic chemicals in plants and the environment (Cook & Baker, 1983). It has also led to the development of fungicide resistance in pathogen populations (Dekker & Georgopolous, 1972). To alleviate these problems, viable supplements or alternatives to chemical control are being sought and the use of microbial antagonists is one of the promising approaches (Cook & Baker, 1983; Papavizas, 1984; Lumsden & Lewis, 1989). The mycoparasite, P. oligandrum, has shown considerable promise as a biocontrol agent for use against a number of important soil-borne plant pathogens in small-scale laboratory as well as field experiments (Lewis et al., 1989). Nevertheless, the information gathered on P. oligandrum is incomplete as various important aspects of the mycoparasite have not been studied systematically. The present investigation was, therefore, carried out to identify nutritional and environmental factors affecting growth, oospore production and germination. Techniques for bulk production of oospores were also developed. Different oospore formulations were prepared and their survival monitored under different temperature regimes and edaphic conditions. These oospore formulations were evaluated for biocontrol efficacy against pathogens causing damping-off in cress. Finally, seeds of cress and sugar beet were coated with oospores using

commercial seed-pelleting and film-coating procedures, and tested against a number of soil-borne plant pathogens. Several important aspects relevant for the development of P. oligandrum as a biocontrol agent of soil-borne diseases have been revealed.

The investigation has identified in detail the nutritional and environmental requirements for growth as well as for oospore production and germination (Chapter III & IV). This information has significance in the development of large-scale production techniques for biocontrol inoculum using inexpensive growth media, and also provides an insight into the range of environmental and nutritional conditions under which growth and biocontrol activity of P. oligandrum may occur in nature.

P. oligandrum was found to grow on most of the carbon sources tested but only the simple sugars glucose, maltose and trehalose were utilized extensively (Table 3). Almost all fungi utilize glucose (Griffin, 1981a), the main exceptions being those that grow in extremely specialized habitats such as Leptomitius lacteus, a member of the Leptomitales that grows in sewage (Gleason, 1968) where glucose is absent. Maltose has been found to be utilized by a wide range of Pythium spp. (Saksena, 1940; Saksena & Mehrotra, 1949) and also by virtually all fungi which have been tested (Griffin, 1981a). Consequently, the utilization of glucose and maltose by P. oligandrum in this study was expected. Trehalose and the sugar alcohol, mannitol, were utilized confirming the findings of Foley & Deacon (1986b) but the amount of growth obtained on the latter was small. Both trehalose and mannitol are translocatable storage carbohydrates in fungi (Cochrane, 1958; Lewis & Smith, 1967) and although all groups of fungal pathogens contain variable amounts of trehalose, members of

the Mastigomycotina generally lack acyclic sugar alcohols (Whipps & Lewis, 1981). The ability to utilize trehalose and mannitol may account for part of the success of P. oligandrum as a mycoparasite of several plant pathogens (Lewis et al., 1989). Once P. oligandrum has penetrated host cell walls by secreting hydrolytic enzymes, it is possible that the mycoparasite can utilize these carbohydrates, if they are present, together with other nutrients to the detriment of the host fungus. The results also show that P. oligandrum was unable to utilize cellulose and therefore corroborate those of other researchers that the mycoparasite is non-cellulolytic (Tribe, 1966; Deacon, 1976; Lewis et al., 1989).

Some points can be made regarding the techniques used to study the effect of carbon sources on growth. The work suffers from the defect that the yields were compared on the basis of a fixed time of harvest. The time chosen for harvest in this study was the time that near-maximum biomass dry weight was attained on glucose in a preliminary experiment. With this approach, no consideration was made for those sources which may have been utilized only after a long lag period. It was also possible that growth on some of the sources may have been completed and followed by extensive autolysis before harvest. Periodic harvesting would have solved this problem. Breakdown of compounds during autoclaving constitutes another problem in studies on carbon nutrition (Cochrane, 1958). Carbon sources tested here were autoclaved separately and added individually to batches of sterile medium, except for oligosaccharides which were added after filter-sterilization. Biomass yields on starch and dextrin were slightly greater than those on the control medium suggesting that P.

oligandrum may have the ability to utilize these two polysaccharides slowly. These results may merely reflect the partial hydrolysis of these polysaccharides during autoclaving to utilizable maltose. Similarly, P. oligandrum was found to grow poorly on xylose. As xylose is broken down to furfural during autoclaving (Cochrane, 1958), the possible non-utilizability of the sugar here should be confirmed with filter-sterilized xylose. Nevertheless, the results obtained from this investigation are useful as they provide valuable information required in developing an inexpensive growth medium for producing inoculum of P. oligandrum. In this respect, natural ingredients should be chosen containing available simple sugars, such as glucose or maltose, which the mycoparasite can readily utilize.

Results from the experiment conducted on the effect of nitrogen source on growth indicate that P. oligandrum may have a preference for utilizing organic nitrogen sources (Table 4). The partial utilization of ammonium nitrate shown here should possibly be viewed with caution since it has been shown by previous researchers that P. oligandrum and other mycoparasitic pythia require organic nitrogen for growth (Leonian & Lilly, 1983; Child et al., 1969; Foley & Deacon, 1986b). The reason for this discrepancy is unknown though it may simply reflect an expression of strain difference or variation. Complex sources of nitrogen were all utilized but to varying degrees. The amount of growth on mycological peptone, which is a complex mixture of peptides and amino acids, was substantial and, in most cases, greater than on any of the single amino acids. This occurrence may be related to the complex nature of mycological peptone, for a mixture of nitrogen sources generally allows greater and more rapid growth of fungi than a

single source although the basis of this physiological phenomenon is unknown (Lilly & Barnett, 1951; Griffin, 1981a).

Amino acids, simple sugars and organic acids as well as other compounds commonly occur in seed and root exudates of many plants (Schroth & Hildebrand, 1964). With the present in vitro work, it was shown that P. oligandrum has the ability to utilize a wide range of these compounds. These results, therefore, provide indirect evidence to suggest that when the mycoparasite is introduced into the soil microbiome, it may be able to utilize a number of nutrients present in both seed and root exudates for growth. In this respect, it has already been shown that cress seed exudates stimulate oospore germination (Lutchmeah, 1985).

The carbon-nitrogen (C:N) ratio of the glucose-asparagine medium had a profound effect on the amount of growth of P. oligandrum (Table 5). Media was adjusted to predetermined C:N ratios of 5:1, 9:1, 16:1, 30:1, 60:1, 90:1 and 130:1 in two ways: firstly by increasing the carbon concentration, secondly by decreasing the nitrogen concentration. Yields in both series showed a direct relation between nitrogen and carbon content until a maximum was reached. Also, when C:N ratios were identical yields in each series were different from those in the other series. Thus it is possible that the more important factors influencing the growth of P. oligandrum are the concentrations of carbon and nitrogen in the medium rather than the C:N ratio per se. Increasing the carbon concentration of the medium (with resultant increase in C:N ratio) gave an increase in the amount of growth but the efficiency at which carbon was utilized declined. Beyond the optimum carbon concentration, there was a reduction in the amount of growth.

This phenomenon may perhaps be ascribed to other constituents of the medium, especially nitrogen, becoming limiting at high carbon concentrations. Alternatively, it is possible that the osmotic potential of the culture medium at these concentrations was unfavourable for growth. In fungi, when the osmotic potential of the external culture medium falls below that of the cell sap, the hyphae become plasmolysed and water is lost from the cytoplasm. With further decreases in medium osmotic potential the protoplast becomes seriously dehydrated and growth may become impossible. Although growth was not inhibited with the highest carbon concentration tested, it is likely that this would eventually happen if growth was tested in media with higher concentrations.

The study has identified the optimum temperature and pH for mycelial growth of P. oligandrum. Assuming that the optimum physical conditions for mycelial growth are similar to those for oospore production, the results obtained have practical significance in producing biocontrol inoculum of the mycoparasite. On most solid and liquid media tested, the optimum temperature for growth was found to be in the range of 20 to 30° and there were very little differences between isolates. Obviously, incubation temperatures within this range need to be used in the production of biocontrol inoculum. It is of interest that growth rates on PDA were slow compared to those on the other solid media (CMA, TWA, SEA). This phenomenon has been previously reported by Whipps (1987a) and is possibly explained by the commercial preparation of PDA containing inhibitors of mycoparasitic Pythium spp. (Foley & Deacon, 1986b). In most cases, growth occurred over the range of pH 4.5 to 9.0 and was optimum between pH 6.0 to 7.5. The moderately narrow optimum

pH range of 6.0 to 7.5 demonstrated has the practical significance that for maximum growth in axenic culture, medium pH may need to be controlled by use of an effective buffer system or simply adjusted with acid or alkali prior to inoculation. The optimum pH for growth found in this study corresponds well with the pH of soils from which P. oligandrum has been commonly isolated. Foley & Deacon (1985) detected P. oligandrum most often in soil samples of pH 5.5 to 6.5 taken from 'disturbed' sites (gardens, arable lands, managed grasslands) and seldom from samples of pH less than 4.5

Physical environmental factors such as pH, temperature and water potential are known to influence the activity of fungal biocontrol agents in soil (Cook & Baker, 1983). Although the cultural studies described here using pH, temperature and water potential systems do not in any way directly simulate the conditions of the natural environment, the results from such studies may give an insight to the likely behaviour of P. oligandrum in the environment. In general, growth and oospore germination occurred over the range of pH 4.5 to 9.0 but were scanty and poor respectively at the extremes of the pH range tested. These observations have important implications for the practical application of P. oligandrum. The results suggest that the antagonist may not be an active biocontrol agent if introduced into soils of high acidity or alkalinity. Nevertheless, if such unfavourable soil conditions are encountered in agricultural and horticultural systems, growers may be able to manipulate the soil pH to favour the biocontrol activity of P. oligandrum. An example illustrating this principle has been used for the control of lily root rot, a disorder thought to be caused by Pythium spp. (Baker & Scher, 1987). Application of sulphuric

acid to lower the growing medium pH has provided complete control of root rot where indigenous Trichoderma spp. are present in the soil. It has been suggested that the antagonists become active biocontrol agents in acid soil but perform poorly under alkaline conditions.

Although such physical factors as pH profoundly influence the activity of biocontrol agents, unfavourable temperatures may be an even more important limiting factor. As stated earlier the optimum temperatures for growth of several isolates of P. oligandrum were found to be in the range of 20 to 30°, with little growth occurring below 10° and at or above 35° (Table 6). Using one isolate (IMI 133857) it was found that oospore germination occurred over the range of 10–35°, whilst the optimum temperatures were 25 to 30° (section 4.4). It would appear from these results that P. oligandrum is ideally suited for use in the glasshouse or plastic tunnels, where temperatures are normally maintained in the region of 15 to 30°. On the other hand, the use of the mycoparasite for biocontrol of diseases of field grown crops may be limited. The mycoparasite, with relatively high optimum temperature requirements, may function in warm climates but may be ineffective in cool soils. This limitation may be overcome by the late planting of crops when temperatures are possibly more conducive for the activity of P. oligandrum or searching for cold-tolerant strains.

The investigation has revealed for the first time the effects of osmotic and matric potentials on the growth and oospore germination of P. oligandrum. Although there were slight differences with media and osmotica, growth generally decreased with decreasing osmotic potential, biomass production and linear growth rates being reduced markedly below -1.0 to -1.5 MPa and ceasing altogether at approximately -2.5 to -3.5

MPa. Similar results were obtained for oospore germination which was maximum and fairly uniform over the range -0.5 to -1.5 MPa. Germination decreased below -1.5 MPa with decreasing osmotic potential and was totally inhibited at -3.5 MPa. Both growth and oospore germination were more affected by low matric than by low osmotic potentials. Furthermore, the matric potential range for growth and oospore germination was markedly narrower than the osmotic range. Again, this information has important implications in the practical use of the mycoparasite. The majority of agricultural and horticultural soils as well as other growing media are maintained naturally or artificially at water potentials higher than the permanent wilting point of mesophytic higher plants, which is approximately -1.5 MPa (Slatyer, 1967). P. oligandrum is therefore likely to function in most commercially used plant growing media providing that other environmental parameters including pH and temperature are conducive for its development. It is also clear that P. oligandrum is able to grow over a similar range of water potentials as two of its potential target pathogens (P. aphanidermatum and P. ultimum). This characteristic is an ideal environmental attribute for a potential fungal biocontrol agent (Cook & Baker, 1983).

Some speculation can be made as to why P. oligandrum is more sensitive to matric potential than to an equivalent low osmotic potential. At low matric potentials, liquid films adsorbed to the substrate may be extremely thin which may reduce diffusion of nutrients. It is possible at low osmotic potentials that hyphae and oospores of P. oligandrum may be able to take up solutes to reduce their internal osmotic potential, a mechanism that may not exist when

water potential is matric. PEG 6000, which was used to adjust matric potential, has a larger molecular size than the solutes NaCl or KCl and, unless broken down into smaller units, would not be able to enter oospores or hyphae. In V8 juice modified with NaCl growth of P. oligandrum was stimulated at -0.7 MPa osmotic potential (Fig. 5, Appendix 1.6b). This may result from uptake of the solute (i.e. NaCl), with either or both of two possible benefits : a lowering of the osmotic potential of the protoplasm to a level more suitable for cellular processes or increased turgor and hence acceleration of growth (Cook & Duniway, 1981). Fungal cell membranes are presumably more permeable to some ions or molecules than to others. Therefore, differences in cell membrane permeability are likely to explain some of the differences in the responses of P. oligandrum hyphal growth to the various solutes used (eg. NaCl, KCl and glycerol).

Biomass yields of P. oligandrum were markedly increased by the addition of thiamine HCl to a basal medium (section 4.1, Table 9) suggesting that the mycoparasite requires an exogenous supply of thiamine or one of its components for growth. The finding is in agreement with those of Foley & Deacon (1986b) and Ridings et al. (1969).

Sterols function as membrane components and exert a striking effect on the growth and reproduction of fungi of the Pythiaceae (Elliot, 1977). In this study, P. oligandrum gave enhanced growth with the addition of cholesterol to a basal medium containing thiamine HCl, and oospores were not produced unless the sterol was present in the medium. The present findings therefore indicate that P. oligandrum requires an exogenous supply of sterols for oospore production. This has

considerable importance when developing media for use in producing biocontrol inoculum. The importance of calcium for oospore development in several species of Pythium has been emphasized previously (Lenney & Klemmer, 1966), and the calcium requirement is probably the explanation of the significantly greater numbers of oospores produced in the basal medium containing cholesterol in combination with calcium.

The carbon-nitrogen (C:N) ratio of glucose-asparagine agar medium was found to have a marked effect on the number of oospores produced by P. oligandrum (section 4.2, Table 11). From the results obtained it is obvious that the C:N ratio is an important nutritional factor to consider if it is desirable to produce an abundant number of oospores in culture. In general, low C:N ratios (5-30:1) were favourable for oospore production whereas high C:N ratios (60:1 and above) were not. These findings were similar to those obtained for Pythium sp. PRL 2142, where C:N ratios of 15-30:1 were optimum for oospore formation and those above 60:1 were inhibitory (Child et al., 1969). The reason for the C:N ratio effect on oospore production of P. oligandrum is unclear. From the experiment on the utilization of D-glucose and L-asparagine in relation to biomass and oospore production (section 4.3), it would appear that the trigger for inducing oospore formation in the defined medium is the production of specific nutrient levels and not total exhaustion (Fig. 8 and Appendix 2.3). In view of these findings, it is possible at low C:N ratios that the trigger for inducing oospore formation occurs before that at high C:N ratios, leading to the production of greater numbers of oospores. This warrants further investigation with a range of isolates.

Results from studies on the use of inexpensive solid growth media

for inoculum production show that oospores were abundantly produced on various media (sections 5.1 & 5.2, Tables 14 & 15). In autoclavable bags oospores production was highest on cracked barley supplemented with a solution of cane molasses and rapeseed oil and gave  $176.0 \times 10^5$  oospores per g. This number of oospores is similar to the amount produced on Polygandron ( $200.0 \times 10^5$  per g), a commercial preparation based on a dried and powdered substrate (Vesely, 1989).

The static and aerated culture techniques utilizing molasses described (Chapter V) are simple and give a very high yield of oospores relative to mycelium. Indeed, between days 14 and 21 in the time-course static culture system, total dry weight increased by 12% whereas oospore numbers increased by 67% (section 5.4, Table 17). This implies that during this period there is a rapid shift from vegetative growth to sexual reproduction, and that during the reproductive phase existing mycelium is utilized to an extent that has rarely, if ever, been observed in other fungi. This indicates remarkable energy conservation by the fungus and provides further evidence that, in nature, this mycoparasite has a highly combative ruderal strategy (sensu Cooke & Rayner, 1984) which is the ideal for a biocontrol organism.

Both static and aerated culture systems are widely used in industry, and P. oligandrum appears doubly suited for production under such conditions as it gives a high oospore yield and can be grown on a medium based on a waste product. Of the systems examined, the aerated culture procedure gave more rapid oospore production than large volume static culture (section 5.7, Table 19). One apparent disadvantage of this liquid formulation is the long period required to produce oospores

in comparison with other industrial processes. In contrast, satisfactory biomass quantities of Trichoderma, Gliocladium and Talaromyces were obtained in 6 to 7 d but this time is still long compared to that for bacteria (Papavizas et al., 1984). However, the long period required to produce oospores is balanced by the extremely high oospore yield. In respect of this, molasses might contain the morphogens required for sexual reproduction. Sexual reproduction in Pythium has been shown to require the presence of sterols (Elliot, 1977) and, in this study, it was shown that oospore production in P. oligandrum was dependent upon the presence of sterols and calcium. Analysis of molasses has revealed the presence of these (United Molasses, Hull; personal communication). Alternatively, during medium utilization a key nutrient may be depleted, or ratios of C:N produced, such that mycelial growth is restricted and reproduction initiated (Cooke & Rayner, 1984). If morphogens are involved then the possibility exists of using molasses as an inexpensive trigger to induce reproduction in commercial systems.

A major advantage of a liquid-produced oospore preparation is that it contains no organic residues that might stimulate growth of fungal pathogens when introduced into soil. This is a risk when solid substratum formulations of biocontrol agents are used. In this respect, incorporation of solid substratum oospore preparations into P. ultimum artificially infested sand had no effect in reducing damping-off and fungal contaminants were present on the sand surface 3 to 4 d post sowing (section 7.2, Table 25). A range of suitable formulations including a CMC seed-coating, alginate pellets, oospore-kaolin dust and an oospore-coated perlite preparation were

prepared from liquid-produced oospore biomass (Chapter VI). Such liquid-produced oospore biomass thus allows considerable flexibility in the preparation of formulations.

The germination of liquid-produced oospores decreased with time under all storage temperature conditions, which could pose a problem for biocontrol (Fig. 11 and Appendix 4.1). However, the assessment employed could not detect those oospores germinating after 16 h and so the figures for germination must be treated as minima. In any case very low oospore dosages have been shown still to protect cress against damping-off (Lutchmeah, 1985) so that low percentage germination per se does not necessarily indicate poor biocontrol performance, particularly if oospores are to be applied to seed in large numbers. Furthermore, coating cress seeds with oospore biomass stored at 15° for 12 months still reduced the incidence of P. ultimum-induced damping-off (Table 27). Alginate pellets and the oospore-perlite preparation survived well in laboratory storage at a range of temperatures for 24 wk (Tables 21 & 22). Oospore dust also survived well in both soil and compost (Fig. 12, Appendix 4.4). In the light of these findings, it would appear that these oospore formulations have excellent storage and survival properties, which are important criteria for an effective biocontrol agent.

The results show that alginate pellet formulations represent a feasible approach to applying P. oligandrum to soil or other plant growing media. The preparation survived well in storage and reduced damping-off of cress in soil naturally infested with Pythium. Moreover, it was shown indirectly that P. oligandrum can grow from the sodium alginate gel in which it was entrapped into soil. The potential

for using alginate pellets, especially in the glasshouse, merits further investigation.

With most of the oospore formulations described in Chapter VII, the level of control of damping-off in artificially infested sand was not as good as that obtained in naturally infested soil. This may simply reflect differences in pathogen inoculum levels and not the relative efficacy of the mycoparasite.

The commercial seed-coating techniques (Chapter VIII) positioned precise quantities of oospores at known locations on the seed achieving 75-95% of the target dose. Oospores survived the extremes of the coating processes which included physical abrasion and temperatures up to 45°, giving levels of germination similar to those found before pelleting. The values of germination (9-19%) were similar to those obtained in section 6.1 and those found by Al-Hamdani *et al.* (1983). Germination has been increased by treatments such as washing with water or myo-inositol solution but subsequently the treated oospores lost viability over 60d (Walther & Gindrat, 1987a). In contrast, the seeds coated in this study still produced mycelia on plating onto CMA after 16 wk storage and those pelleted by Lutchmeah & Cooke (1985) were viable after 4 yr storage (K Lewis & J.M. Whipps, unpublished). This isolate of P. oligandrum produces oospores with low levels of germination but great longevity, a useful commercial attribute.

The new P. oligandrum seed-coatings also gave control of Pythium damping-off on cress and sugar beet under a range of conditions, confirming previous results with non-commercial P. oligandrum seed-coating systems (Al-Hamdani *et al.*, 1983; Vesely & Hejdaneck, 1984, Lutchmeah & Cooke, 1985; Martin & Hancock, 1987). Control of Pythium

damping-off on cress with P. oligandrum pelleted treatments in compost was less effective than that obtained in sand. The compost has a more complex physicochemical and microbiological nature than sand and may account for the differences. These factors are worthy of further study in relation to the use of P. oligandrum in the field.

In contrast to P. ultimum, the control of R. solani was not as good as that achieved with fungicides (Table 35). This may reflect differences in the susceptibility of R. solani hyphae in comparison with Pythium hyphae (Deacon, 1976; Walther & Gindrat, 1987a, b), antagonism between R. solani and P. oligandrum or different modes of action (Lewis et al., 1989). Interactions in the spermosphere and rhizosphere are likely to be intrinsically different to those observed on agar plates. This investigation also presents the first observation of P. oligandrum controlling A. cochlidioides damping-off in naturally infested soil. Although numerous investigations describe the control of damping-off in sugar beet by P. oligandrum they have always concerned Pythium spp. or Phoma betae (Vesely & Hejdaneck, 1984; Martin & Hancock, 1987; Walther & Gindrat, 1987a). There is a single report of P. oligandrum attacking Aphanomyces laevis de Bary in vitro (Vesely, 1978).

Both coating systems on their own gave some control of Pythium damping-off in sugar beet in artificially infested sand and soil with a low inoculum potential of Pythium. Coating alone gave no control of damping-off in soil with a high inoculum potential of Aphanomyces. P. ultimum has been shown to colonize seed pericarps of sugar beet within 4 h under favourable conditions in soil and to infect the true seed 14 h after germination. By 2 d, 55% of the true seed can be infected

(Osburn et al., 1989). These coatings may then act as simple barriers to early Pythium infections.

The control of A. cochlioides damping-off in sugar beet is clearly dependent on the inoculum potential of the soil (Tables 43 & 44). Neither the standard hymexazol fungicide nor P. oligandrum treatments gave control at high inoculum potentials of A. cochlioides. This may well explain some of the problems commonly associated with field trials of other fungal biocontrol agents. Generally the field tests are carried out in situations with guaranteed high levels of disease or with artificially increased disease levels. Under these conditions, the disease incidence may well be too high for chemical or biological control to work. Thus field trials should cover as wide a range of disease levels as possible rather than a few localized plots.

In the U.S.A., seedling loss in sugar beet is mainly due to pre-emergence damping-off caused by P. ultimum (Martin & Hancock, 1987). Under these conditions, seed-coatings of P. oligandrum controlled P. ultimum pre-emergence damping-off as effectively as fenaminosulf treatment but did not control post-emergence damping-off as well. In Switzerland, seed-coatings of oospores of P. oligandrum also controlled pre- and post-emergence damping-off caused by soil-borne P. ultimum and seed-borne Phoma betae (Walther & Gindrat, 1987a). In the U.K. soil tested here, A. cochlioides post-emergence damping-off was the main cause of sugar beet seedling loss and P. oligandrum gave control of the disease equivalent to the hymexazol treatment. P. oligandrum thus has the potential to control pre- and post-emergence damping-off of three of the major sugar beet diseases.

P. oligandrum does not appear to be rhizosphere competent on cress

and sugar beet (section 8.5). This is in contrast to some isolates of other seed applied fungal biocontrol agents such as Trichoderma spp., strains of which have been shown to spread along roots giving the potential for longer term protection from root-infecting pathogens (Chao et al., 1986, Ahmad & Baker, 1987a, 1988). In part this may be due to the absence of cellulolytic ability in P. oligandrum (Tribe, 1966; Deacon, 1976), considered to be a key attribute for rhizosphere competence in T. harzianum Rifai (Ahmad & Baker, 1987b). Consequently, the biocontrol activity of P. oligandrum applied to seed must be due to its localized activity around the seed, the hypocotyl and the root adjacent to the seed.

The use of P. oligandrum as a biocontrol agent of damping-off diseases, particularly of sugar beet, has now reached a stage requiring field testing on numerous sites with a range of pathogen inoculum potentials. Despite the lack of rhizosphere competence it has a considerable number of commercial and ecological attributes in its favour, the fungus has a natural worldwide distribution; it can control most of the major damping-off pathogens of sugar beet; it can be simply produced and coated on seed; it can survive coating and long term storage at room temperature; it is non-pathogenic to over 15 species of economic crop plants (Klemmer & Nakano, 1964; Kilpatrick, 1968; Lutchmeah & Cooke, 1985; Martin & Hancock, 1987). Finally, it can act both as a mycoparasite and a competitor of pathogens rather than producing antibiotics (Martin & Hancock, 1986, 1987; Lewis et al. , 1989) and thus appears a safe organism to release in quantity to the environment.

## APPENDICES

### Appendix 1.1

Effect of incubation period on the biomass yield (mg) of Pythium oligandrum in glucose-asparagine liquid medium at 25°.

Each figure is the mean of four replicates with SEM.

---

Incubation period (days)	Dry weight biomass (mg)
3	9.9±0.5
6	23.9±1.1
9	33.9±0.7
12	47.3±1.4
15	52.0±0.6
18	48.0±0.5
21	46.0±2.1
LSD (P=0.05)	3.3

---

Appendix 1.2

Effect of pH on the biomass yield (mg) of Pythium oligandrum in glucose-asparagine liquid medium after 12 d incubation at 25°.

Each figure is the mean of four replicates with SEM.

---

pH	Dry weight biomass (mg)
4.0 (3.9)*	N.G.**
4.5 (4.4)	N.G.
5.0 (5.0)	12.5±1.9
5.1*** (5.8)	14.8±1.2
5.5 (5.6)	44.8±1.5
6.0 (6.2)	51.5±1.6
6.5 (6.7)	55.0±1.3
7.0 (6.8)	53.5±1.5
7.5 (7.8)	50.8±2.8
8.0 (8.2)	31.5±1.2
8.5 (8.7)	31.8±1.0
9.0 (9.0)	10.8±1.6
9.5 (9.5)	N.G.
LSD (P=0.05)	4.7

---

\* Values in parentheses are the pH of spent medium.

\*\* N.G. - no growth.

\*\*\* Unbuffered medium.

Appendix 1.3

Colony radial growth rates (mm/d) of Pythium oligandrum on solid media of different pH.

Each figure is the mean of four replicates with SEM.

---

<u>pH</u>	<u>CMA</u>	<u>pH</u>	<u>TWA</u>
4.0	N.G.*	4.0	N.G.
4.4	9.0±0.4	4.4	N.G.
5.0	13.5±0.3	4.9	13.2±0.5
5.6	16.2±0.5	5.4	18.5±0.6
5.8**	19.5±0.5	6.0	24.0±0.4
6.1	26.3±0.6	6.5	23.0±0.4
7.1	27.8±0.5	7.1	21.5±0.3
7.6	19.8±0.5	7.3**	18.0±0.4
7.9	8.8±0.5	7.6	16.2±0.5
8.5	N.G.	8.1	9.2±0.6
9.0	N.G.	8.6	N.G.
		9.0	N.G.
LSD (P=0.05)	1.3	LSD (P=0.05)	1.4

---

\* N.G. - No growth

\*\* Unbuffered control.

#### Appendix 1.4

Effect of temperature on the biomass yield (mg) of Pythium oligandrum in glucose-asparagine liquid medium after 12 d incubation.

Each figure is the mean of four replicates with SEM.

---

Temperature (°C)	Dry weight biomass (mg)
5	N.G.*
10	12.2±1.1
15	23.8±1.5
20	36.2±2.4
25	52.8±1.6
30	63.5±1.6
35	55.8±1.2
40	N.G.
LSD (P=0.05)	4.8

---

\* N.G. - No growth

## Appendix 1.5

### Determination of media water potential

All liquid and solid agar media were autoclaved and adjusted to pH 6.5 with sterile N-NaOH or N-HCl. Cooled agars were poured thinly into 9 cm diam Petri dishes which were then wrapped individually in catering grade 'Cling-film' to prevent water loss. The water potentials of media were determined with a 100 channel automated thermocouple psychrometer (Stevens & Alcock, 1976). Individual pieces of agar (5 mm diam, c. 2 mm thick), or 0.5 ml aliquots of liquid media were placed in 10 replicate chambers within the psychrometer. After an equilibration period of c. 3 h water potentials (-MPa) were recorded:-

	<u>Liquid media</u>	<u>Solid agar media</u>
Glucose-asparagine	0.39±0.01*	0.45±0.02
Soil extract	0.49±0.01	0.51±0.01
V8 juice	0.22±0.06	-
Cornmeal	-	0.48±0.01

\* Each figure is the mean of four replicates with SEM.

- Not determined.

Appendix 1.6a

Amounts of solute (g) added per l of water to give final osmotic potentials (-MPa) of glucose-asparagine and V8 juice broth at 25°. Medium components were dissolved in the appropriate volume of osmotically maintained solution.

---

Glucose-asparagine			V8 juice broth		
Osmotic potential(-MPa)	NaCl	Glycerol	Osmotic potential(-MPa)	NaCl	Glycerol
0.4*	-	-	0.2*	-	-
0.7	4.4	9.2	0.5	2.9	9.2
1.0	8.8	23.0	1.0	10.5	31.3
1.5	11.7	41.4	1.5	17.5	47.9
2.0	20.5	61.7	2.0	23.4	64.5
2.5	26.3	76.4	2.5	29.2	82.9
3.0	32.2	110.5	3.0	36.3	101.3
3.5	38.0	113.3	3.5	41.5	119.7

---

\* Unmodified growth medium.

Appendix 1.6b

Effect of osmotic potential, modified with NaCl and glycerol on the biomass yield (mg) of Pythium oligandrum in liquid media after 12 d incubation at 25°.

Each figure is the mean of four replicates with SEM.

Glucose-asparagine			V8 juice broth		
Osmotic potential(-MPa)	NaCl	Glycerol	Osmotic potential(-MPa)	NaCl	Glycerol
0.4*	55.4±1.8	49.5±0.9	0.2*	59.2±1.4	57.2±2.0
0.7	54.3±2.0	36.2±1.8	0.5	65.9±1.0	55.9±1.0
1.0	55.1±2.3	31.6±0.7	1.0	55.5±0.6	50.6±2.5
1.5	43.6±0.8	16.1±0.7	1.5	42.6±1.8	33.2±0.5
2.0	36.1±1.2	9.1±0.9	2.0	33.7±0.8	18.4±1.0
2.5	23.5±1.5	N.G.	2.5	25.9±1.8	N.G.
3.0	15.5±1.3	N.G.	3.0	N.G.	N.G.
3.5	N.G.**	N.G.	3.5	N.G.	N.G.
LSD (P=0.05)		3.8	LSD (P=0.05)		4.0

\* Unmodified growth medium.

\*\* N.G. - No growth.

Appendix 1.7a

Amounts of solute (g) added per l of water to give final osmotic potentials (-MPa) of GAA and CMA at 25°.

Medium components were dissolved in the appropriate volume of osmotically maintained solution.

---

GAA			CMA		
Osmotic potential(-MPa)	NaCl	KCl	Osmotic potential(-MPa)	NaCl	KCl
0.4*	-	-	0.5*	-	-
0.9	7.6	9.3	0.7	2.9	3.7
1.4	12.9	17.2	1.0	7.6	9.7
1.8	18.1	23.9	1.5	12.9	17.2
2.2	23.4	29.8	2.0	18.7	24.6
2.6	28.1	37.3	2.5	26.9	33.6
3.2	35.7	47.0	3.0	33.3	42.5
3.5	38.0	52.2	3.5	38.6	51.5

---

\* Unmodified growth medium.

Appendix 1.7b

Effect of osmotic potential, modified with NaCl and KCl, on the linear growth rate (mm/d) of Pythium oligandrum on solid media at 25°.

Each figure is the mean of four replicates with SEM.

GAA			CMA		
Osmotic potential(-MPa)	NaCl	KCl	Osmotic potential(-MPa)	NaCl	KCl
0.4*	24.2±1.3	28.4±0.5	0.5*	28.5±1.7	30.9±1.2
0.9	26.9±0.7	21.4±0.6	0.7	23.8±0.6	26.4±0.4
1.4	25.8±0.5	22.6±0.5	1.0	17.0±1.2	18.2±0.8
1.8	22.8±0.7	21.6±0.3	1.5	11.9±0.4	14.8±0.5
2.2	21.9±0.8	18.4±1.2	2.0	3.8±0.7	5.1±0.6
2.6	18.9±0.8	17.1±0.7	2.5	4.9±0.1	6.8±3.8
3.2	N.G.**	13.9±0.9	3.0	2.0±0.2	1.8±1.0
3.5	N.G.	N.G.	3.5	N.G.	N.G.
LSD (P=0.05)	2.1		LSD (P=0.05)	2.4	

\* Unmodified growth medium.

\*\* N.G. - No growth.

Appendix 1.7c

Amounts of solute (g) added per l of water to give the final osmotic potentials (-MPa) of SEA at 20°.

Double-strength SEA was added to the appropriate volume of osmotically maintained solution to give the required medium osmotic potential.

---

Osmotic potential (-MPa)	NaCl	KCl	Glycerol
0.5*	-	-	-
0.7	2.9	3.7	7.3
1.0	7.0	10.4	18.4
1.5	12.9	17.9	36.8
2.0	20.5	25.4	73.7
2.5	26.9	34.3	78.3
3.0	33.9	42.5	92.1
3.5	39.8	50.7	110.5

---

\* Unmodified growth medium.

Appendix 1.8a

Formula derived by Michel & Kaufmann (1973):-

$$\psi_m = -(1.18 \times 10^{-2}) C - (1.18 \times 10^{-4}) C^2 \\ + (2.67 \times 10^{-4}) CT + (8.39 \times 10^{-7}) C^2 T$$

where  $\psi_m$  is the matric potential in bars, C is the concentration of PEG 6000 in g/kg H<sub>2</sub>O and T is the temperature in °C. For specific values of  $\psi_m$ , and T the equation becomes a simple quadratic and C may be obtained as the positive value in a quadratic solution.

Amounts of PEG 6000 (g/kg H<sub>2</sub>O) added to water to give final matric potentials (-MPa) of SEA. Double-strength SEA was added to the appropriate volume of matrically maintained solution to give the required medium matric potential.

---

Matric potential (-MPa)	PEG 6000 (g/Kg H <sub>2</sub> O)
0.6	78
0.8	152
1.0	203
1.5	296
2.0	368

---

Water potential of SEA = -0.51 MPa.

Appendix 1.8b

Radial growth rates (mm/d) and dry weights (mg) of 72-h-old colonies of Pythium oligandrum at different matric potentials on cellophane discs 'floating' on polyethylene glycol (PEG) 6000-amended SEA media at 25°. Each figure is the mean of four replicates with SEM.

---

Matric potential (-MPa)	Radial growth rate (mm/d)	Colony dry weight (mg)
0.6	21.4±0.6	1.90±0.07
0.8	21.1±0.2	1.82±0.02
1.0	15.5±0.5	1.22±0.05
1.5	7.2±0.4	0.62±0.05
2.0	N.G.*	N.G.
LSD (P=0.05)	1.5	0.155

---

\* N.G. - No growth.

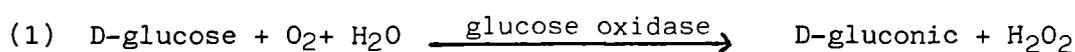
Water potential of SEA = -0.51 MPa.

## Appendix 2.1

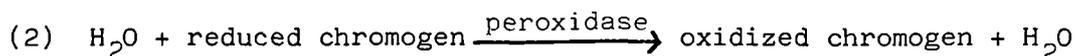
### Enzymic determination of D-glucose (Lloyd & Whelan, 1969)

#### 1. Principle

D-glucose is oxidized to D-gluconic acid and hydrogen peroxide by the enzyme glucose oxidase (1).



The hydrogen peroxide generated in reaction (1) is utilized by horseradish peroxidase to oxidize o-dianisidine dihydrochloride (reduced chromogen) to yield a brown-coloured product (oxidized chromogen) and H<sub>2</sub>O (2).



#### 2. Reagents

##### i. Tris-phosphate-glycerol buffer

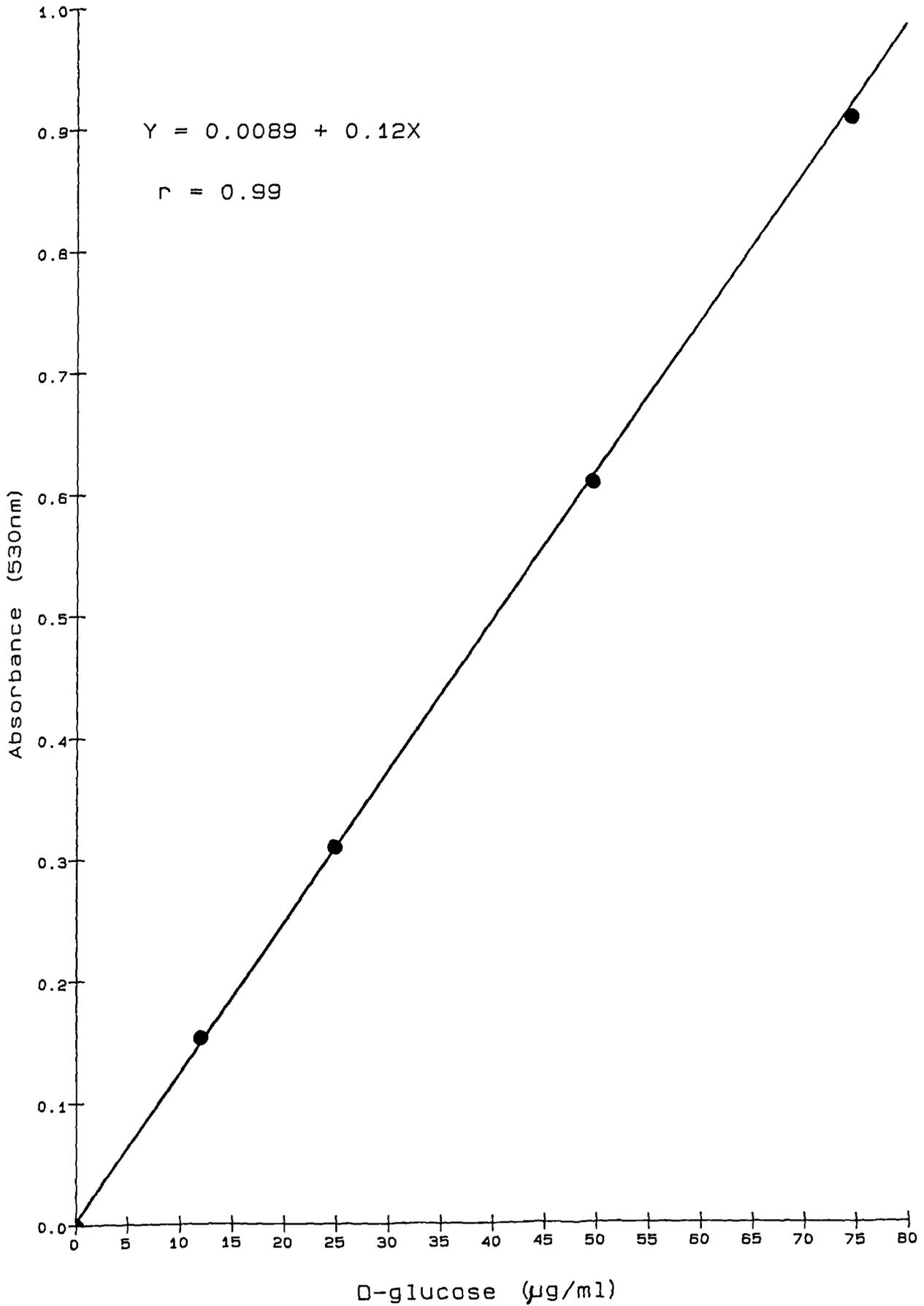
Tris(hydroxymethyl)methylamine (3.63 g) and NaH<sub>2</sub>PO<sub>4</sub> (5.9 g) were dissolved in c. 30 ml water. Glycerol (40 ml) was added to this solution which was then made up to 100 ml with water. The pH was adjusted to pH 7.0 using solid NaH<sub>2</sub>PO<sub>4</sub>.

##### ii. Glucose oxidase reagent

The following were dissolved in 100 ml Tris-phosphate-glycerol buffer: glucose oxidase, type VII\*, 10 mg; horseradish peroxidase\*, 2.5 mg; o-dianisidine dihydrochloride\*, 22.5 mg. This reagent was used within one month of storage at 5°.

\* Supplied by Sigma Chemical Company.

Standard curve for D-glucose estimation

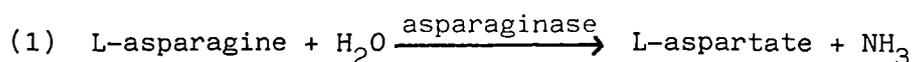


## Appendix 2.2

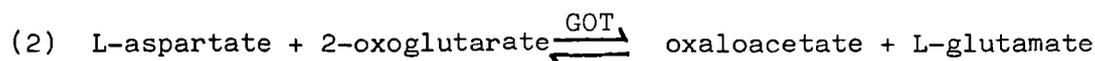
### Enzymic determination of L-asparagine (Möllering, 1985)

#### 1. Principle

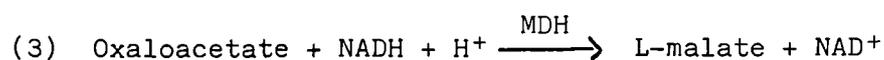
L-asparagine is hydrolysed to L-aspartic acid (aspartate) and ammonia by the enzyme L-asparaginase (1).



In the presence of the enzyme glutamate-oxaloacetate transaminase (GOT), L-aspartate is converted to oxaloacetate with 2-oxoglutarate (2).



In the reaction catalyzed by malate dehydrogenase (MDH), oxaloacetate is reduced by reduced nicotinamide-adenine dinucleotide (NADH) to L-malate (3).



At first, aspartate is determined according to equations (2) and (3). The amount of NADH oxidized in reaction (3) is stoichiometric with the amount of L-aspartate. Addition of asparaginase leads to a further decrease of the absorbance of NADH which is stoichiometric with the asparagine concentration (1). The decrease of NADH is determined by means of its absorbance at 340 nm.

## 2. Reagents

### i. Phosphate buffer

Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O, A.R. (3.2 g) and KH<sub>2</sub>PO<sub>4</sub>, A.R. (0.95 g) were dissolved in water. The solution was made up to 1-l with water and had a final pH of 7.2.

### ii. Reduced nicotinamide-adenine dinucleotide solution, NADH

NADH-Na<sub>2</sub>\* (30 mg) and NaHCO<sub>3</sub>, A.R. (60 mg) were dissolved in 6 ml redistilled water. The solution was used within 4 wk of storage at 4°.

### iii. 2-Oxoglutarate solution

2-Oxoglutarate disodium salt\* (230 mg) was dissolved in 10 ml redistilled water. The solution was used within 4 wk of storage at 4°.

### iv. Malate dehydrogenase, MDH\*

### v. Glutamate-oxaloacetate transaminase, GOT\*

### vi. L-asparaginase\*

\* Supplied by Boehringer Mannheim GmbH.

## 3. Calculation

The general formula for calculating the concentration (c) is:

$$c = \frac{V \times MW}{\epsilon \times d \times v \times 1000} \times A \text{ (g/l), where}$$

A = absorbance difference

V = final volume (ml)

v = sample volume (ml)

MW = molecular weight of L-asparagine (g)

$\epsilon$  = adsorption coefficient of NADH at 340 nm = 6.3 (m mol<sup>-1</sup> × cm<sup>-1</sup>)

It follows for

L-asparagine:

$$c = \frac{2.75 \times 132.1}{\epsilon \times 1 \times 0.1 \times 1000} \times A = 3.633 \times A \text{ (g L-asparagine per l sample solution)}$$

The assay measures levels of L-asparagine between 0.03 and 0.65 g/l.

Sample solutions must therefore be sufficiently diluted.

Appendix 2.3

Utilization of D-glucose and L-asparagine by Pythium oligandrum in relation to biomass and oospore production.

Media had initially 10 g D-glucose and 1.5 g L-asparagine per l.

Each figure is the mean of four replicates with SEM.

Incubation period (days)	Dry weight biomass (mg)	Total No. oospores ( $\times 10^5$ )	Amount in culture filtrate (g/l)	
			L-Asparagine	D-Glucose
0*			1.52±0.04	10.02±0.11
2	8.1±0.5	-**	1.21±0.05	9.06±0.18
4	23.6±0.9	1.42±0.17	0.85±0.04	8.28±0.12
6	30.1±1.0	6.58±0.53	0.67±0.03	7.51±0.06
8	44.1±2.4	8.02±0.71	0.15±0.01	1.30±0.10
12	40.7±0.4	10.92±0.49	0.01±0.001	0.01±0.001
16	33.0±1.3	13.65±0.45	0	0
21	26.7±1.1	15.22±0.35	0	0

\* Uninoculated original media

\*\* Oospores not present

Appendix 2.4

Germination (%) of Pythium oligandrum oospores after 12 h at 25° on SEA maintained at a range of osmotic potentials with NaCl and KCl.

Figures in parentheses are means with SEM from four replicates calculated after angular transformation of percentage data.

---

Osmotic potential (-MPa)	NaCl	KCl
0.5*	21.6 (27.7±1.0)	22.8 (28.5±0.8)
1.0	20.1 (26.6±0.9)	21.8 (27.9±0.7)
1.5	20.4 (26.8±0.5)	20.7 (27.0±1.2)
2.0	17.8 (24.9±0.8)	15.9 (23.4±0.9)
2.5	8.9 (17.3±1.2)	10.2 (18.6±0.6)
3.0	5.3 (5.3±1.9)	2.3 (7.2±2.9)
3.5	-**	-
LSD (P=0.05)		(3.7)

---

\* Unmodified media

\*\* Germination not detected after 12 h incubation at 25°.

### Appendix 2.5

Germination (%) of Pythium oligandrum oospores after 12 h at 25° in soil extract broth at a range of matric potentials with PEG 6000.

Figures in parentheses are means with SEM from four replicates calculated after angular transformation of percentage data.

---

Osmotic potential (-MPa)	Germination (%)
0.6	18 (24.7±1.1)
0.8	16 (23.3±1.1)
1.0	8 (15.7±2.2)
1.5	5 (12.4±1.1)
2.0	—*
2.5	—
LSD (P=0.05)	(4.5)

---

\* Germination not detected after 12 h incubation at 25°.

### Appendix 3.1

Modified selective medium of Martin & Hancock (1986) contained per l of distilled water:

17 g Oxoid conrmeal agar \*

0.1% Tween 20 \* (polyoxyethylene sorbitan monolaurate)

0.1 g penicillin G \*

0.2 g vancomycin \*

0.02 g pimaricin \*

50 µg rose bengal

0.02 g benomyl (50% ai, WP, Du Pont)

\* Supplied by Sigma Chemical Company

Tween 20 was added to medium immediately after autoclaving. Antibiotics were prepared as water solutions and added after the media had cooled to 40°.

Appendix 4.1

Effect of storage temperature on germination of oospores from biomass of Pythium oligandrum on CMA after 16 h incubation at 25°. Figures in parentheses are means from nine counts with SEM, three for each of three vials, calculated after angular transformation of percentage data.

		Storage temperature (°C)			
Storage period (wk)	5	15	20	25	30
0	23 (28.6±0.4)	23 (29.0±0.2)	22 (27.9±0.3)	19 (25.8±0.5)	23 (28.7±0.4)
3	19 (26.2±0.4)	20 (26.7±0.4)	10 (18.9±0.4)	9 (17.8±0.6)	9 (17.4±0.5)
6	14 (22.0±0.4)	15 (22.8±0.5)	8 (16.4±0.4)	6 (14.1±0.5)	5 (13.5±0.5)
9	6 (14.2±0.4)	6 (14.5±0.4)	3 ( 9.8±0.6)	2 ( 8.8±0.4)	2 ( 8.4±0.6)
16	3 (9.3±0.4)	4 (11.2±0.6)	1.5 (6.9±0.4)	1.4 (6.7±0.4)	1.2 (6.2±0.5)
LSD (P=0.05)		(1.8)			

#### Appendix 4.2

A soil characteristic curve relating water potential of a medium loam soil (collected from the University of Sheffield Experimental Garden) to the actual water content, was determined by psychrometry. For the adsorption curve, different amounts of water were added to c. 500 g samples of soil and allowed to equilibrate for 48 h with regular mixing. Subsamples were then placed in an automated psychrometer (Stevens & Alcock, 1976) and subsequently dried in an oven at 80° for 24 h to determine actual water content.

The relationship between soil water potential and actual water content was as follows:-

---

Water content (g H <sub>2</sub> O/100g soil)	Soil water potential (-MPa)
5	5.82±0.27*
7.5	2.51±0.05
10.0	1.32±0.02
12.5	1.04±0.04
15.0	0.52±0.01
20.0	0.40±0.02
25.0	0.39±0.02

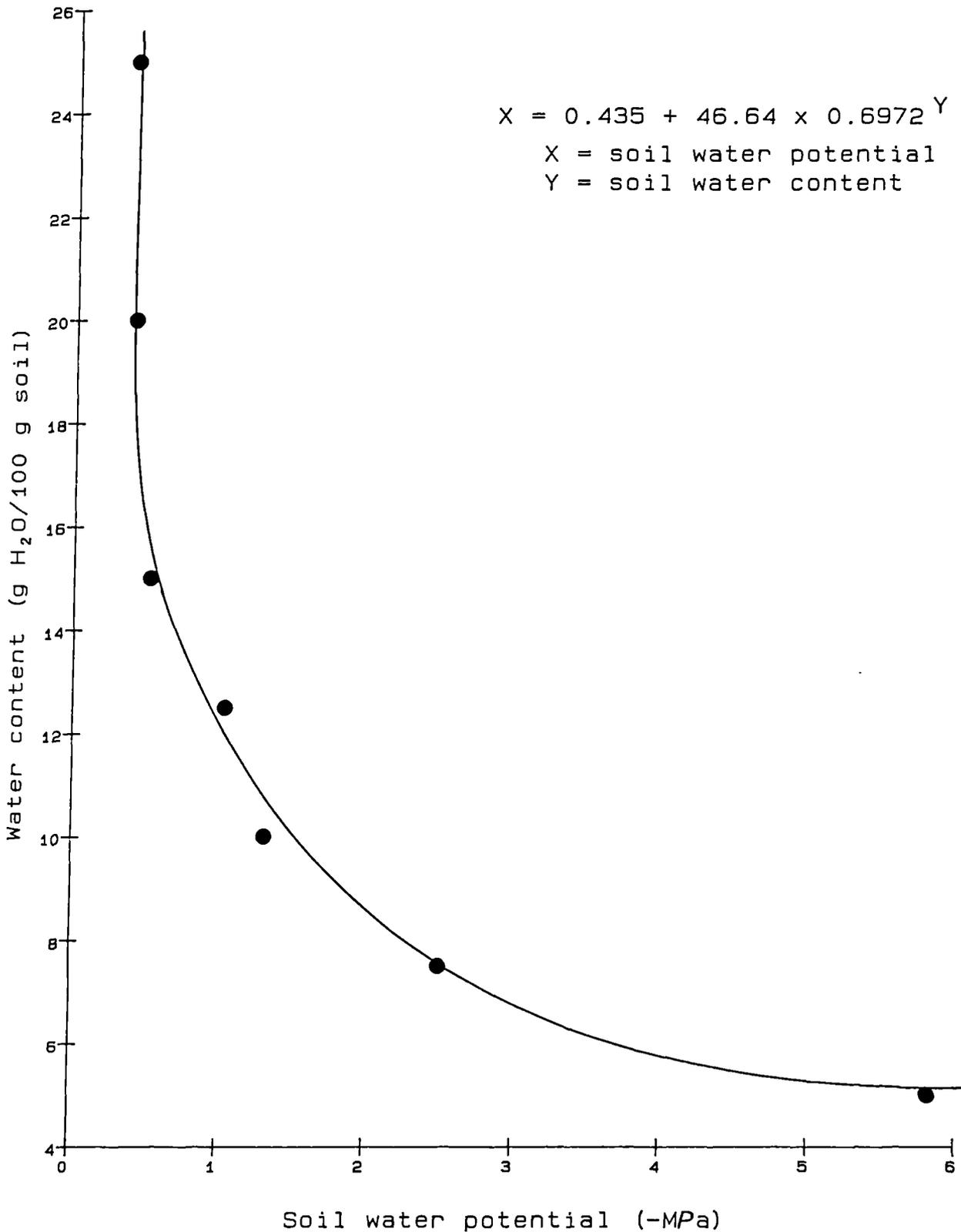
---

\* Each value is the mean of ten replicates with SEM.

The adsorption curve is shown in Appendix 4.3. It was used as the main guide for maintaining water content since the experimental soil was initially dry and had to be wetted up to the required levels.

Appendix 4.3

The relationship between water potential (-MPa) and water content (g H<sub>2</sub>O/100g soil) (adsorption) for medium loam soil collected from the University of Sheffield Experimental Garden



#### Appendix 4.4

Effect of storage on the numbers of colony-forming units of Pythium oligandrum from oospore biomass dust in a medium loam soil and potting compost.

Each value is the mean log. colony-forming units per g dry weight soil or compost with SEM in three replicate glass containers stored at 15°C.

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Colony-forming units (log. cfu/g)

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Storage period (wk)	Soil	Compost
0	4.39±0.01	4.38±0.01
1	4.45±0.01	4.48±0.01
4	4.34±0.03	4.45±0.02
8	4.12±0.08	4.25±0.06
12	3.92±0.03	4.02±0.03
16	2.94±0.02	3.85±0.08
LSD (P=0.05)	0.12	0.13

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