BEHAVIOUR OF ZOOSPORES OF *PYTHIUM* SPECIES

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DECLARATION

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

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ABSTRACT

Pythium aphanidermatum (Edson) Fitz., *Pythium catenulatum* Matthews and *Pythium dissotocum* Drechs. were compared for responses to amino acids and sugars in tests involving taxis, zoospore encystment and cyst germination *in vitro*. Comparisons were made between the fungi and for each fungus at these three stages of development, which are parts of the normal sequence leading to infection of host roots or colonisation of non-living substrata from zoospores. Other substances tested for induction of encystment or germination *in vitro* were cellulose film, crab shell, uronic acids or uronate-containing compounds, partially characterised polysaccharides from plants, root mucilages and cations. Motility attributes of the fungi were compared and, for *P. aphanidermatum* only, were characterised in the presence of several ions and compounds that interfere with calcium-mediated events (EGTA, dibucaine, trifluoperazine, lanthanum, verapamil, amiloride, A23187 and TMB-8). Amino acids were tested for competitive effects in chemotaxis assays *in vitro*. Calcium and EGTA were also tested for effects on adhesion of encysted spores to glass slides. Accumulation and encystment of *P. aphanidermatum* on detached wheat roots was studied on microscope slides, with or without prior treatment of roots with calcium alginate gel, methylene blue, alcian blue, India, ink ruthenium red and lectins that bind residues of fucose, D-glucose, D-mannose, N-acetyl-D-glucosamine and N-acetyl-D-galactosamine. Many experiments involved video-recording zoospore responses and subsequent analysis of videotapes.

Evidence is presented for an effect of calcium and other divalent cations on zoospore motility patterns, cyst adhesion and induction of germination. Several amino acids and sugars elicited zoospore taxis, encystment, or cyst germination but sometimes differently for different fungi or at these different stages of development. Competition experiments enabled some of these responses to be related to proposed receptor functions, and in some cases amino acids could overcome inhibition of germination caused by the presence of calcium-modulators. The use of alginate gel, dyes and lectins provided no evidence of the involvement of fucosyl residues in encystment on roots; rather, the evidence was compatible with the involvement of polyuronates in encystment. A link was demonstrated between adhesion of cysts and their ability to germinate without an organic nutrient trigger; a model involving an autonomous calcium-mediated signalling system is proposed to explain this.

TABLE OF CONTENTS

1. INTRODUCTION

1.1. General introduction

Fungal zoospores are asexual, flagellate, wall-less, motile spores that are dispersed in the presence of free water. They are produced by fungi in the Chytridiomycetes, Oomycetes, Plasmodiophoromycetes, and Hypochytridiomycetes and are receiving increasing attention for their roles in plant pathogenesis and colonisation of non-living substrates in natural environments.

In the absence of external stimuli, zoospores can remain motile for many hours, utilising lipid reserves (Bimpong, 1975), but frequently show random changes of direction with the result that they travel relatively short net distances. They can, however, respond to a wide range of stimuli by chemotaxis (Khew & Zentmyer, 1973), rheotaxis (Katsura & Miyata, 1971), geotaxis (Cameron & Carlile, 1977) and electrotaxis (Khew & Zentmyer, 1974) thus accumulating at sites suitable for colonisation or avoiding environments unsuitable for colonisation. Typically, when they accumulate at localised sites they encyst. This involves loss of motility and the rapid production of a cell wall. The cyst germinates by producing a germ-tube which may show tropism towards the source of stimulation. But in some fungi such as the Plasmodiophoromycetes the cyst germinates to release a protoplast that invades a host cell.

The behavioural and developmental responses of zoospores can be separated into at least four stages, during which they respond to external stimuli: swimming leading perhaps to accumulation via taxis, encystment, adhesion of the cyst, germination and orientation of the germ-tube. Surface recognition and zoosporic colonisation of substrates, leading to infection, may be reduced or eliminated by disrupting these key stages. The natural sequence of events and the factors involved in each of these phases are reviewed below.

1.2. Motility and accumulation

1.2.1 Motility

Most fungal spores reach their potential sites for future growth by passive

means such as movement in air or water. However, in addition, zoospores are actively motile and can accumulate locally at specific sites after they have been passively dispersed by other means.

The type, position and number of flagella are characteristic of different groups of the lower fungi. Chytridiomycetes typically have zoospores with a single whiplash flagellum (although it may also be inserted anteriorly or even laterally; Barr, 1983). Hypochytridiomycetes have a single, anteriorly inserted, tinsel flagellum. Oomycetes produce biflagellate spores, with an anterior tinsel type flagellum and a posterior whiplash type flagellum. Plasmodiophoromycetes have biflagellate zoospores with two whiplash-type flagella (Aist & Williams, 1971).

In Oomycetes the flagella are of two types- a posterior "whiplash" (smooth or naked) and an anterior "tinsel" flagellum that is lined with tubular hairs called mastigonemes. The anterior flagellum generates nearly 90% of the thrust whilst the posterior flagellum steers the spore, acting mainly as a rudder (Carlile, 1983). Both anterior and posterior flagella propagate sinusoidal waves that begin at the base of the flagellum and progress towards the tip (Hickman, 1970; Holwill, 1982) and the spore is propelled forward in a helical motion (Allen & Newhook, 1973) frequently interrupted with random changes of direction. During forward swimming both of the flagella extend lengthwise along the axis of the helix but during a random turn the posterior flagellum stops beating momentarily and comes to lie almost perpendicular to the soma (body) of the spore.

Both uniflagellate and biflagellate zoospores are able to swim for several hours at speeds often in excess of 100μm sec^{-1} and cover distances of several centimetres (Allen & Newhook, 1973; Duniway, 1976; Lange & Olson, 1983). However, zoospores respond to and are attracted towards external stimuli by a change in the normal swimming pattern, largely due to a suppression of random turning causing the spore to follow a straight path (Allen & Newhook, 1974). Similarly, modelling of taxis of other micro-organisms (Schnitzer *et al.,* 1990) demonstrated that this would cause spores to drift up a gradient of attractant, provided that the cells are able to compare a recently experienced concentration of attractant with one encountered previously.

Recently, two calcium binding proteins have been shown to be

present on the flagella of *Phytophthora cinnamomi* zoospores (Gubler *et al.*, 1990; Hardham, 1992). Calmodulin is found localised at high concentrations at the base of the anterior flagellum whereas centrin is present on the basal body connecting fibres and microtubules of the anterior microtubular rootlet. It is possible, therefore, that calcium as in cells of other flagellate organisms (Ordal, 1977; Boitano & Omoto, 1992) may play a role in motility of fungal zoospores.

1.2.2. Accumulation

The term accumulation describes the aggregation of zoospores in response to stimuli. It comprises two events, attraction and trapping in the immediate vicinity of the stimulant. Attraction, which is a tactic response, refers to a directional movement by most zoospores when they sense a gradient of stimulus (Fraenkel & Gunn, 1940; Royle & Hickman, 1964b; Carlile, 1983). Attraction in this sense can also be described as topotaxis. An alternative or additional form involves kinesis, subdivided into orthokinesis and klinokinesis. Orthokinesis is defined as the change in linear velocity which occurs in response to a source of stimulation. Klinokinesis is the change of direction (or angular velocity) of an organism in response to a stimulus.

Both orthokinesis and klinokinesis can result in positive accumulation of zoospores around a source of stimulus or can elicit an avoidance reaction. Negative orthokinesis, a decrease in speed of an organism, leads to accumulation of organisms around a source. If they slow down, they will spend more time near the zone of stimulation and *vice-versa*. Similarly, positive klinokinesis will result in trapping of an organism at a source of attraction. Zoospores may also be trapped in a zone to which they have not been attracted but merely entered by chance (Royle & Hickman, 1964b).

1.2.2.1. Different types of taxis. Zoospores can respond by taxis to a wide array of stimuli and thus act as site-selection agents. Such responses are reviewed below.

Geotaxis. Ho & Hickman (1967a) found that zoospores of *Phytophthora megasperma* accumulated just below a water-air interface. Investigating this, a possible effect of aerotaxis was excluded because the spores were unresponsive to air or carbon dioxide in capillary tubes, so negative geotaxis was invoked

(Cameron & Carlile, 1977). But Carlile (1983) argued that this is probably not a true sensory response; rather it results from the cell shape coupled with asymmetrical flagellation and the location of the centre of gravity, causing zoospores to tilt upwards as they swim. The significance of this for zoospores of *Phytophthora* and other plant pathogenic Oomycetes may be that it helps keep zoospores near the soil surface in proximity with host plant rootlets and would also counteract the effects of percolating water in saturated soils.

Rheotaxis. Katsura & Miyata (1971) found that zoospores of *Phytophthora capsici* swam towards the mouth of a capillary tube from which a water current emerged, but accumulated at a distance from the tube corresponding to a current of 130-140 μm sec^{-1}. This result was suggested to be due to positive rheotaxis, and its effect would be like that of negative geotaxis when water percolates through soil (Carlile, 1983).

Electrotaxis. The effects of electric currents on zoospore motility have been studied by Troutman & Wills (1964), Ho & Hickman (1967b), Katsura & Miyata (1971), Khew & Zentmyer (1974) and Miller *et al.* (1988). In general, when an electric current is passed through a zoospore suspension, zoospores accumulate around the negative pole, followed by encystment and germination depending on the fungal species and current intensity applied. Attraction seems to be due to a suppression of turning by zoospores swimming to the anode, probably caused by an alteration of the zoospore transmembrane potential.

Troutman & Wills (1964) suggested that electrotaxis could also occur *in vivo* and contribute to the accumulation of zoospores at root tips which generate an electrical field. Miller, *et al.* (1988) demonstrated that roots of *Nicotiana tabacum* generate transcellular ion currents which traverse developing and wounded tissues and that root zones with the strongest fields are the most attractive to zoospores. However, the same zones are also major sites of release of soluble nutrients known to attract zoospores, and both Hickman (1970) and Zentmyer (1970) concluded that electrical attraction has little or no role in the accumulation of zoospores on plant roots.

Adelphotaxis. Adelphotaxis refers to attraction between zoospores which may result in auto-aggregation in response to chemical signals released from aggregating spores (Porter & Shaw, 1978; Thomas & Peterson, 1990). Motile zoospores of the water mould *Achlya* were attracted, at a rate of approximately

4-10 x10^3 spores min^{-1}, directly to a swarm of aggregating spores rather than by an exogenous attractant (Thomas & Peterson, 1990). Such aggregation, favoured by high concentrations of zoospores, is also a time dependent process. A heat-stable factor, detected in the vicinity of aggregates and in zoospore or cyst supernatants, was found in chemotaxis assays significantly to attract zoospores. In natural conditions zoospore aggregation may be a dual chemotactic mechanism whereby aggregating spores triggered by exogenous attractants might release amplifying chemical signals which perhaps reinforce the effect of the exogenous attractant. Similarly, bacteria have been shown to aggregate in response to a self-released attractant which is detected via the aspartate chemoreceptor (Budrene & Berg, 1991).

Chemotaxis. Chemotaxis is the most widely studied aspect of zoospore behaviour and is therefore considered in detail below.

1.2.2.2. Chemotaxis to plant roots and soluble compounds. Although the movement of zoospores over long distances is due mainly to physical carriage in flowing water (Goode, 1956; Carlile, 1983) zoospores can accumulate near root tips and on root wounds in saturated soils (Hickman, 1970) and thus can locally accumulate in response to stimuli. The many reports of this have been extensively reviewed (Hickman & Ho, 1966; Hickman, 1970; Zentmyer, 1970; Carlile, 1983). The effect can be reproduced experimentally, using root diffusates or root extracts in capillaries.

In spite of variations attributable to factors such as host vigour, the type of root, the medium in which the roots have been grown, the concentration and the rate of motility of the zoospore suspension (Zentmyer, 1970) responses of many zoosporic pathogens towards roots are similar to those described by Royle & Hickman (1964a) for *Pythium aphanidermatum* on pea roots. In the absence of stimuli zoospores displayed a helical flowing motion with apparent random changes of direction. When unwounded pea roots were added to zoospore suspensions, the spores responded in a very short time and converged on the root surface. Attraction was mainly to the zone of elongation, just behind the root tip, where the zoospores became trapped, by orthokinesis and klinokinesis, and then rapidly encysted. Zoospores showed little accumulation at the extreme root tip or on root hairs and they were indifferent to glass splinters used in control experiments. Other workers have reported similar attraction primarily to the root elongation zone (Zentmyer, 1961; Cunningham &

Hagedorn, 1962; Royle & Hickman, 1964a; Ho & Hickman, 1967b; Chi & Sabo, 1978; Hinch & Clarke, 1980; Longman & Callow, 1987). In contrast, however, Jones *et al.* (1991) observed that after a period of time, accumulation of *Pythium aphanidermatum* spores was primarily to the root hair zone of wheat. But spores often show even stronger and preferential accumulation at wound sites, especially if the wound extends to the vascular cylinder (Pearson & Parkinson, 1961; Schroth & Snyder, 1961; Royle & Hickman, 1964a; Chi & Sabo, 1978). On the cut ends of roots they accumulate preferentially over the stelar tissues (Royle & Hickman, 1964a). All these attractive zones are probably the major sites of nutrient release. But there are as yet unexplained exceptions to the normal findings. For example Goldberg *et al.* (1989) reported that zoospores of *Pythium dissotocum* were attracted exclusively to the root cap region and in some cases to individual cells. They were not attracted to pin-prick wound sites on roots.

Several workers have studied zoospore responses to root exudates, either in their crude forms or after fractionation, incorporated in capillary tubes placed in zoospore suspensions (reviewed by Carlile, 1983). If agar is used in the capillaries then spores accumulate at the capillary mouth; if agar is omitted, the spores swim into the tubes- the "swim-in" test. Spores can then be counted and compared to numbers in control (water-filled) capillaries.

Allen & Newhook (1973) proposed two other methods to study chemotaxis. First the "swim-out" test whereby capillaries filled with zoospores are submerged in a "pond" of test material. Second, a variant of this- the "rhizosphere model"- in which capillaries connect a large attractant source with a pond of zoospores. Under these conditions a steady state can be achieved and a uniform gradient set up and maintained. With the aid of all these model systems attempts have been made to mimic the attraction to crude exudate by recombination of its fractions, with generally inconsistent findings (Royle & Hickman, 1964b; Rai & Strobel, 1966; Chang-Ho & Hickman, 1970).

Zoospores are found to be attracted to individual amino acids, sugars and organic acids, the ability of a compound to attract zoospores, in general, increasing with its concentration. Royle & Hickman (1964b) found that glutamic acid at pH 5.4 (similar to that of root exudate) was the single most attractive compound for *Pythium aphanidermatum*, an observation confirmed for *Phytophthora* spp. by Khew & Zentmyer (1973). Similarly Jones *et al.* (1991)

demonstrated that zoospores of *P. aphanidermatum* were attracted towards glutamic acid, glutamine, asparagine, aspartic acid and alanine whereas Machlis (1969) found that a combination of leucine and lysine induced chemotaxis of *Allomyces arbuscula* and *Allomyces macrogynus*. In addition, zoospores of *P. aphanidermatum* have been demonstrated to show chemotaxis to the sugars mannose, maltose, fructose and sucrose (Jones *et al.,* 1991). Similarly, zoospores of *Neocallimastix frontalis* are attracted to a wide range of carbohydrates including glucose, sucrose and mannose (Orpin & Bountiff, 1978). Bimpong & Clerk (1970) also showed that *Phytopthora palmivora* was attracted to individual sugars and amino acids, which induced encystment and germination with germ tubes directed uniformly towards the source of attractant. In contrast, Barash *et al.* (1965) and others have demonstrated that ^{14}C-labelled root exudates, sugars and amino acids, despite being metabolised, did not alter motility of zoospores of *Phytophthora drechsleri*.

Besides amino acids and sugars, Khew & Zentmyer (1973) found vitamins, organic acids and nucleotides to cause accumulation of *Phytophthora* spp. but the spores accumulated by immobilisation and trapping with no evidence of directional movement. Recently zoospores of *Phytophthora sojae* (Morris & Ward, 1992) have been shown to display chemotaxis towards isoflavones which are released from seeds and roots of many plants.

In general, the same simple organic compounds such as sugars and amino acids attract zoospores of several fungi thus explaining a common lack of specificity in taxis of zoospores towards plant roots (Cunningham & Hagedorn, 1962; Royle & Hickman, 1964a; Carlile, 1983). But this lack of host-specific taxis may be due in part to the use of fungi with characteristically broad host ranges- for example *P. cinnamomi* and *P. aphanidermatum* have very wide host ranges (van der Plaats-Niterink, 1981). Moreover studies have involved root exudates as opposed to root extracts which may have much larger nutrient contents and a wider range of components. Detailed accounts of taxis to root exudates are those by Royle & Hickman, (1964a, b) and Chang-Ho & Hickman, (1970) involving *P. aphanidermatum*. Of all the many individual components of pea root exudate tested by Royle & Hickman (1964b) very few could mimic the ability of root material to induce zoospore accumulation. On fractionation of the exudate the cationic fraction containing amino acids was the most attractive, followed by the neutral fraction (containing sugars) and very weak attraction

occurred to the anionic fraction containing carboxylic acids. Recombination of the fractions did not elicit responses equivalent to those of the crude exudate (Royle & Hickman, 1964b; Rai & Strobel, 1966; Chang-Ho & Hickman, 1970). This is consistent with many reports that mixtures of compounds, especially amino acids and sugars, are much more attractive than are individual components (*e.g.* Royle & Hickman, 1964b; Chang-Ho & Hickman, 1970). Although possible explanations, such as the balance of the mixture (Carlile, 1966; Zentmyer, 1966) or synergism among the components of the mixture (Machlis, 1969; Chang-Ho & Hickman, 1970; Halsall, 1976), have been offered, a more likely possibility is that some key root exudate components are lost during the fractionation process. Such components may be alcohols or other volatile compounds (Cameron & Carlile, 1978).

Zentmyer (1970) reported that environmental conditions can alter the nature of the exudate released from plant roots. One such condition is soil saturation in which roots may become temporarily anaerobic and it is notable that epidemics by zoosporic root pathogens often occur in these conditions (although the zoospores themselves require oxygen for continued activity). In periods of temporary waterlogging, roots are likely to release fermentation products (Rovira, 1969; Bolton & Erickson, 1970). Indeed Bolton & Erickson (1970) have shown that tomato roots in waterlogged soils may exude ethanol at a concentration of 300 ppm after 24h.

Allen & Newhook (1973, 1974) were the first to recognise the significance of this for taxis. They found that zoospores of *P. cinnamomi* showed a significant reduction in the number of turns (klinokinesis) coupled with a directional movement indicative of chemotaxis, up a concentration gradient of ethanol. In swim-in tests the spores showed increasing accumulation to ethanol over the range 5 to 25mM concentration. Methanol, n-propanol, n-butanol and acetaldehyde were also found to be chemoattractants.

Halsall (1976) extended this study to other *Phytophthora* spp. Ethanol was the best attractant tested for *P. cinnamomi* although it was not the most effective for other species and some did not respond to it at all. Cameron & Carlile (1978) further tested *P. palmivora* which was attracted to a range of aldehydes, organic acids and alcohols including ethanol, but not to methanol, propanol or butanol. Cameron & Carlile (1978) found that the most potent attractants had 4-6 carbon atoms, and that some volatile compounds were

attractive at much lower threshold concentrations than ethanol. The most powerful attractant was isovaleraldehyde, with a threshold of 10^{-6} M for eliciting a response (Cameron & Carlile, 1978, 1981). They also tested the ability of other compounds to displace [^{3}H] isovaleraldehyde that had been adsorbed by zoospores. Only unlabelled isovaleraldehyde and closely related ligands, for example valeraldehyde, which were also known to be chemoattractants could displace [^{3}H] isovaleraldehyde. Other attractants such as asparagine could not displace [^{3}H] isovaleraldehyde suggesting that they act via different receptors.

It is known that volatile compounds closely related to isovaleraldehyde can be present in root exudates: Arkad'eva (1963) demonstrated that axenically grown roots of corn (*Zea mays L.*) produce large quantities of valeric acid. Young *et al.* (1979) extended the earlier work of Allen & Newhook (1973,1974) by examining the responses of zoospores to ethanol in "ideal soils" represented by glass columns containing glass beads. Capillaries containing test solution (5mM ethanol in water or 1mM NaCl for comparison) were inserted through the sides of the columns. Approximately eight times as many zoospores accumulated in tubes containing ethanol as opposed to control tubes containing NaCl when there was no flow of water through the column.. Zoospores also accumulated in side tubes containing ethanol (about six fold increase over controls) when the spore suspension was allowed to flow through the column at a rate less than the individual speed of swimming zoospores.

It is possible, therefore, that ethanol and other volatile compounds released by fermentation in roots in a waterlogged environment can act as powerful zoosporic attractants.

1.2.3. Mechanisms of chemotaxis

The first step in a chemotactic response is the interaction of the attractant or the repellent with the cell surface. Bacteria have proteinaceous receptors which are located in the plasma membrane or in the periplasmic space (Adler, 1975; Ames *et al.*, 1988; Koshland *et al.*, 1988; Bischoff & Ordal, 1992). These receptors can specifically detect some amino acids and sugars (Mesibov & Adler, 1972; Adler, 1975). Sensory adaptation in *Escherichia coli* requires methyl esterification of the receptor- the methyl- accepting chemotaxis proteins (MCPs). The MCP transducers are organised into two structural domains. The

amino-terminal half comprises the periplasmic receptor and the carboxylic terminus the cytoplasmic signalling domain flanked by methylation sites (Ames *et al.*, 1988). *Escherichia coli* responds chemotactically by controlling its swimming pattern via direction of the flagellar motor. In the absence of stimuli, the MCPs are non-methylated, the flagella rotate clockwise resulting in a series of tumbles with abrupt changes of direction. However, when stimulated the bacteria tumble less frequently and swim in a more direct line displaying a "biased random walk" towards an attractant. In this state the MCPs are methylated and the flagella rotate counter-clockwise (Ames *et al.*, 1988). Thus bacteria sense attractants *per se*: the cells are equipped with chemosensors that "measure" changes in concentration of attractants and report the changes to the flagella (Adler, 1975).

Unfortunately, few such studies have been done with eukaryotic organisms. However, it is likely that zoospores have a receptor-mediated system for chemotaxis. To date the only demonstration of a zoosporic receptor, for an attractant eliciting chemotaxis, was for an isovaleraldehyde receptor of *P. palmivora* (Cameron & Carlile, 1981).

Allen & Newhook (1973) have observed that zoospore flagella, which have an extended conformation, require the presence of a strong force opposing the natural tendency for all proteins to adopt a random coil configuration of high entropy (Allen & Harvey, 1974). The most probable force is electrostatic repulsion between adjacent negative charges along the flagellum surface. If this force is strong enough, the rod-like configuration can be maintained, but diminution of this force would result in collapse of the flagellum and loss of normal swimming ability. However, when *Phytophthora* spores exhibit chemotaxis to ethanol spontaneous random turns are suppressed and the spore hyperpolarises (Allen & Newhook, 1974). Similarly, Doetsch (1972) observed that in bacteria ethanol affects chemotactic reactions by affecting membrane-mediated reactions that lead to the reorientation of flagella by altering the distribution of electrostatic charges on the flagellar membranes. If major changes in charge distribution result in flagellar re-orientation, then a consequent change in zoospore movement and chemotactic responses to nutrients could involve changes in charge distribution of the intraflagellar compartment. It is known that depolarisation of the cell membrane in *Paramecium* causes a similar response (Van Houten, 1979).

Carlile (1980) proposed that movement away or towards a stimulus may involve spatial sensing whereby two or more spatially separated chemoreceptors allow comparison of stimulus intensity at various points on the body surface as well as direction. The zoospore whilst exhibiting chemotaxis may turn in the direction of those receptors receiving the greater stimulation until the chemoreceptors are stimulated symmetrically with respect to the zoospore long axis; it may then proceed directly up the chemical gradient.

However, Hardham (1992) has suggested that zoospores, as in the case of bacteria, adopt a temporal method of detecting attractants whereby the spore monitors the concentration of chemoattractants in its environment over a period of time. In addition, it is proposed that chemoreceptors may be located on the flagella (Hardham, 1992) forming part of a signal transduction pathway interacting with calcium binding proteins on the flagella to elicit directional changes. In support of this, transmembrane signalling occurs in both cilia and flagella (Bloodgood, 1991) and the effects of calcium in altering both flagellar movement and motile patterns of behaviour have been described for bacteria and sperm cells (Ordal, 1977; Carlile, 1980; Boitano & Omoto, 1992). However, equivalent information on this and other aspects of flagellar behaviour is still lacking for fungal zoospores.

1.3. Encystment

Zoospores have complex arrays of membranes, organelles and cytoskeletal elements (Truesdell & Cantino, 1971). These undergo rapid reorganisation during encystment. The flagella are retracted or shed, the nucleus is relocated, the cell becomes spherical and shrinks in volume, a glycoprotein cyst coat is released and a microfibrillar cyst wall forms beneath the cyst coat (Truesdell & Cantino, 1971). These events are reviewed below.

1.3.1. Induction of encystment

The cytoskeleton is important in maintaining the shape of wall-less zoospores. The difference in shape of Oomycete and Chytridiomycete zoospores, for example, probably reflects differences in the arrangement of their microtubules and microfilaments that comprise the cytoskeleton (Held, 1972; Cantino & Mills, 1976; Holloway & Heath, 1977). Such a structure involves one or more sets of microtubular rootlet systems, usually connected at one end to the

kinetosomes of the flagella and at one point to the nuclear membrane of the zoospore giving it characteristic shape and maintaining rigidity (Grove & Bracker, 1978).

Zoospores encyst in response to treatment with agents that degrade cytoplasmic microtubules such as ice, temperature, hydrostatic pressure, cupric ions and colchicine (Held, 1972). However, treatments known to promote encystment, for example mechanical disturbance, dilution of the suspension and frequent contact with solid surfaces (Ho & Hickman, 1967b; Tokunaga & Bartnicki-Garcia, 1971a,b) do not degrade microtubules. It seems likely, however, that encystment induced by these harsh treatments is part of a survival strategy in an attempt to ensure the survival of the spore.

Nevertheless, zoospores also encyst in the presence of nutrients, particularly those present in roots- for example pea root exudate can induce encystment of *P. aphanidermatum* spores (Royle & Hickman, 1964a). The plant compound pectin, which is rich in uronic acids, induces encystment of *P. cinnamomi* and *P. palmivora* zoospores (Irving & Grant, 1984; Grant *et al.*, 1985) with galacturonate residues being implicated (Zhang *et al.*, 1990). However, chemical modification of pectin, by reduction or periodate oxidation, destroys its capability to induce encystment (Zhang *et al.*, 1990). In addition, cations such as calcium and strontium have been demonstrated to elicit encystment of *Phytophthora* zoospores (Grant *et al.*, 1986; Griffith *et al.*, 1988).

Saturation of zoospore receptors as would occur for example during adhesion to a root (Hinch & Clarke, 1980; Longman & Callow, 1987) may result in membrane depolarization influenced by ions such as potassium (Jen & Haug, 1981), leading to osmotic water uptake, a reduction in water pump activity, (Grant *et al.*, 1986) and swelling of the zoospore leading to breakdown of the cytoskeleton and encystment. In particular, fucosyl residues present in root polysaccharides have been implicated in triggering encystment of *P. aphanidermatum* (Longman & Callow, 1987; Estrada-Garcia *et al.*, 1990b) and *P. cinnamomi* (Hinch & Clarke, 1980) on roots of cress and maize respectively. However, Jones *et al.* (1991) contested these findings by being unable to prevent encystment of zoospores on roots double coated with alginate gel which should have masked exposed saccharide residues.

Zoospores of *P. cinnamomi* can be induced to encyst non-specifically

by lectin and monoclonal antibody binding to the cell surface (Hardham & Suzaki, 1986; Hardham, 1989). Concanavalin A (Con A) binds to the entire surface of *P. cinnamomi*, including both flagella, resulting in 70% encystment- an effect annulled by preincubation of the lectin with its hapten sugar, methyl-D-mannoside. Furthermore, binding of a monoclonal antibody, Zf-1, specific for both flagella, induces encystment whereas antibodies raised against the whole zoospore surface, including the two flagella, were not specific enough to induce this response, perhaps suggesting that specific receptors involved in triggering of encystment are located on the flagella surface. It has been observed that flagella interact with insoluble substrates and host plants prior to encystment (Mitchell & Deacon, 1986b ; Shishkoff, 1989, Jones *et al.,* 1991). Tests show that the component on the flagella that binds Zf-1 is conserved throughout the *Phytophthora* and *Pythium* genera, suggesting the components binding Zf-1 trigger encystment at the surface of a potential host during infection. Similarly, monoclonal antibodies that bind to the whole zoospore surface have been found to induce encystment of *P. aphanidermatum* zoospores (Estrada-Garcia *et al.,* 1990b).

Recently, Zhang *et al.* (1992) have shown that phosphatidic acid, at levels as low as 3μM, induced encystment of zoospores of *Phytophthora palmivora.* It is thought that production of phosphatidic acid arises via a stimulus-activated phospholipase D and may act as a secondary messenger inducing encystment.

However, whatever the trigger for encystment, the disruption of the microtubular cytoskeleton, either directly or indirectly, seems to induce the sequential events in encystment.

1.3.2. Flagellar loss (or retraction) and organelle rearrangement

Following cessation of zoospore motility the flagellum or flagella disappear. The cytoskeletal stucture as well as maintaining shape provides a firm anchor for flagellar beating. Thus degeneration of the anchoring mechanism during encystment would permit the flagellum to be detached or retracted onto or into the zoospore surface. Koch (1968) proposed that flagellar retraction, apart from detachment, involves four distinct patterns which he termed "lash around", "body twist", "vesicular" and "straight in". Truesdell & Cantino (1971) concluded

that straight-in retraction is merely a mechanical process. During retraction the axoneme, along with its membraneous sheath comprising the flagellum, coils up along the inside of the periphery of the encysting spore. After the axoneme has been withdrawn, the sheath is retained as part of the plasmamembrane.

Fuller (1966) studied lash-around retraction in *Blastocladiella emersonii* and proposed that the flagellum wraps itself around the zoospore body with resultant fusion of the flagellar sheath and cell membrane, a view suggested by Koch (1968). In contrast, Cantino & Mills (1976) proposed that the flagellum wraps itself around the spore and the sheath pulls away from one side of the axoneme to become part of the plasmalemma. Body twist, retraction and vesiculation, outwith Koch's proposals (1968), are not well documented.

Cantino & Mills (1976) hypothesised that the retraction mechanisms with lash-around may be due to *in vitro* conditions because *B. emersonii*, whilst undergoing the latter form of flagellar retraction, may die instead of undergoing the normal encystment process.

During encystment due to cytoskeletal disruption zoosporic organelles such as the nucleus, nuclear cap (if present), kinetosomes, lipid sac and mitochondria are reshaped and repositioned.

1.3.3. Rounding up and formation of a cyst wall

The fully encysted zoospore has an electron-opaque cell wall resulting from discharge, through the cell membrane, of vesicles containing wall material. Lunney & Bland (1976) characterised five major vesicle types present in Oomycete zoospores, some of which are common in other zoospores. The vesicle types are discussed below.

Peripheral vesicles These vesicles, which are situated at the cell periphery and contain a fibrous material, discharge their contents extracellularly. They have been implicated in adhesion of encysting zoospores to solid surfaces (Sing & Bartnicki-Garcia, 1975a, b) and in primary cell wall deposition.

Peripheral cisternae These are seen at the cell periphery as elongated or flattened vesicles and are thought to function in initial wall deposition.

Phospholipid or "Fingerprint" vesicles These have also been described as liposomes (Williams & Webster, 1970) dense body vacuoles (Gay *et al.,* 1971) gamma particles or endosomes (Cantino & Mills, 1976). The term "fingerprint vesicle" is derived from their striking striate or lamellate appearance resembling a fingerprint. After encystment they break down, forming vacuoles containing membraneous components. It is proposed that they function as a protective package for sequestering key zoospore ingredients, such as chitin synthetase, which are transported in carrier vesicles budded off from the gamma particle membrane to the plasmalemma following encystment (Cantino & Mills, 1976). Components suggested to be transported to the plasmalemma include 4s RNA, a DNA satellite molecule and chitin synthetase. Cantino and Mills (1976) termed such a vesicle an endosome.

Microbodies These are also referred to as parastrasomes (Williams & Webster, 1970) or X-bodies (Sing & Bartnicki-Garcia, 1975a). In Oomycetes they are spherical structures, containing crystalline inclusions, bound by a single membrane. Bimpong (1974) suggested in *Phytophthora* these structures may be glyoxysomes. Recently, Gubler & Hardham (1988) proposed that in *P. cinnamomi* these vesicles, secreted during encystment, contain the adhesive material.

Cell wall vesicles These are present throughout zoosporogenesis, encystment and germination and may play a role in deposition of the electron-dense wall material.

Encysting zoospores undergo surface changes. For example *Phytophthora palmivora* spores in the presence of pectin display "blebs" and "microvilli" on both cell and flagellar surfaces. The appearance of bulges and pits on the spore, by 40 sec, and subsequent formation of a smooth surface is consistent with exocytotic fusion of secretory vesicles with the surface to form a cyst coat (Paktitis *et al.,* 1986). Similarly, fusion of flocculant vesicles with the cell surface of *P. aphanidermatum* has been reported (Grove & Bracker, 1978). After a post induction period of 5-10 min cysts are encased by a cell envelope, comprising at least two layers, the cyst coat and cell wall, believed to result from the peripheral cisternae which disappear during this period. Hegnauer & Hohl (1973) propose that the cyst wall of *P. palmivora* is also formed from peripheral cisternae. The cyst wall is composed of interwoven fibrils, embedded on the inner side of the wall in an amorphous matrix, and contains predominantly 1-3

linked glucose and C-4 linked residues with branched C-3 and C-6 linked residues containing non-reducing terminal units (Tokunaga & Bartnicki-Garcia, 1971a). Tokunaga & Bartnicki-Garcia (1971b) showed that cyst wall microfibrils are formed during encystment from endogenous reserves within the zoospore cytoplasm by enzymes probably derived from "endocystosomes".

Surface components of *P. cinnamomi*, before and after encystment, have been analysed by lectin (Bacic *et al.*, 1985; Hardham, 1989) and monoclonal antibody binding studies (Hardham, 1989). Staining with ruthenium red and the lectin Con A, which binds D-glucosyl and D-mannosyl residues, shows a glycocalyx over the zoospore surface. During encystment the array of molecules on the cell surface changes drastically, with a decline in material binding Con A but with a surgence of material binding Soybean agglutinin (SBA) indicative of N-acetyl-D- galactosamine residues (Hardham, 1989). Similarly Hohl & Balsiger (1988) demonstrated the presence of galactose and fucose binding sites on the outer surface of germinated cysts of *Phytophthora megasperma* and *Phytophthora infestans.*

Although encystment seems to involve release or synthesis of saccharide residues it does not seem to require manufacture of RNA or protein (Lovett, 1968; Leaver & Lovett, 1974). Therefore, proteins required for structural changes and rearrangements during encystment must already exist within the zoospore. However, it seems that cyst germination requires synthesis of new proteins for completion (Leaver & Lovett, 1974).

1.4. Adhesion of encysted zoospores

During encystment the cyst becomes sticky due to the release of an adhesive, allowing attachment to solid surfaces (Sing & Bartnicki-Garcia 1975a). Once adhered, the spores are not easily dislodged by washing or mild chemical treatment. However, the adhesive phase is short-lived, in some cases lasting for only 1-4 min after induction of encystment (Gubler *et al.,* 1989), but adhesiveness can be restored to relatively old cysts by addition of calcium. Contrastingly, adhesion, which correlates closely with the release of glycoproteins (Sing & Bartnicki-Garcia, 1975b; Gubler *et al.,* 1989) can be inhibited by glycoprotein treatment with pronase (Gubler & Hardham, 1988) trypsin (Longman & Callow, 1987) and lectin binding treatments (Gubler *et al.,* 1989) as well as sequestering of available calcium (Gubler *et al.,* 1989).

It was generally believed that the adhesive was released during rapid exocytosis of large peripheral vesicles that lie beneath the zoospore plasma membrane (Sing & Bartnicki-Garcia, 1975 a, b). However, antibodies raised against the adhesive material suggest that the adhesive is contained in small vesicles in the cell periphery of *P. cinnamomi* (Gubler & Hardham, 1988). More specifically, two types of small vesicles that lie just beneath the plasmamembrane, termed dorsal and ventral vesicles, are secreted coincidentally with the acquisition of adhesion. Immunolabelling revealed that the dorsal vesicle material forms a coating on the hemispherical part of the cell that faces away from the root surface whereas the ventral vesicle material lies between the cyst and the root surface (Gubler & Hardham, 1991). This suggests that the ventral vesicles, which lie adjacent to the root immediately prior to encystment (Hardham & Gubler, 1990) because of the orientation in which zoospores encyst (Mitchell & Deacon, 1986c), contains the adhesive material in zoospores of *Phytophthora*. Similarly, ventrally located vesicles, termed K-bodies, store the adhesive in *Saprolegnia* zoospores (Lehnen & Powell, 1989). In contrast, however, in support of the work of Sing & Bartnicki-Garcia (1975a,b), immunocytochemical studies suggest that the adhesive of *P. aphanidermatum* may be located in the large peripheral vesicles which are present throughout the zoospore periphery (Estrada-Garcia *et al.* 1990a).

It has been suggested that adhesion, *in vivo* involves interaction between proteinaceous components on the cyst surface and sugar residues, in particular fucose, present in root slime. Adhesion of *P. cinnamomi* and *P. aphanidermatum* zoospores to roots of maize and cress, respectively, is abolished by treatment with periodate, exofucosidase and fucose-binding protein (Hinch & Clarke, 1980; Longman & Callow, 1987). In addition Kelleher *et al.* (1990), using fluorescent latex beads coated with fucose, were able to demonstrate the presence of fucose-specific binding proteins on the surface of zoospores, cysts and germinated cysts of *P. cinnamomi*. Similarly, in other studies encystment of *P. graminicola* and *P. aphanidermatum* zoospores was almost eliminated by treatment of roots with methylene blue, which binds mucilage, and periodate oxidation (Mitchell & Deacon, 1986a). The secreted adhesive, however, is non-specific in its ability to attach to surfaces. Zoospores have a tendency to become firmly adhered to any surface, such as glass or plastic, on to which they settle. However, there is some evidence of specific adhesion. Cysts of *P. graminicola*, which is a specialised grass pathogen, adhered

better to roots of grasses than non-grasses when compared to the non-specialised pathogen, of wide host range, *P. aphanidermatum* (Mitchell & Deacon, 1987).

1.5. Germination of encysted zoospores

Cysts germinate in almost every case from the zoospore side nearest to the attractant source and the germ-tube grows towards this source. It was thought that the point of germ-tube outgrowth was random, being influenced by external stimuli, thus representing a case of tropism. However, germ-tubes seem to arise from a predetermined point (Mitchell & Deacon, 1986c; Paktitis *et al.,* 1986) and zoospores are observed to swim along the surface of a root with their ventral groove facing down, to settle with this point nearest to the host. Paktitis *et al.* (1986) proposed that attachment of flagella to the host surface, prior to encystment, may orientate the cyst to germinate from a point nearest to the host due to contraction of flagellar axonemes following adhesion.

Cations such as calcium and strontium (Grant *et al.,* 1986), the amino acids glutamate, aspartate and asparagine (Jones *et al.,* 1991), various carbohydrates such as sucrose, glucose and galactose (Byrt *et al.,* 1982b), pectin (Irving & Grant, 1984) and uronic acids (Irving & Grant, 1984) have all been found to induce germination of Oomycete cysts. In other conditions, such as distilled water, if environmental conditions are not suitable for growth, then germination is delayed and in some cases a secondary zoospore is released by breakdown of the germ-tube tip.

After stimulation of germination, germ-tubes of pre-encysted spores emerging randomly in relation to an attractant source can reorientate themselves by tropism to nutrients, oxygen (Mitchell & Deacon, 1986c), some amino acids (Musgrave *et al.,* 1977; Manavathu & Thomas, 1985) and in electrical fields (McGillivary & Gow, 1986). The sequence of events leading to cyst germination commences before any outward signs of germination. After 30 min a slight bulge appears, due to accumulation of vesicles with granular contents, near the cyst periphery closest to the zone of attachment to the host surface (Held, 1973; Grove & Bracker, 1978). The bulge beside the peripheral cluster of vesicles heralds the first visible sign of germination. With time a germ-tube appears, containing a cluster of vesicles at its tip.

After encystment, zoospores require new protein synthesis for germination (Hemmes & Hohl, 1971; Penington *et al.,* 1989) and rapid production of new RNA has also been observed (Penington *et al.,* 1989). At least some of the energy required to fuel germ-tube growth, in the case of *Phytophthora*, is believed to originate from breakdown of the large peripheral vesicles which store protein, thus supplying a nitrogen source (Gubler & Hardham, 1990) at a time when it is unlikely that the spore has infected and derived nutrients from a host root. At the germling stage, however, cysts are able to transport amino acids and sugars into the spore, presumably coinciding with infection of a root *in vivo* (Penington *et al.,* 1989).

With reference to the germ-tube, the new hyphal wall is continuous with the cyst wall but not with the cyst coat which remains as a remnant on the cyst surface. The germ-tube walls differ from cyst walls in that their two-layered structure consists of a microfibrillar internal region and a granular amorphous layer on the outside (Tokunaga & Bartnicki-Garcia, 1971a; Hegnauer & Hohl, 1973).

In general, two types of germ-tube are distinguished (Hawker, 1966). The first is a simple extension of the cyst or spore wall and the second formed from a new wall inside of the existing coat prior to germination. Hegnauer & Hohl (1973) propose that in cysts with a clear demarcation, at the germ-tube base where the cyst walls merge into the germ- tube, an intermediate system is involved. In the intermediate system of *P. palmivora* and *Botrytis cinerea* a new wall is formed by apposition on the inside of the existing wall at the site of granular vesicle accumulation, and an overlapping of the regions of cyst and germ-tube walls occurs. In contrast, Grove & Bracker (1978) propose that, for *P. aphanidermatum*, the germ-tube is continuous with the cyst wall. Such differing interpretations perhaps reflect different effects of the various preparation and staining techniques for electron microscopy.

1.6. Accumulation of zoospores on insoluble substrata

Willoughby (1962) examined populations of *Saprolegnia* spp. in lake water and found that all fungal propagules were motile or encysted zoospores. It is widely reported that zoosporic fungi are isolated from natural environments by baits of insoluble substrata over soil (Couch, 1939; Ward, 1939; Stanier, 1941; Sparrow,

1957; Willoughby, 1959) or in streams (Park, 1976, 1980) or lakes (Willoughby, 1957, 1959)

Willoughby & Redhead (1973) found *Pythium* spp. to be amongst the earliest colonisers of cellulosic baits placed in streams, attributable to passive propagule accumulation (Park, 1974). Park (1974), however, showed that the chytrid *Rhizophlyctis rosea*, known to grow on cellulose, accumulated more on filter paper than on glass fibre exposed in streams, whereas fungi with non-motile propagules occurred to similar degrees on the two types of substratum. This suggests that the chytrid preferentially selected the cellulosic bait. Mitchell & Deacon (1986b) examined the selective accumulation of Chytridiomycetes and Oomycetes on cellulose and chitin in laboratory conditions and found that zoospores of *R. rosea* accumulated on cellulose, whereas *Chytridium confervae*, *P. aphanidermatum* and a *Saprolegnia* species accumulated only on chitin, and *Allomyces arbuscula* and *P. graminicola* accumulated on both materials. Accumulation could be reduced by prior treatment of cellulose and chitin with dyes or wheat germ agglutinin suggesting that surface recognition events mediate zoospore accumulation. Similarly, zoospores of the mosquito-pathogenic fungus *Lagenidium giganteum* preferentially attach to and encyst in response to derivitives of chitin and chitosan present in insect cuticles (Kerwin *et al.*, 1991). These ecologically significant results suggest that zoospores may select suitable substrates for encystment and growth of a fungus.

1.7. Aims and objectives

The literature survey above reveals that most recent progress in understanding the host-locating and substrate-locating mechanisms of zoosporic fungi had come from study of the stages after zoospore taxis, especially zoospore encystment and cyst germination. Such studies revealed host- and substrate-differential effects on encystment. They had also revealed that fungi can differ in their chemoresponses at different stages of the sequence, *i.e.* zoospore taxis, encystment, cyst germination and germ-tube tropism. The aim of this study was to extend these findings to a comparison of three different fungi, while further investigating individual aspects of behaviour of one fungus *Pythium aphanidermatum*, in greater depth.

The specific objectives were as follows.

1. To study the factors associated with host roots that influence the pre-infection behaviour of *Pythium aphanidermatum* by (a) attempting to block responses to roots and (b) investigating the factors that influence zoospore motility, encystment and cyst germination of this fungus *in vitro*.

2. To compare the chemoresponses of *Pythium aphanidermatum*, *Pythium catenulatum* and *Pythium dissotocum* to a range of compounds at different stages of the pre-infection response sequence- zoospore taxis, encystment and cyst germination- *in vitro*. Thereby, to determine the amount of variability between species, which might be related to their previously reported differences in behaviour on plant roots.

2. MATERIALS AND METHODS

2.1. Fungal culture and production of zoospores

Pythium aphanidermatum (Edson) Fitz. (CBS 634.70), isolated from *Lycopersicon esculentum*, was as used by Jones *et al.* (1991); *Pythium dissotocum* Drechs. and *Pythium catenulatum* Matthews, isolated from lettuce and spinach respectively, were supplied by M.E. Stanghellini and used by Goldberg *et al.* (1989). The fungi were grown on potato-dextrose agar (PDA) and maintained in sterile water at 4°C.

Production of zoospores was essentially as described by Goldberg *et al.* (1989). Cultures for zoospore production were maintained on V8 agar, comprising 100ml V8 juice (Campbell's Soups Co.), 20g Oxoid number 3 agar and 1l distilled water. Squares, 15mm, were cut from 3, 5 and 7 day-old cultures of the respective fungi on plates of V8 agar incubated at 25°C. Six blocks were placed in an empty Petri dish containing 20ml of sterile 0.01M phosphate buffer (pH 7) and incubated on a laboratory bench at 16-20°C.

After 6h, the medium contained at least 1x 10^5 zoospores ml^{-1}. Unless stated, for standardization the suspensions were adjusted to $2x10^4$ zoospores ml^{-1} (confirmed by a haemocytometer) by addition of sterile phosphate buffer.

2.2. Experiments with wheat roots

2.2.1 Seedling growth and isolation of root mucilage

Seeds of wheat (*Triticum aestivum* L. cv. Mardler) and tomato (*Lycopersicon esculentum* Mill cv. Moneymaker) were surface sterilised with 0.5% sodium hypochlorite solution for 5 min and washed 3 times in sterile distilled water (SDW) then germinated on moist filter paper in darkness at room temperature. In all cases 5-day-old wheat and 10- day-old tomato roots that were not in contact with the filter paper were excised 2cm behind their tips and used in experiments.

Isolation of root mucilage was essentially as described by Longman & Callow (1987). Roots were excised from over 500 wheat and tomato seedlings

and were stirred in 200 ml SDW for 3h at 4°C. The solution was then filtered through a glass fibre, GF/A filter and the filtrate reduced to 10 ml by rotary evaporation at 25°C. Polysaccharides were precipitated by addition of 4 volumes of ethanol and left overnight at 4°C. The precipitate was collected by centrifugation at 10,000g for 20 min and the pellet was washed, suspended in a small volume of water, and dialysed for 24h at 8°C. Aliquots were frozen until required. The total carbohydrate content of the sample was assayed for by the method of Dubois *et al.* (1956). Test sample (100μl) was added to 100μl of aqueous phenol then 500μl of conc. H_2SO_4 all, in an acid-washed cuvette. The solution was mixed thoroughly and left for 15 min at room temperature after which the absorbance was read at a wavelength of 490 nm. The carbohydrate content of the mucilage was then determined by reference to a standard for glucose solutions.

2.2.2. Zoospore responses to wheat roots

Wheat roots approximately 25mm long were treated with dyes in an attempt to block zoospore encystment. India ink (commercial stock solution, Watersons Ltd, Edinburgh), Methylene blue (0.1%), ruthenium red (0.1%) and alcian blue (1.0%), all from BDH chemicals, were prepared in distilled water. Wheat roots were incubated in dye for 45 min, and the excess dye was removed in three successive washings of phosphate buffer. Control (untreated) roots were treated identically but in phosphate buffer alone. In all experiments the roots were inserted into observation chambers which were made by gluing two strips of glass to a microscope slide to form a chamber 2mm deep, 10mm wide and 25mm long. A coverslip was placed on top and the chamber was filled with 0.4ml of a zoospore suspension, from one of the open ends. Roots were then inserted with their cut ends projecting from the chambers. Further tests involved adding wheat roots to suspensions to which various compounds had been added previously. Such compounds included a nutrient solution comprising bacteriological peptone (0.075%, Oxoid), L-glutamate (2.625mM, Sigma) D-galacturonic acid (1.875%, Sigma) and D-glucuronic acid (1.875mM, Aldrich Chemical Co) prepared in 0.01M phosphate buffer (pH 7.0). In all cases after 10 min the numbers of motile and encysted zoospores in the vicinity of roots were counted, a distinction being made between spores within 300μm of the root surface and those between 300-900μm from the root. After 20 min the number of encysted spores on the root, in the first 20mm, was also determined. Data

were analysed by t-test or chi-squared test as appropriate. The swimming speeds and patterns of response were followed throughout the experiments by video microscopy.

In further tests wheat roots approximately 25mm long were excised 2cm behind their tips. Some were coated with a double layer of calcium alginate gel by dipping them (30 sec) in a 1% (w/v) solution of sodium alginate (BDH Chemicals Ltd) and then (30 sec) in a 3% solution of calcium chloride; then the process was repeated. The gelled roots were then immersed for 3 min in distilled water. Both gelled and non-gelled roots were subsequently treated with lectin from *Tetragonolobus purpureas* or wheat germ agglutinin (which bind to L-fucose and N-acetyl-D-glucosamine respectively) both of the lectins being labelled with fluorescein isothiocyanate (FITC). The lectins were prepared at 125 $\mu g ml^{-1}$ in 0.1M phosphate buffer pH 6.8. Half of each lectin preparation was incubated with an excess of the complementary hapten sugar in accordance with the technical data supplied by Sigma Chemical Co. Gelled and non-gelled roots were immersed in the lectin solutions for 2-3 min and then in phosphate buffer (0.01M pH 7.0), for the same period, before being viewed by incident fluorescence microscopy. In addition, calcium alginate beads, produced by dropwise addition of a 1% (w/v) solution of sodium alginate into a 1% calcium chloride solution, were also treated with lectins and examined as above.

In a third series of experiments, gelled and non-gelled roots were immersed in lectin, dye or enzyme solutions for 45 min before being rinsed in 3 successive transfers of phosphate buffer. The lectins used were from *Tetragonolobus purpureas*, *Triticum vulgaris*, *Pisum sativum*, *Phaseolus limensis*, and Concanavalin A (all Sigma Chemical Co.) which were prepared at 125μg ml^{-1} in 0.1M phosphate buffer pH 6.8. Similarly, roots were incubated in trypsin (prepared at 100μg ml^{-1} in 0.01M phosphate buffer, pH 7.6) or exofucosidase (1 IU in $1ml^{-1}$) and in the case of gelled roots the dyes methylene blue and India ink. Control (untreated roots) were treated identically but in phosphate buffer or distilled water, alone. Non-gelled roots were inserted into observation chambers containing a suspension of zoospores, and after 10 min the number of motile spores in the vicinity of roots was assessed, by video microscopy; the numbers of encysted spores on the roots were determined after 20 min. For gelled roots the number of encysted spores on the alginate coating encompassing both sides of the root was determined after 20 min.

2.2.3. Video microscopy

A colour video camera (Panasonic WVP100E) was attached by a photographic extension tube to a Leitz Orthoplan microscope. The camera was attached to a Toshiba DV-80B video recorder, via a Panasonic WV-PS01AE/B power supply unit, which was attached to a Sony Trinitron 14 inch colour television. The camera contained an integral time date facility (min/sec/tenths of sec) which superimposed times on the video tape recordings. Zoospores were visualised using x4, x10 and x25 phase contrast objectives and the patterns of behaviour were analysed by playback of video tapes aided by the use of digitised frame-freeze and frame-shift facilities on the video recorder. In addition, behaviour of zoospores was followed by tracing on a cellulose acetate overlay on the video screen or by comparison of polaroid photographs, using a Mitsubishi P61-B video copy processor.

2.3. Zoospore responses to nutrients and cations

In all experiments, amino acids, sugars, uronic acids, root mucilage and ethanol were prepared 7 days earlier in phosphate buffer (pH 7.0) and checked periodically for pH with readjustment (using NaOH) if necessary. In the case of cations these were prepared as chloride salts in distilled water and adjusted to pH 7.0 with NaOH.

2.3.1. Zoospore taxis

Zoospore suspensions were carefully introduced into an observation chamber until the chamber was filled (ca 0.4ml) and was held by menisci at both ends. Then a 2μl microcapillary (Horwell Ltd, London), filled with a potential attractant, was inserted to about 3mm distance at one open end and a control capillary containing phosphate buffer (0.01M pH 7.0) was inserted at the other end. After 20 min the number of spores in each capillary was recorded by means of video microscopy.

In experiments designed to block or reduce chemotaxis towards L-glutamate (7mM), zoospores (final concentration $2x10^4$ spores ml^{-1}) were preincubated, for 90 sec, in background concentrations (7mM) of amino acids. When L-glutamate itself was used as a "blocking" agent it was supplied at only 2mM background (with 7mM L-glutamate in the test capillary). Similarly, L-aspartic acid was supplied at a background concentration of 3mM with 3mM L-

glutamate in the test capillary. These reduced concentrations were necessary to prevent premature encystment. The background amino acids were added to spore suspensions in Eppendorf tubes, mixed by slow inversion, and pre-incubated for 90 sec before the suspension was added to the observation chambers. Different tests were compared simultaneously. In one investigation the zoospores were pre-incubated in the presence of an amino acid with both the test and control capillaries containing the background compound, the only difference being that the test capillary also contained L-glutamate. In a matching test the zoospores were preincubated in phosphate-buffered pond water (Jones *et. al.,* 1991) whereas the test and control capillaries contained L-glutamate and phosphate buffer respectively. In a similar experiment background concentrations of cations, in addition to the calcium chelation agent EGTA (Schmid & Reilley, 1957), were tested for their effects on chemotaxis towards L-glutamate (7mM) diffusing from a capillary. Zoospores of *P. aphanidermatum* were preincubated for 90 sec in background concentrations of Ca^{2+} (500μM), Fe^{3+} (250μM), Na^{+} (250μM) and EGTA (500μM) all of which were prepared in distilled water. After 20 min the number of zoospores in test capillaries containing L-glutamate and control capillaries containing phosphate buffer was analysed as before.

2.3.2. Encystment

Aliquots (100μl) of amino acids (40mM final concentration), sugars (25mM final concentration), uronic acids (250-25,000 μgml^{-1} final concentration) or cations (1-25mM final concentration) were added to 100μl of zoospore suspension contained in an Eppendorf tube. The contents were then mixed by slow inversion, and were withdrawn after 1h or 1.5h to assess the number of encysted spores by microscopy. Controls were treated as above except that phosphate buffer or SDW were added to spores in place of nutrients or cations respectively.

In another experiment L-glutamic acid and L-aspartic acid were tested at successively lower concentrations to determine the threshold concentration eliciting encystment of *P. aphanidermatum* spores.

In another series of experiments zoospores were preincubated, for 5 min, in a background concentration of amino acids (final concentration 40mM) and the numbers of zoospores induced to encyst by subsequent addition of L-

glutamate (7mM final concentration) was determined. As before, L-glutamic acid and L-aspartic acid were also used as background solutions but at 2mM and 3mM, respectively, thus preventing premature encystment.

Zoospore accumulation and encystment in response to cellulose and chitin was examined by a method essentially like that of Mitchell & Deacon (1986c). Transparent cellulose film (Rayophane PU 525) was cut into pieces 4 x 4mm and autoclaved; similarly sized pieces of chitin (purified crab shell, Sigma Chemical Co) were autoclaved then both materials were rinsed in three changes of SDW before use. Test material was inserted into an observation chamber containing a suspension of zoospores. After 45 min, the number of motile and encysted zoospores that had accumulated on the test material in a microscope field of view (400x 300μm) was compared to the number in a randomly selected field not containing the test material.

2.3.3. Germination

Zoospore suspension was added to Eppendorf tubes and the spores were encysted by holding them against a vortex mixer for 70 sec. Aliquots (100μl) of amino acids (20mM), sugars (50mM) and uronic acids (500-50,000 μgml^{-1}) were added to an equivalent volume of zoospore suspension. The contents of the tube were mixed by inversion, and after 16h were transferred to welled cavity slides. The number of germinated spores were compared with those in controls treated similarly with phosphate buffer. In all cases, cells were recorded as germinated when the germ-tube length was equal to, or exceeded, the cyst diameter (ca 10μm).

In similar experiments 150μl of cation solution was added to 150μl of zoospore suspension (at a final concentration of 1-25mM) and incubated in Eppendorf tubes. After 1.5h, 150μl of cyst suspension was transferred to welled cavity slides and the number of germinated spores were counted. The slides were then incubated for a total 16h in moist chambers and germination was reassessed, all comparisons being made with similarly treated water controls. In a repeat of this experiment 150μl of motile zoospore suspension was incubated with an equivalent volume of cation solution (1-25mM final concentration), polysaccharide solution (100μgml^{-1} final concentration) or root mucilage of wheat or tomato (100μgml^{-1} final concentration). As above, the numbers of encysted and germinated spores were assessed after 1.5 and 16h for both the

cation and root mucilage tests whereas encystment was analysed after 1h in the case of the polysaccharide solutions.

To investigate the effects of sugars and amino acids on germ-tube growth of zoosporic cysts, aliquots (150μl) of zoospores were added to welled cavity slides immediately after encystment by agitation in a vortex. After 10 min, the cysts were rinsed with phosphate buffer (2x 300μl) to remove non-adhered spores. Each slide then received 150μl of an amino acid or sugar solution (10 mM). Slides were then incubated in moist chambers for 16h before germ-tube lengths were measured and compared to controls treated similarly with buffer.

2.4. Zoospore responses to Ca^{2+} modulators

2.4.1. Experiments analysing the relationship between adhesion and germination

Encystment of *P. aphanidermatum* (1ml suspension) was induced by holding an Eppendorf tube against a vortex mixer for 70 sec. Aliquots (80μl) were then added to welled slides, from a standard position in the tube, immediately (t_0) or 10 min after induction of encystment (t_{10}). Spores added to slides at t_0 or t_{10} were immediately treated with 80μl aliquots of either $CaCl_2$ solution or, EGTA [ethylene glycol bis (beta-aminoethyl ether)- N,N,N',N'-tetraacetic acid], both at 7mM final concentration, or SDW as controls. An additional treatment involved adding EGTA to spores, which were added to slides immediately after induction of encystment, after a 10 min delay. The total number of cells on each slide and the numbers germinating were recorded after 1.5h. Then the liquid and any non-adhering cells were removed by pipette, 160μl of SDW was added and similarly removed, this was repeated, and 160μl SDW was finally added before the slides were re-examined to determine the number of remaining cells that had germinated.

In a further experiment to investigate the relationship between adhesion and germination, pre-encysted spores, were added to welled slides at t_0 and then rinsed, as above, to remove non-adhering cysts. SDW was added (160μl) and the wells were scraped with a sharp scalpel to dislodge some cells. The water (160μl) containing detached spores was transferred in equal amounts to untreated slides (80μl) or slides (80μl) coated with the adhesive polylysine (Sigma; methodology in accordance to the supplier's technical data sheet). The

original slide, containing adhered spores, then received 80μl SDW and all three treatments were incubated for 1.5h. The same procedure was used for spores added to slides 10 min after induction of encystment, except that these were rinsed immediately to remove non-adhering spores (the majority). In all cases the numbers of germinated spores were assessed, after 1.5h, and the slides were then rinsed to determine the number of adhered spores. In order to ensure that enough spores were present in each treatment a dense zoospore suspension (2 x 10^5 ml^{-1}) was used for this experiment.

2.4.2. The effect of EGTA on germination

The period after induction of encystment when cells lose their ability to germinate was analysed by adding 80μl of vortexed spores of *P. aphanidermatum*, as above, to slides immediately after induction of encystment (t_0) or at 1 min intervals up to 10 min. At the time of spore transfer 80μl of EGTA (final concentration 7mM) or 80μl of SDW were simultaneously added. In each case the number of germinated cysts for each treatment was assessed after 2h.

The effect of EGTA on germination of three *Pythium* spp. was also tested in the presence of cations. Samples (80μl) of vortexed spore suspensions of *P. aphanidermatum*, *P. catenulatum* or *P. dissotocum* were transferred to Eppendorf tubes, which then received an equal volume of either EGTA, EGTA+ $CaCl_2$, EGTA+ $MgCl_2$, EGTA+ $SrCl_2$, EGTA+ KCl, EGTA+ NaCl (all at 7mM final concentration) or SDW (as a control). In all cases the salts and EGTA had been mixed and incubated for 24h prior to use. The number of germinated spores was assessed after 1.5 and 16h.

In a similar experiment, treatments as above were applied to an equivalent volume (80μl) of a motile zoospore suspension. After 1.5h the contents were transferred to a welled slide and the percentage of encysted and germinated spores was assessed after both 1.5 and 16h.

2.4.3. The effect of lanthanum and EGTA on cyst germination in the presence or absence of nutrients or cations

A suspension of *P. aphanidermatum* (0.5ml), encysted by agitation 10 min previously, was incubated in Eppendorf tubes for 10 min in the presence of 0.5ml SDW (control) or 0.5ml $LaCl_3$ solution (1.5mM final concentration).

After an additional 10 min (t_{20}), 80μl aliquots were transferred to separate tubes which received one of the following (all at final concentrations shown) : $CaCl_2$ (10mM), $SrCl_2$ (10mM), $MgCl_2$ (10mM), $CaCl_2$ (10mM) + $LaCl_3$ (1.5mM), $LaCl_3$ (1.5mM), MEP (3% malt extract and 1% peptone) or SDW (control). Germination was assessed after 1.5h by transferring cells to welled slides and was re-assessed after 16h on the slides. In a similar experiment, cysts were centrifuged at t_{20} (3 min in a microfuge at 15000 rpm) and the pellet rinsed twice with SDW, to remove lanthanum then resuspended and treatments were applied as above.

In identical experiments, cysts were pre-treated, at t_{10}, with $LaCl_3$ (1.5mM final concentration), EGTA (7mM final concentration) or SDW. At t_{20}, 80μl aliquots of these suspensions were post-treated with $CaCl_2$ (10mM), L-asparagine (10mM), L-aspartic acid (10mM), L-glutamic acid (10mM), L-glutamine (10mM), L-lysine (10mM), all at final concentrations, or SDW.

2.4.4. The effect of Ca^{2+} modulators on cyst germination

Cysts of *P. aphanidermatum* were treated, 10 min after induction of encystment (t_{10}), with a range of Ca^{2+} modulators (all Sigma; final concentration in parentheses): verapamil hydrochloride (30μM), amiloride hydrochloride (20μM), dibucaine hydrochloride (50μM), trifluoperazine dihydrochloride (TFP; 5μM), 8-(diethyl-amino)-octyl-3,4,5,-trimethoxybenzoate hydrochloride (TMB-8; 30μM), A23187 (5μM), a mixture of Ca^{2+} modulators (comprising TFP, dibucaine, verapamil, amiloride, at the above concentrations, plus $LaCl_3$ (at a final concentration of 0.75mM) or SDW. At t_{20} the cells were post-treated with $CaCl_2$, $MgCl_2$, $SrCl_2$, L-asparagine, L-lysine (all at 10mM final concentration), SDW or the individual Ca^{2+} modulators. The number of germinated spores was then assessed after 16h. All the Ca^{2+} modulators except A23187 were dissolved in SDW and adjusted to pH 7 with NaOH. A23187 was initially dissolved in dimethylsulphoxide (DMSO) and then diluted to 0.05% (v/v) DMSO in water which alone at this concentration had no effect on cyst germination.

In another experiment the same procedure as above was followed except that vortexed spores of *P. aphanidermatum* and *P. dissotocum* were used. At t_{10} cells were treated with dibucaine, verapamil or SDW and were post-treated at t_{20} with Ca^{2+} (10mM), L-asparagine (10mM), L-alanine (10mM),

dibucaine (50μm), verapamil (30μm) or SDW. The number of germinated spores for each treatment was then assessed after 16h.

All experiments were incubated in darkness because the compounds are light sensitive

2.5 Effects of cations, Ca^{2+} modulators and nutrient solutions on zoospore motility characteristics

A 100μl aliquot of zoospore suspension was added to an Eppendorf tube containing an equal volume of test solution to give a final concentration of 2x 10^{-4} zoospores ml^{-1}. The contents were then withdrawn from the tube and added to a glass cavity slide. Zoospore motility characteristics were analysed by video microscopy in the first 10 min following addition to the cavity slide and then reassessed after 50 min.

A range of cations and Ca^{2+} modulators were compared to water controls for their effects on motility. Selected amino acids (7mM) and ethanol (25mM) were similarly examined. Where applicable, the spores were assessed for swimming speed, amplitude and wavelength of the swimming helix and incidence of random changes of direction of the motile spores. From these assessments the velocity and frequency could be calculated (Figure 4.2; Allen & Newhook, 1974). Motility characteristics were analysed from video-recordings onto an acetate overlay on the video monitor and using frame-freeze and frame-shift facilities of the video recorder, together with an integral timer in the video camera, which superimposed min, sec and tenths of sec on the recordings.

3. RESPONSES OF ZOOSPORES TO ROOTS, ROOT COMPOUNDS AND INSOLUBLE SUBSTRATA

Some of the work described in this section has been published (Jones *et al.*, 1991).

3.1 Introduction

Fungal zoospores exhibit chemotaxis towards root exudates (Royle & Hickman, 1964a, b) and are attracted, in general, primarily to the root zone of elongation (Cunningham & Hagedorn, 1962; Ho & Hickman, 1967b; Chi & Sabo, 1978; Hinch & Clarke, 1980; Longman & Callow, 1987). However, if host-specific colonisation of plants and substrates occurs at all then it occurs when the spore is induced to encyst, lose its motility and adhere to a substratum (Mitchell & Deacon, 1986a,b). The process of encystment is a stimulus-mediated event (Irving & Grant, 1984) which is believed to involve an interaction between zoosporic surface receptors (Hardham & Suzaki, 1986; Estrada-Garcia *et al.*, 1990b; Kelleher *et al.*, 1990) and root surface components. Reports by Hinch & Clarke (1980) and Longman & Callow (1987) have implicated fucosyl residues, present in root surface polysaccharides, as being involved in the encystment of zoospores of, respectively, *P. cinnamomi* on maize roots and *P. aphanidermatum* on roots of cress. Pectin and other uronic acids, present in root surface mucilage, have also been reported to induce encystment and germination of zoospores of *Phytophthora* (Irving & Grant, 1984; Grant *et al.*, 1985) and *Pythium* (Jones *et al.*, 1991). The latter workers suggested that encystment *in vivo* involves synergism between chemoattractants and root surface components.

The aim of the experiments in this section was to determine the nature of the compounds that are responsible for eliciting accumulation, encystment and subsequent differentiation of fungal zoospores primarily on roots and on insoluble substrates. This approach involved the attempted saturating of zoospore chemoreceptors, in order to prevent zoospore accumulation, and also treatment of roots with gels, dyes, lectins or enzymes, in an attempt to block access of the zoospore to root surface components that elicit encystment. In addition, the effects of root- associated compounds, such as uronic acids and root mucilage, and insoluble substrates such as chitin and cellulosic material were examined.

3.2 Effects of root treatment with dyes or pre-treatment of zoospores with a nutrient solution on accumulation and encystment of zoospores of *P. aphanidermatum* on wheat roots

Root diffusates are widely implicated in causing zoospore accumulation and encystment (Royle & Hickman, 1964a; Hickman, 1970). In addition polyuronates, including pectin, which are present in root surface mucilage have been implicated in the encystment of *Phytophthora* zoospores (Grant *et al.*, 1985).

Tests were undertaken with various solutions in an attempt to block zoosporic encystment on roots. Wheat roots were pre-treated with the following as: (1) methylene blue, which binds to mucilage (Oades, 1978); (2) India ink, a stain of bacterial capsules and slime; (3) ruthenium red, which stains pectin and (4) alcian blue a stain of bacterial polysaccharides (Gurr, 1960). In addition to these treatments zoospores were pre-incubated for 3 min in a solution comprising peptone (0.075%), L-glutamic acid (2.625mM), D-galacturonic acid (1.875mM) and D-glucuronic acid (1.875mM). Each type of treatment was compared with designated controls (no treatment of roots or spore suspension). The tests, with single replicates were repeated five times so that treatments and controls could be compared by paired-samples t-tests.

The results (Table 3.1) show that pre-treatment of roots with methylene blue and India ink or addition of nutrients to the zoospore suspension significantly reduced the number of zoospores that encysted on wheat roots compared to control roots treated with phosphate buffer. Ruthenium red caused some reduction it was not significant; alcian blue had no effect. In all cases the dyes except India ink were noted to stain the roots uniformly; India ink stained the zone of elongation poorly. However, zoospores were noted to encyst preferentially in the root hair zone which contributed most to the counts in Table 3.1. Counts of motile spores in regions 0-300μm and 300-900μm around the roots (Table 3.2) showed that none of the treatments prevented accumulation in the immediate root vicinity. There was a consistently greater ($P = 0.001$) number of zoospores close to the roots (0-300μm) than further away (300-900μm). The swimming speeds of spores were also assessed by repeatedly playing and rewinding the video recordings to identify spores that showed uninterrupted paths of attraction to the roots as they entered the microscope field of view. Individual spores were then assessed for

Table 3.1 Numbers of zoospores of *Pythium aphanidermatum* that encysted during 20 min on wheat roots pretreated with dyes or on untreated roots in the presence or absence of nutrients in the zoospore bathing solution; means ± S.E.M. for 5 replicates

	No of encysted spores	
Type of treatment	Treated	Control treatment
Nutrients in zoospore suspension+; root pre-incubated in phosphate buffer	105 ± 24.9*	427 ± 123.3
Roots pre-incubated in:		
Methylene blue (0.1%)	119 ± 41.0**	770 ± 43.8
India ink	111 ± 22.5***	656 ± 88.9
Ruthenium red (0.1%)	382 ± 79.0	628 ± 112.8
Alcian blue (1.0%)	712 ± 123.1	789 ± 158.7

+ peptone (0.075%), L-glutamic acid (2.625mM), D-galacturonic acid (1.875mM), D-glucuronic acid (1.875mM) in zoospore bathing solution.

Significance of difference of encystment between treatments and phosphate buffer controls: *, 0.05; **, 0.01; ***, 0.001 by paired-samples t-test.

Table 3.2 Accumulation of motile spores after 10 min in regions 0-300μm and 300-900μm around wheat roots treated with dyes or around untreated roots in the presence or absence of nutrients in the zoospore bathing medium after 10 min

Test-treatment [+]	No of accumulated spores			
	Test treatment		Control treatment	
	0-300μm	300-900μm	0-300μm	300-900μm
Nutrients in zoospore suspension	260 ± 45.9 --*--	166 ± 37.8	295 ± 43.7 --*--	156 ± 15.6
Methylene blue (0.1%)	354 ± 37.5 --*--	194 ± 18.5	394 ± 65.6 --*--	217 ± 47.7
India ink	269 ± 61.4 --*--	130 ± 32.8	293 ± 73.2 --*--	142 ± 41.7
Ruthenium red (0.1%)	443 ± 56.9 --*--	179 ± 25.3	298 ± 71.4 --*--	150 ± 39.8
Alcian blue (1%)	318 ± 73.9 --*--	158 ± 35.5	403 ± 69.1 --*--	214 ± 52.5

[+] see Table 3.1.

* Accumulation of zoospores in the two different root regions does not represent the expected random distribution of spores but the number of zoospores in the two different cross sectional areas (0-300μm and 300-900μm) differ significantly from one another at P= 0.001 by Chi-squared analysis.

swimming speed in the regions 900-300μm and 300-0μm from the root surface. Results for several such spores were averaged for each root, and repeated for 9 replicate roots of each treatment (Table 3.3). There was no significant change in swimming speed as the spores approached the roots in different treatments, nor between the distances 900-300μm and 300-0μm during the spores's approach to the roots.

3.3 Effects of lectin or enzyme treatment of alginate-coated and uncoated wheat roots on zoospore accumulation and encystment

Adhesion and encystment of zoospores, on roots, is thought to be mediated by carbohydrates, in particular fucosyl residues, present in root slime (Hinch & Clarke, 1980; Longman & Callow, 1987). However, Jones (1989) disputed the role that exposed saccharide residues may play in zoosporic encystment on roots. This was because he was able to encase roots and, therefore, entrap root polysaccharides, in a double layer of alginate gel which had no effect on accumulation or encystment of *Pythium* zoospores on roots of tomato and wheat. However, it was possible that Jones (1989) had not completely prevented exposure of zoospores to root slime components if some of these had diffused through the calcium alginate gel (which is a highly porous material) and been deposited in its interstices or on its surface.

In an attempt to exclude these possibilities, wheat roots encased in a double layer of calcium alginate gel were post-treated with lectins or enzymes which should, respectively, block or remove exposed saccharide residues that mediate encystment. The lectins used were Concanavalin A (Con A, which specifically binds D-glucosyl and D-mannosyl residues), wheat germ agglutinin (WGA, which binds N-acetyl-D-glucosamine (NAG)), fucose binding protein from *Tetragonolobus purpureas*, pea (*Pisum sativum*) lectin (P. sat, which binds D+ mannose), and bean (*Phaseolus limenis*) lectin (P. lim, which binds N-acetyl-D-galactosamine). The enzymes used were the protease trypsin and α-L-exofucosidase which cleaves fucosyl residues. In addition, gelled roots were post-treated with the dyes, methylene blue or India ink. Encystment of zoospores on the roots was examined as before (Table 3.4).

Post-treatment of gelled and non-gelled wheat roots (Table 3.4), with lectins, enzymes or dyes had no effect on the number of encysted spores or the pattern of encystment in any case. In addition, no treatment prevented zoospore accumulation in the immediate vicinity of non-gelled roots (Table 3.5).

Table 3.3 Zoospore swimming speeds of *P. aphanidermatum* towards wheat roots pre-treated with dyes or untreated, or in the presence and absence of nutrients in the zoospore bathing medium

	Speed (μm sec^{-1})*	
Pre-treatment of root+	away from root	near root
Methylene blue (0.1%)	116 ± 3.8	114 ± 3.8
India ink	111 ± 5.0	118 ± 5.0
Ruthenium red (0.1%)	113 ± 3.5	116 ± 4.8
Alcian blue (1.0%)	117 ± 3.5	116 ± 3.0
None (control with phosphate buffer)	119 ± 3.8	115 ± 3.7
Phosphate buffer, but with nutrients in zoospore suspension	118 ± 3.9	113 ± 5.4

+ Except where noted, all tests were done with zoospores suspended in phosphate buffer; see Table 3.1 for nutrient supplement to zoospore suspension.

* Speeds were averaged for zoospores of *Pythium aphanidermatum* on each root; results are presented as means ± s.e. for 9 replicate roots. Each zoospore was followed over 1000μm distance as it approached a root, and speeds are shown for the distance 0-300μm (near root) and 300-1000μm (away from root).

Table 3.4 Numbers of zoospores that encysted during 20 min on alginate-coated and non-coated wheat roots that were post-treated with lectins, enzymes or dyes, compared to corresponding control roots treated with phosphate buffer*

Post-treatment roots	Coated roots	Non-coated
Concanavalin A	1468 ± 409.3	725 ± 217.8
Phosphate buffer	1429 ± 255.9	548 ± 54.8
P. sativum	1404 ± 264.3	577 ± 111.1
Phosphate buffer	1184 ± 307.8	1081 ± 290.6
T. vulgaris	1740 ± 127.8	1081 ± 290.6
Phosphate buffer	1184 ± 307.8	1211 ± 129.4
T. purpureas	1399 ± 206.9	499 ± 81.4
Phosphate buffer	1429 ± 255.9	434 ± 86.5
P. limensis	1291 ± 364.3	478 ± 52.8
Phosphate buffer	1426 ± 235.7	434 ± 86.5
Trypsin	1613 ± 529.0	1200 ± 126.9
Phosphate buffer	1426 ± 235.7	1211 ± 129.4
Fucosidase	1197 ± 158.0	370 ± 86.4
Phosphate buffer	1410 ± 165.2	336 ± 81.6
Methylene blue (0.1%)	2004 ± 406.5	n.d.
Phosphate buffer	1633 ± 245.1	n.d.
India ink	1138 ± 166.7	n.d.
Phosphate buffer	1284 ± 193.4	n.d.

* Means ± s.e. for 5 replicates. No significant difference was found between coated/non-coated or post-treatment/phosphate buffer comparisons in any instance; n.d. = not determined

Table 3.5 Numbers of motile zoospores of *Pythium aphanidermatum* after 10 min in regions 0-300μm and 300-900μm from wheat roots treated with lectins or enzymes compared to corresponding control roots treated with phosphate buffer+

	Distance	
Treatment	0-300μm	300-900μm
Concanavalin A	613 ± 81.3*	636 ± 157.4
Phosphate buffer	656 ± 181.4*	681 ± 183.4
P. sativum	523 ± 71.2*	660 ± 83.8
Phosphate buffer	656 ± 181.4*	681 ± 183.4
T. vulgaris	838 ± 61.2*	922 ± 148.5
Phosphate buffer	689 ± 100.7*	796 ± 181.2
T. purpureas	646 ± 86.9*	415 ± 123.7
Phosphate buffer	798 ± 184.3*	470 ± 195.8
P. limensis	608 ± 153.2*	339 ± 134.9
Phosphate buffer	798 ± 184.3*	470 ± 195.8
Trypsin	884 ± 161.4*	926 ± 268.6
Phosphate buffer	689 ± 100.7*	796 ± 181.2
Fucosidase	971 ± 115.1*	903 ± 103.3
Phosphate buffer	808 ± 93.7*	610 ± 96.6

+ Means ± s.e. for 5 replicates. Accumulation of zoospores in the two different root regions does not represent the expected random distribution of spores but the number of zoospores in the two different cross sectional areas (0-300μm and 300-900μm) denoted by * differ significantly from one another at P= 0.001 by Chi-squared analysis.

In this experiment zoospores were seen to accumulate at the alginate surface overlying both the root cap (Figure 3.1) and the zone of young root hairs within 90 sec. However, by 10 min zoospores had vacated the root cap region, without encysting (Figure 3.2), and swam towards the root hair zone where rapid encystment occurred (Figures 3.3, 3.4). In addition zoospores orientated themselves prior to encystment such that their fixed point of germ-tube outgrowth was immediately adjacent to the root (Figures 3.5, 3.6). This behaviour was also observed with non-gelled wheat roots (Figures 3.7, 3.8), and zoospores were induced to encyst at the base of root hairs (Figure 3.9) irrespective of treatment.

In a further experiment, lectins were used to analyse the distribution of exposed sugar residues on wheat roots, to determine whether localised regions of specific saccharide residues could account for localised zones of zoospore encystment on roots. Both gelled and non-gelled wheat roots were treated with FITC-labelled WGA and FBP, to analyse the distributions of NAG and fucose respectively, both of these sugars being known to be present on wheat roots (Moody *et al.,* 1988). The roots were then examined by fluorescence microscopy. For both lectin treatments, non-gelled roots displayed intense fluorescence of the root cap and uniform fluorescence along their surfaces. When the lectin-treated roots were retained in darkness for 3-4h, fluorescence disappeared from the root cap region but was retained along the length of the root. Alginate-coated roots showed uniform but diffuse fluorescence along their lengths. However, calcium alginate beads treated with lectins showed similar fluorescence, indicating that the lectins were trapped in the gel rather than being bound to specific glycosyl residues. In all cases, preincubation of the lectin with its specific hapten sugar almost completely eliminated lectin binding to the roots. In no case, therefore, was there evidence of localised distribution of sugars, at least for NAG and fucose, which could account for localised encystment of zoospores in the zone of young root hairs.

3.4 Zoospore responses to root mucilage

Root mucilage, and its components, have been implicated in accumulation (Mitchell & Deacon, 1986a), encystment, and adhesion (Hinch & clarke, 1980; Longman & Callow, 1987) and germination of zoospores (Grant, *et al.,* 1985). The aim of this experiment was to determine whether root mucilage was capable of eliciting accumulation and germination of *Pythium* zoospores. Root

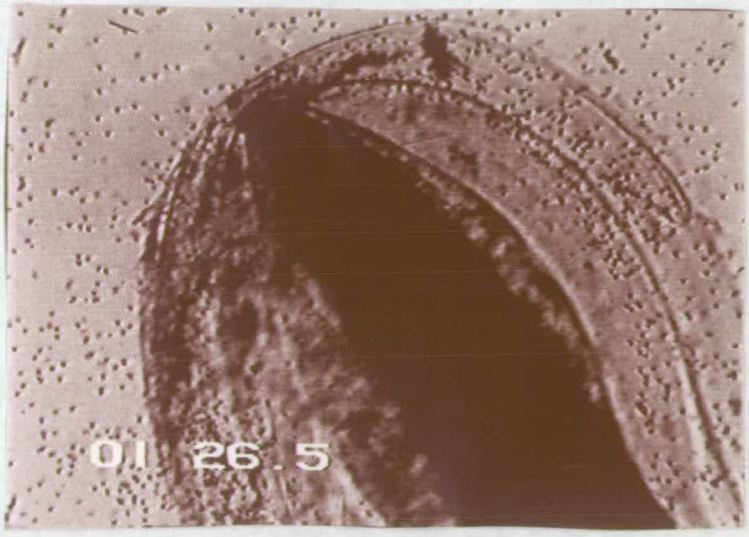


Figure 3.1 Accumulation of zoospores of *P. aphanidermatum* around wheat root tips coated with alginate gel, 90 sec. after immersion of the root in zoospore suspension

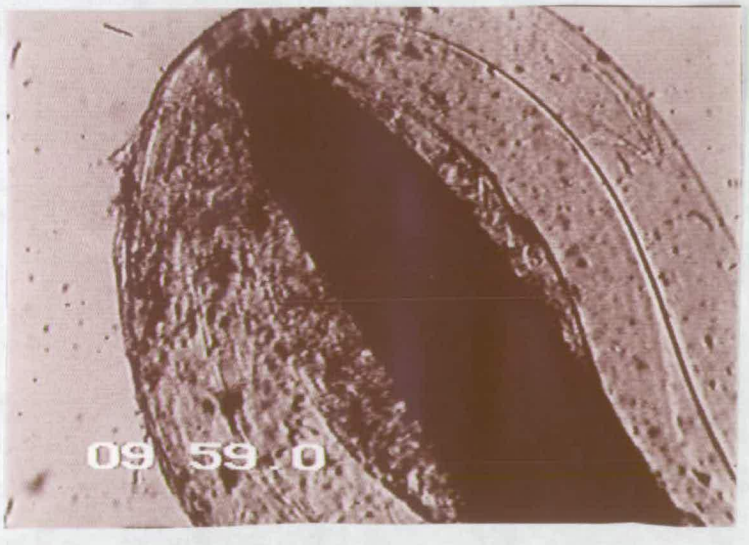
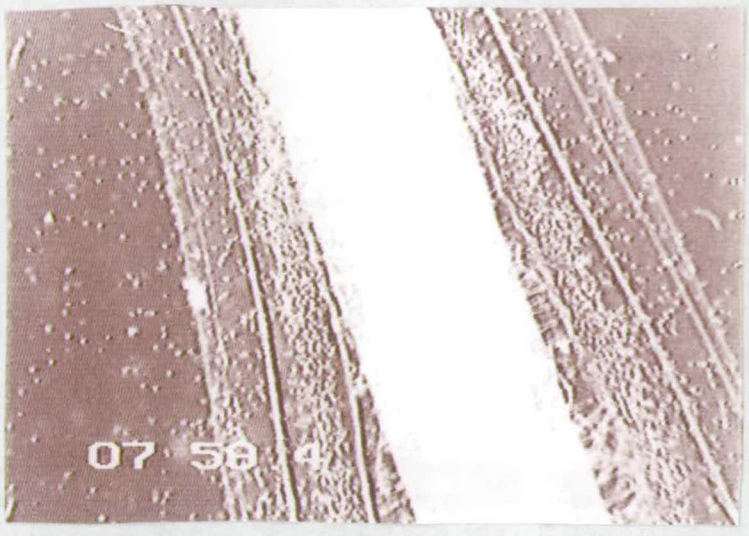
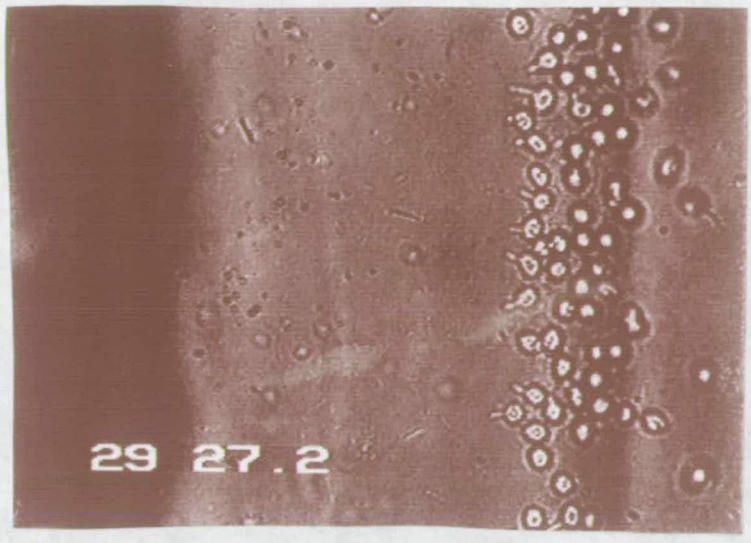
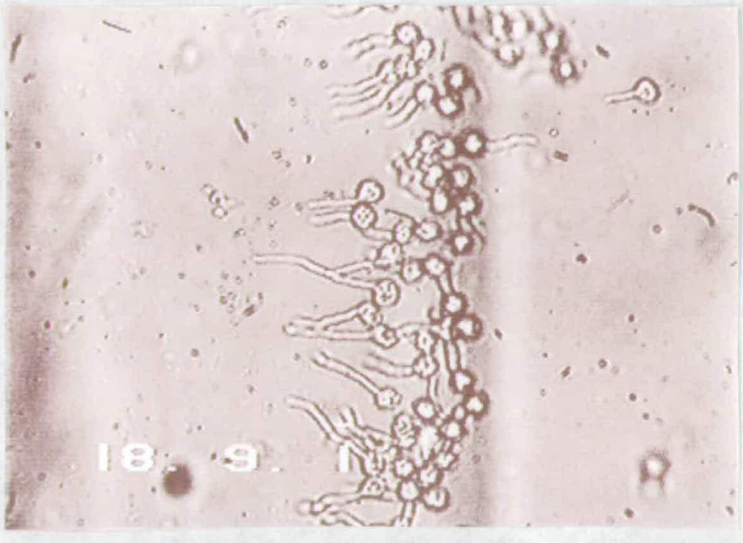


Figure 3.2 As Figure 3.1 but after 10 min: zoospores have vacated the root cap region without encysting.



Figures 3.3, 3.4. Accumulation and encystment of zoospores of *P. aphanidermatum* overlying the root hair zone on wheat roots coated with alginate gel, at ca 8 min after immersion of roots in zoospore suspension





Figures 3.5, 3.6. Zoospore cysts of *P. aphanidermatum* have encysted and germinated over the root hair zone of wheat roots coated with calcium alginate, and show germ-tube outgrowth towards a root (extreme left of photographs). Photographs taken at ca 30 min and 90 min after immersion of roots in zoospore suspension.

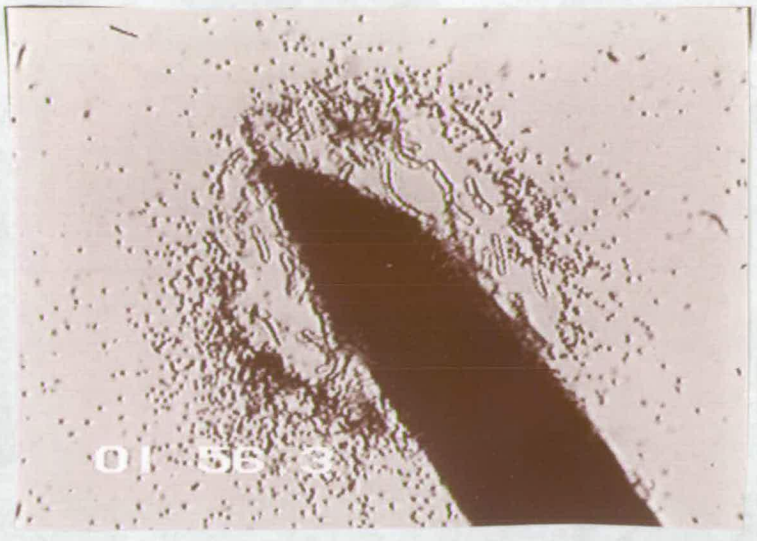


Figure 3.7 Accumulation of zoospores of *P. aphanidermatum* around wheat root tips (non-gelled) at ca 2 min after immersion of root in zoospore suspension.

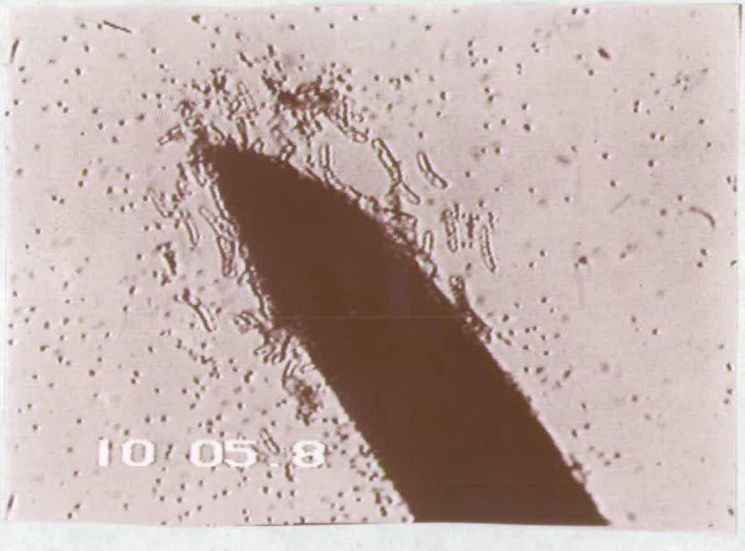


Figure 3.8 As figure 3.7 but after 10 min; zoospores have vacated the root cap region without encysting.

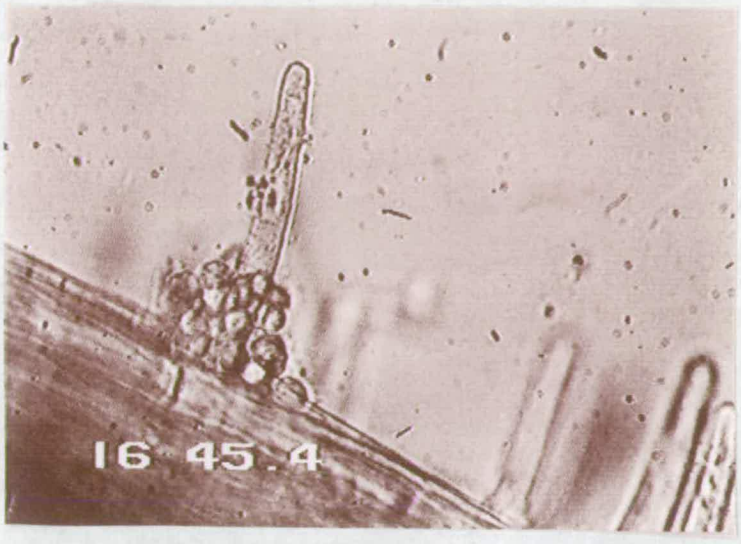


Figure 3.9 Zoospores of *P. aphanidermatum* encysted at the base of a wheat root hair.

mucilage of both wheat and tomato were prepared as described in section 2.2.1 and dialysed against distilled water for 24h to remove any low molecular weight soluble materials. Then they were introduced into microcapillaries, with control capillaries containing phosphate buffer, and inserted in zoospore suspensions (Table 3.6).

Both tomato and wheat root mucilage (each at 100μgml^{-1}) elicited chemotaxis of all three fungi when compared to capillaries containing only phosphate buffer.

In a further experiment, the effects of root mucilage on encystment and germination of motile spores were examined by incubating zoospores and root mucilage together in Eppendorf tubes (Table 3.7). After 1.5h, wheat root mucilage had elicited significant encystment and germination of all three *Pythium* spp. Tomato root mucilage had elicited significant encystment of *P. catenulatum* and *P. dissotocum* but not *P. aphanidermatum* spores although all three fungi were induced to germinate by tomato root mucilage. At 16h, nearly all the spores had encysted, even in controls, and again both tomato and wheat root mucilage enhanced germination of all three fungi.

3.5 Effect of uronic acids on encystment and germination of *Pythium* zoospores

Uronic acids are reported to stimulate encystment of zoospores of *P. aphanidermatum* (Jones *et al.,* 1991) and to enhance germination of *P. palmivora* (Grant *et al.,* 1985). In order to study this various uronic acids were tested for their effects on encystment and germination of three *Pythium* species. Different concentrations of sodium alginate, D-galacturonic acid, D-glucuronic acid, and poly-D-galacturonic acid were mixed with spore suspensions in Eppendorf tubes (Table 3.8). All four substances were found to significantly enhance levels of encystment above phosphate buffer controls, but at different minimum effective final concentrations. Sodium alginate was most effective, causing significant encystment by all three fungi at 250-500μgml^{-1} whereas poly-D-galacturonic and D-galacturonic acid were effective at about 1000μgml^{-1} and D-glucuronic acid was effective at only 5000μgml^{-1}. There was also evidence of differential behaviour of the fungi, *P. dissotocum* responding, in general, to a higher concentration of each compound than did *P. aphanidermatum* and *P. catenulatum* (Table 3.8). At the higher concentrations polygalacturonic acid and D-galacturonic acid induced lysis of a substantial proportion of the spores of *Pythium* spp., whereas D-glucuronic acid and sodium alginate caused only a

Table 3.6 Numbers of zoospores in test capillaries containing wheat or tomato root mucilage ($100\mu gml^{-1}$) compared with capillaries containing phosphate buffer (control) at 20 min after capillaries were inserted into a zoospore suspension+

Fungus	Tomato mucilage	Control	Wheat mucilage	Control
P. aphanidermatum	34 ± 3.8*	5 ± 1.0	58 ± 8.4*	6 ± 1.3
P. catenulatum	57 ± 7.7*	3 ± 0.6	42 ± 7.0*	4 ± 1.1
P. dissotocum	29 ± 4.8*	3 ± 0.8	38 ± 5.2*	3 ± 1.0

+ means ± s.e. for 9 replicate tests. * Matching mucilage and buffer controls differ significantly ($\underline{P}$= 0.001) from one another by paired-samples t-tests.

Table 3.7 The effects of wheat and tomato root mucilages on encystment and germination by *Pythium* zoospores[+]

Fungus	Treatment	Per cent encystment (E)		Per cent germination (G)		G/E (%)	
		1.5h	16h	1.5h	16h	1.5h	16h
P. aphanidermatum	Tomato	13 ± 1.7	100 ± 0	7 ± 1.5***	58 ± 5.1***	49 ± 4.5***	58 ± 5.1***
	Wheat	24 ± 2.4***	100 ± 0	10 ± 1.9***	51 ± 5.5***	41 ± 6.4*	51 ± 5.5***
	Buffer	10 ± 1.1	99 ± 1.0	3 ± 1.1	19 ± 2.6	29 ± 7.2	20 ± 2.6
P. catenulatum	Tomato	70 ± 5.9***	99 ± 1.0	38 ± 4.7***	64 ± 4.1***	53 ± 4.9***	65 ± 4.1***
	Wheat	47 ± 3.9***	100 ± 0	27 ± 2.6***	58 ± 4.2***	57 ± 3.3***	58 ± 4.2***
	Buffer	11 ± 1.5	100 ± 0	1 ± 0.6	22 ± 2.0	8 ± 4.9	22 ± 2.0
P. dissotocum	Tomato	73 ± 4.7***	99 ± 1.0	41 ± 5.4***	60 ± 5.6***	55 ± 5.8***	61 ± 5.7***
	Wheat	60 ± 7.2***	100 ± 0	39 ± 6.4***	67 ± 3.9***	64 ± 3.9***	67 ± 3.9***
	Buffer	13 ± 1.9	100 ± 0	1 ± 0.3	24 ± 2.6	8 ± 1.9	24 ± 2.6

[+] Means ± s.e. for 9 replicates, based on counts of 100 spores per replicate, assessed after 1.5 and 16h. Significance of difference between mucilage treatments and matched phosphate buffer controls by paired-samples t-tests: *, 0.02; **, 0.01; ***, 0.001.

Table 3.8 (part) The effects of uronic acids on percentage encystment or lysis of *Pythium* zoospores ; means ± s.e. for 7 replicates based on assessment of 100 spores per replicate after 1.5h[+]

Treatment	concentration	*P. aphanidermatum*		*P. catenulatum*		*P. dissotocum*	
	(μgml^{-1})	Lysis	Encystment	Lysis	Encystment	Lysis	Encystment
Poly-D-galacturonate	None (control)	0	32 ± 3.6	0	26 ± 4.7	0	27 ± 2.3
	250	0	32 ± 4.7	0	32 ± 7.7	0	18 ± 3.5*
	500	0	51 ± 10.6	0	63 ± 9.4*	0	33 ± 10.2
	1000	0	68 ± 7.1***	6 ± 3.2	74 ± 5.4***	1 ± 1.0	48 ± 7.8**
	5000	6 ± 2.3	79 ± 3.8****	15 ± 4.5**	85 ± 4.5****	4 ± 2.7	65 ± 9.7***
	10,000	45 ± 7.0****	55 ± 7.0*	43 ± 5.4****	57 ± 5.4***	36 ± 8.9****	64 ± 8.9**
	25,000	72 ± 4.7****	28 ± 4.7	41 ± 7.9****	59 ± 7.9*	52 ± 6.8****	48 ± 6.8*
Sodium alginate	None (control)	0	34 ± 3.1	0	39 ± 6.1	0	22 ± 2.7
	250	0	72 ± 5.4***	0	65 ± 3.4***	0	27 ± 3.8
	500	0	93 ± 3.5****	0	67 ± 4.4***	0	42 ± 4.6*
	1000	0	100 ± 0****	0	100 ± 0****	0	92 ± 3.1****
	5000	0	100 ± 0****	0	100 ± 0****	0	100 ± 0****
	10,000	0	100 ± 0****	0	99 ± 1.0****	0	100 ± 0****
	25,000	5 ± 1.7	95 ± 1.7****	0	98 ± 1.0****	4 ± 2.4	96 ± 2.4****

[+] Significance of difference between uronic acid treatments and matched buffer controls by paired-samples t-test : *, 0.05; **, 0.02; ***, 0.01; ****, 0.001.

Table 3.8 (Continued) The effects of uronic acids on percentage encystment or lysis of *Pythium* zoospores ; means ± s.e. for 7 replicates based on assessment of 100 spores per replicate after 1.5h[+]

Treatment	concentration (μgml^{-1})	*P. aphanidermatum*		*P. catenulatum*		*P. dissotocum*	
		Lysis	Encystment	Lysis	Encystment	Lysis	Encystment
D-glucuronate	None (control)	0	9 ± 2.0	0	10 ± 1.6	0	13 ± 3.2
	250	0	9 ± 2.4	0	13 ± 3.1	0	16 ± 4.1
	500	0	15 ± 3.0	0	13 ± 2.8	0	17 ± 3.5
	1000	0	14 ± 2.4	0	13 ± 2.3	0	18 ± 3.5
	5000	0	57 ± 5.3****	0	44 ± 6.2****	0	25 ± 4.3*
	10,000	0	93 ± 3.0****	4 ± 1.9	96 ± 1.9****	0	32 ± 8.2
	25,000	9 ± 1.6***	91 ± 1.6****	5 ± 2.0	95 ± 2.0****	0	100 ± 0****
D-galacturonate	None (control)	0	8 ± 1.3	0	13 ± 3.2	0	22 ± 3.4
	250	0	8 ± 2.0	0	14 ± 4.0	0	27 ± 4.1
	500	0	16 ± 5.4	0	13 ± 2.3	0	27 ± 4.6
	1000	0	47 ± 7.7***	0	38 ± 7.2**	0	27 ± 7.7
	5000	0	29 ± 6.8**	0	41 ± 6.3****	0	31 ± 2.8
	10,000	0	86 ± 3.7****	44 ± 5.0****	56 ± 5.0***	1 ± 1.0	46 ± 8.9
	25,000	58 ± 12.2***	42 ± 12.2*	68 ± 4.2****	32 ± 4.2**	14 ± 2.8***	86 ± 2.8****

[+] Significance of difference between uronic acid treatments and matched buffer controls by paired-samples t-test : *, 0.05; **, 0.02; ***, 0.01; ****, 0.001.

small amount of lysis, this being at the highest tested concentrations.

In addition after 16h, sodium alginate enhanced germination of spores, of all three *Pythium* spp, pre-encysted by agitation in a vortex, the minimum effective concentration being 500-1000μgml^{-1} and with no evidence of toxicity at even 25,000μgml^{-1} (Table 3.9). Polygalacturonic acid slightly stimulated germination of *P. aphanidermatum* and *P. catenulatum* at intermediate concentrations but was markedly inhibitory to all three species at the higher concentrations. The monomers, D-galacturonic acid and D-glucuronic acid, caused no significant enhancement of germination, and D-galacturonic acid, in particular, was markedly inhibitory at the higher concentrations.

3.6 Effects of cellulosic and chitin-containing materials on accumulation and encystment of *Pythium* zoospores

Zoospores are commonly isolated from natural environments by floating baits such as cellulose over streams and lakes (Couch, 1939; Willoughby, 1957). In possible explanation of this, Mitchell & Deacon (1986b) reported that insoluble substrata, such as cellulosic and chitin-containing materials, can selectively cause accumulation and encystment of fungal zoospores. This study was extended to the three *Pythium* spp., using pieces of transparent cellulose film and purified crab shell (Table 3.10).

Motile zoospores of *P. catenulatum* (Table 3.10), were found to accumulate on both cellulose and chitin, but to encyst only on cellulose. Spores of *P. dissotocum* accumulated and encysted on both cellulose and chitin, whereas, spores of *P. aphanidermatum* did not accumulate significantly on either material but were induced to encyst on chitin. In all cases where accumulation occurred around cellulose and chitin there was no change of directional swimming towards the material. Rather, zoospores encountered the materials at random and, where appropriate, were retained in the vicinity of the substrate by both a reduction in the swimming speed (negative orthokinesis) and an increase in the frequency of turning (positive klinokinesis).

Table 3.9 Effects of uronic acids on percentage germination of *Pythium* zoospores ; means ± s.e. for 7 replicates, based on assessment of 100 spores per replicate after 16h[+]

Treatment	conc.(μgml^{-1})	*P. aphanidermatum*	*P. catenulatum*	*P. dissotocum*
Poly-D-galacturonate	0 (control)	25 ± 1.9	21 ± 2.2	23 ± 1.4
	250	24 ± 1.8	24 ± 1.4	20 ± 1.9
	500	22 ± 1.6	20 ± 1.2	25 ± 2.9
	1000	19 ± 3.1	35 ± 3.3***	23 ± 2.9
	5000	31 ± 2.6*	39 ± 4.3**	22 ± 2.0
	10,000	15 ± 2.0***	18 ± 2.2	21 ± 2.2
	25,000	5 ± 0.8****	9 ± 2.4****	9 ± 1.2****
Sodium alginate	0 (control)	23 ± 1.2	28 ± 1.0	23 ± 1.3
	250	30 ± 3.0	24 ± 2.3	26 ± 2.7
	500	45 ± 5.8**	39 ± 3.7**	28 ± 4.3
	1000	52 ± 4.2****	53 ± 4.9***	58 ± 4.9****
	5000	76 ± 4.3****	46 ± 2.9***	72 ± 3.2****
	10,000	65 ± 7.6***	65 ± 5.0***	70 ± 3.9****
	25,000	68 ± 6.7****	60 ± 4.8****	53 ± 4.1****
D-glucuronate	0 (control)	24 ± 1.9	20 ± 1.6	26 ± 1.4
	250	20 ± 1.5	24 ± 2.1	24 ± 1.6
	500	26 ± 1.6	22 ± 1.7	26 ± 2.2
	1000	22 ± 2.3	19 ± 1.7	22 ± 1.4
	5000	23 ± 1.8	22 ± 1.6	26 ± 2.0
	10,000	19 ± 2.3	22 ± 2.5	24 ± 1.8
	25,000	5.3 ± 1.1****	20 ± 2.1	22 ± 1.2
D-galacturonate	0 (control)	21 ± 1.6	24 ± 1.6	24 ± 1.4
	250	25 ± 2.0	22 ± 2.7	24 ± 1.4
	500	21 ± 1.7	25 ± 2.2	21 ± 1.6
	1000	19 ± 2.0	15 ± 1.9****	21 ± 1.8
	5000	9 ± 1.7***	16 ± 1.7*	20 ± 1.7
	10,000	4 ± 1.0****	9 ± 1.4****	11 ± 1.9**
	25,000	2 ± 1.6****	10 ± 1.2****	8 ± 1.4****

[+] Significance of difference between uronic acid treatments and matched buffer controls by paired-samples t-test : *, 0.05; **, 0.02; ***, 0.01; ****, 0.001.

Table 3.10 Numbers of fungal zoospores swimming or encysted, after 45 min, in microscope fields centred on pieces of cellulose or chitin (test) or in randomly selected control fields[+]

	Cellulose		Chitin	
	Test	Control	Test	Control
P. aphanidermatum				
Swimming	63 ± 7.7	70 ± 6.4	56 ± 9.8	49 ± 5.0
Encysted	4 ± 1.0	2 ± 0.8	35 ± 5.2*	5.3 ± 1.2
P. catenulatum				
Swimming	73 ± 6.1*	22 ± 4.3	55 ± 5.9*	22 ± 2.5
Encysted	25 ± 2.4*	3 ± 0.9	2 ± 0.8	4 ± 0.8
P. dissotocum				
Swimming	101 ± 12.4*	41 ± 4.3	90 ± 8.9*	35 ± 6.8
Encysted	40 ± 5.0*	3 ± 1.2	35 ± 5.9*	6 ± 2.2

[+] Means ± s.e. for 7 replicates ; * denotes significance of difference between test and matched control fields ($\underline{P}$= 0.001) based on Chi-squared analysis.

3.7 Effects of polysaccharides on encystment and germination of zoospores of Pythium spp.

Zoospores of *Pythium* spp. are chemotactically attracted to a wide range of plants but can be specifically induced to encyst on host, as opposed to non-host, roots (Mitchell & Deacon, 1986a). Similarly, cellulosic and chitin-containing materials, although not attractive, selectively encyst zoospores of a wide range of fungi (Mitchell & Deacon, 1986b). Zoospores, *in vivo*, are reported to encyst in response to saccharide-containing residues present in root mucilage (Hinch & Clarke, 1980; Longman & Callow, 1987). In order to extend these investigations, a defined range of polysaccharides were examined to determine whether sugar-containing compounds could specifically induce encystment of *Pythium* zoospores *in vitro*.

The polysaccharides shown in Table 3.11 were added to Eppendorf tubes containing zoospores and the percentage encystment was assessed after 1h. Only gum arabic, isolated from *Acacia* and containing ß-D-galactose, α-L-arabinose and D-glucuronic, acid elicited significant encystment of all three *Pythium* spp. A mixed- linkage glucan, composed of 1,4 and 1,3 linked ß-D-glucose, induced encystment of both *P. catenulatum* and *P. dissotocum* but not *P. aphanidermatum*. Both glucuronoxylan, containing ß-D-xylose and 4-O-Me-α-D-glucuronic acid, and arabinoxylan, composed of ß-D-xylose and α-L-arabinose, specifically induced encystment of *P. catenulatum* and *P. aphanidermatum* respectively. Non-fucosylated xyloglucan, comprising ß-D-glucose, α-D-xylose and ß-D-galactose, elicited encystment only of *P. dissotocum* whereas both a xyloglucan polymer, containing additional fucose, and fucoidan (sulphated fucose) elicited encystment only of *P. catenulatum*. An arabinogalactan polymer, containing ß-D-galactose and α-L-arabinose, had no effect on encystment of any of the *Pythium* spp.

After 16h all the spores had encysted and some had germinated in response to the polysaccharides (Table 3.11). Gum arabic, which induced encystment of all three *Pythium* spp., stimulated germination of *P. catenulatum* and *P. aphanidermatum* cysts, whereas arabinoxylan and xyloglucan (non-fucosylated) enhanced germination of *P. aphanidermatum* and *P. dissotocum* cysts respectively. Methylglucuronoxylan, in addition to inducing encystment, stimulated germination of *P. catenulatum*. The fucosylated xyloglucan and the mixed linkage glucan stimulated germination of *P. aphanidermatum* despite

Table 3.11 Effects of polysaccharides on encystment and germination of zoospores of *Pythium* spp.[+]

Treatment ($100\mu gml^{-1}$)	*P. catenulatum*		*P. aphanidermatum*		*P. dissotocum*	
	Encyst	Germinate	Encyst	Germinate	Encyst	Germinate
None (control)	12 ± 1.7	24 ± 2.6	10 ± 1.4	22 ± 3.5	25 ± 1.8	41 ± 3.9
Arabinoxylan	18 ± 1.8	22 ± 3.9	61 ± 9.0***	33 ± 2.7*	26 ± 1.9	36 ± 4.3
Methylglucuronoxylan	54 ± 5.8***	76 ± 5.9***	10 ± 0.7	28 ± 3.7	23 ± 2.2	49 ± 4.3
Xyloglucan (non-fucosylated)	17 ± 1.2	25 ± 2.9	10 ± 0.4	25 ± 3.8	73 ± 7.0***	63 ± 6.3*
Xyloglucan (fucosylated)	31 ± 2.4***	23 ± 3.4	8 ± 1.0	45 ± 5.7**	26 ± 2.3	41 ± 5.6
Mixed linkage glucan	45 ± 5.5***	26 ± 4.1	11 ± 1.2	36 ± 3.3*	39 ± 3.4**	25 ± 2.9
Fucoidan	54 ± 5.9***	26 ± 3.2	7 ± 1.1	21 ± 2.0	25 ± 2.1	37 ± 4.4
Gum arabic	69 ± 8.4***	52 ± 3.2***	37 ± 6.4***	67 ± 6.4***	65 ± 5.4***	48 ± 4.0
Arabinogalactan	19 ± 2.1	25 ± 5.5	8 ± 1.0	35 ± 7.6	27 ± 2.8	54 ± 7.2

[+] Means ± s.e. for 5 replicates, based on counts of 100 spores per replicate; encystment and germination assessed 1h and 16h, respectively, after treatments were applied.

Significance of difference between polysaccharide treatments and matched phosphate buffer controls by paired-samples t-tests: *, 0.05; **, 0.01; ***, 0.001.

having no corresponding effect on encystment. No other polysaccharide tested had any effect on germination.

3.8 Discussion

The experiments in this section demonstrate that zoospores of *P. aphanidermatum* display localised accumulation and encystment on wheat roots, primarily in the zone of young root hairs. In addition, the *in vitro* studies demonstrate that zoospores of *Pythium* spp. are induced to undergo differentiation by root mucilage, polysaccharidesand uronic acids whereas chitin and cellulose can selectively elicit accumulation and encystment.

Fungal zoospores are attracted by exudates leading to trapping and encystment (Hickman & Ho, 1966; Hickman, 1970; Zentmyer, 1970). In general, zoospores tend to encyst on roots in the zone of elongation (Cunningham & Hagedorn, 1962; Royle & Hickman, 1964a; Chi & Sabo, 1978; Hinch & Clarke, 1980; Longman & Callow, 1987). It was believed that localised encystment was due to interaction between a proteinaceous receptor on the zoospore surface and carbohydrate residues, in particular fucose, present in root slime. In two reports, localised encystment of *Phytophthora cinnamomi* (Hinch & Clarke, 1980) and *Pythium aphanidermatum* (Longman & Callow, 1987) on roots of maize and cress, respectively, was abolished by enzymic removal of fucosyl residues as well as by treatments of roots with fucose binding lectin. Recently, a fucose binding protein on the surface of *P. cinnamomi* zoospores has been demonstrated (Kelleher *et. al.,* 1991) and supports an earlier report that trypsinisation of *P. aphanidermatum* zoospores abolished encystment by removing a fucosyl receptor on the zoospore surface (Longman & Callow, 1987).

However, in contrast to these reports, the experiments here with *P. aphanidermatum* revealed no evidence that saccharide residues specifically mediate localised accumulation and encystment of zoospores- at least on wheat roots. These roots initially attracted zoospores to both the root cap and the region of young root hairs. However, by 10 min the zoospores had vacated the root cap region, without encysting, and swam to the zone of young root hairs where they were rapidly induced to encyst (Figures 3.7-3.9). This series of events, confirming the work of Jones *et al.* (1991), was also observed with alginate-coated roots even although any saccharide residues, of the root slime,

would be expected to be trapped by the alginate layer and, therefore, inaccessible to zoospores (Figures 3.1-3.4). Attempts were made to demonstrate that sugar residues were not protruding from alginate-coated roots. In this case gelled roots were treated with FITC-labelled lectins, specific for fucosyl and N-acetyl-D-glucosaminosyl (NAG) residues which are present on wheat roots; Moody *et al.,* (1988), as probes for exposed saccharide residues. However, these results were not clear-cut because even root-free calcium alginate beads showed fluorescence, when treated with these lectins, indicating that the lectins were trapped in the gel. A similar uniform pattern of fluorescence along the length of alginate coated roots was observed with non-gelled wheat roots and in both cases was abolished by pre-incubation of the lectin with its hapten sugar. This suggests some degree of binding specificity but it was not localised to specific root regions. Thus, although fucose is reported to elicit encystment of both *P. cinnamomi* and *P. aphanidermatum* (Hinch & Clarke, 1980; Longman & Callow, 1987) whereas chitin, which is composed of NAG residues, elicits encystment of *P. aphanidermatum* (Table 3.10; Mitchell & Deacon, 1986b), yet in neither case could the observed distribution of these glycosyl residues account for localised encystment of *P. aphanidermatum* in the zone of young root hairs. This contrasts with the work of Hinch & Clarke (1980) and Longman & Callow (1987) who found coincidental binding of fucose-specific lectin (FBP) and zoospores in the zone of root elongation of cress. Treatment of gelled and non-gelled wheat roots with exofucosidase or FBP, to remove or block exposed fucosyl residues, had no effect on the number of zoospores encysting on roots (Table 3.4). Similarly, treatment with the lectins Con A, WGA and *P.limensis* which should bind exposed residues of glucose/mannose, NAG and N-acetyl-D-galactosamine, respectively, had no effect on the amount or distribution of zoospore encystment (Table 3.4). In this respect glucose, mannose, and NAG are known to be present on wheat roots (Moody *et al.,* 1988). The work here confirm the findings of Longman & Callow (1987) that trypsinisation of roots to remove protein-containing components, had no effect on encystment. In addition, no treatment reduced accumulation of zoospores in the immediate vicinity of non-gelled roots, indicative of chemotaxis, and in all cases similar numbers accumulated around roots treated with lectins or enzymes compared to phosphate buffer controls (Table 3.5).

From all the work here, there is no reason to believe that the localisation of encystment of *P. aphanidermatum* on wheat roots is a response to the localised distribution of specific glycosyl residues on the root surface. This is

in stark contrast to the claims (Hinch & Clarke, 1980; Longman & Callow, 1987) that localised encystment of *P. cinnamomi* on maize or of *P. aphanidermatum* on cress, respectively, is mediated by specific glycosyl residues. Despite this conclusion there was evidence that surface mediated events, in general, were involved in encystment because this was reduced by treatment of non-gelled roots (Table 3.1) with India ink, which is commonly used as a negative stain for bacterial polysaccharides, and methylene blue which stains root mucilage (Oades, 1978). These treatments had no effect on encystment on alginate- coated roots (Table 3.4).

Mitchell & Deacon (1986a) similarly reported that methylene blue abolished encystment of *P. aphanidermatum* and *P. graminicola* on roots of maize, tomato and wheat and suggested that this was due to staining of the mucilage. In support of this, zoospores on non-gelled roots typically encysted at the base of root hairs which are known to have thick deposits of mucilage (Foster, 1986). In, addition pre-incubation of zoospores in a nutrient solution comprising peptone, L-glutamate and the uronates, galacturonic acid and glucuronic acid (both of which are present in wheat root slime (Moody *et al.*, 1988) reduced encystment on roots (Table 3.1). It is unlikely that the amino acid-rich peptone and glutamate *per se* prevent encystment because zoospores preincubated in amino acids still encysted on subsequent addition of a higher concentration of glutamate (Chapter 5). Therefore, the uronates exerted their effects alone or cooperated in conjunction with the amino acids to interfere with zoospore receptors which detect root compounds mediating encystment.

The role that uronic acids play in encystment and germination, and therefore potentially in surface recognition, was further analysed (Tables 3.8, 3.9). Each of the tested uronic acids was capable of eliciting encystment of the *Pythium* spp. (Table 3.8) but differential responses were observed with regard to germination of cysts (Table 3.9). For each of the three *Pythium* spp., sodium alginate, which is composed of guluronic acid and mannuronic acid (Haug, 1974), significantly enhanced both encystment and germination over a wide range of concentrations, with little or no cell damage when compared to phosphate buffer controls. Glucuronic acid elicited encystment of spores with minor levels of cell damage but had little effect on germination except that it inhibited *P. aphanidermatum* at the highest tested concentration ($25 mgml^{-1}$). In contrast D-galacturonic acid, which also significantly enhanced encystment, elicited relatively high levels of cell lysis, with increasing concentration, and also inhibited germination of all three fungi. Similarly, polygalacturonic acid

enhanced both encystment and lysis (Table 3.8), but at the highest tested concentrations inhibited germination of all three *Pythium* spp. (Table 3.9). However, polygalacturonate at intermediate concentrations enhanced germination of *P. aphanidermatum* and *P. catenulatum* spores.

These results partly confirm those of other workers. Jones *et al.* (1991) demonstrated that sodium alginate and polygalacturonic acid (albeit at a higher concentration) caused encystment of spores of *P. aphanidermatum* whereas D-glucuronic and D-galacturonic acid had no effect at the highest concentration tested ($485\mu gml^{-1}$). Similarly, Grant *et al.* (1985) demonstrated that polygalacturonic acid, sodium alginate, glucuronic acid and galacturonic acid elicited encystment of *P. palmivora* spores but, in contrast, found that increasing concentrations of sodium alginate ($>100\mu gml^{-1}$) reduced the number of germlings whereas galacturonic acid stimulated germination. In addition, Grant *et al.* (1985) have demonstrated that pectin elicits both encystment and germination of *P. palmivora* and have identified the component galacturonic acid residues as being essential for complete differentiation of the zoospore.

These effects of uronyl residues could be relevant to encystment on root surfaces, because uronyl residues are a major constituent of root surface mucilage of many plants (Moody *et al.,* 1988). Although few detailed analyses have yet been made, the evidence suggests that the root mucilage of graminaceous plants has a relatively low uronate content, and much of this is composed of glucuronic acid rather than galacturonic acid. In contrast, the root slime of dicotyledons has a high content of galacturonic acid. Consistent with this, the amorphous contents of the middle lamella of grasses have a relatively low uronate content- especially low in galacturonic acid- whereas the pectin-like middle lamella of dicotyledons is rich in galacturonic acid (Moody *et al.,* 1988). Foster (1981) reports that much of the root slime- at least in the young regions of roots- is derived by breakdown and extrusion of the components of the primary walls of the root epidermis.

The role that uronic acids may play in surface- recognition could explain the observation that methylene blue was able to block encystment on non-coated roots (Table 3.1). The binding of fungal spores to roots is thought to be essentially ionic, involving calcium bridging between polyanionic groups on fungal surfaces and polyuronic acid residues on roots (Northcote & Gould, 1989). In addition, adhesion of zoospore cysts is also dependent on divalent cations (Gubler *et al.,* 1989), suggesting an ionic component in binding to roots and other surfaces. Methylene blue is a basic dye (Gurr, 1960) and could,

therefore, prevent encystment if this is induced ionically by binding to a polyanionic surface, suggested by Jones *et al.,* 1991).

Other work on the induction of encystment of zoospores is relevant to this discussion. In particular, Longman & Callow (1987) found that pectinase treatment of cress roots increased encystment of *P. aphanidermatum* spores. In addition, fractionation studies involving cress root mucilage demonstrated that a fraction relatively low in uronic acid residues, but containing 5% fucose, was more effective in inducing encystment of *P. aphanidermatum in vitro* than was a uronate-rich fraction (Estrada-Garcia *et al.,* 1990b).

The use of monoclonal antibodies has indicated the presence of specific encystment mediating domains on zoospore surfaces. Of several monoclonal antibodies raised against *P. cinnamomi*, only one, which bound specifically to the surface of both flagella, induced zoospore encystment when applied at relatively low concentrations (Hardham, 1989). This monoclonal was also found to induce encystment of zoospores of a wide range of *Phytophthora* and *Pythium* spp., including *Pythium drechsleri* (Hardham, 1989). In similar work, Estrada-Garcia *et al.* (1989) raised monoclonal antibodies to *P. aphanidermatum* and found that one monoclonal antibody, which bound to the surface of both flagella as well as to the surface of the zoospore soma, specifically induced encystment. This monoclonal was species-specific and did not bind to other *Pythium* spp. It is difficult at present to reconcile this finding and that of Hardham & Suzaki (1986). But at least in both cases the evidence points to a role of flagellar surface components in the encystment process. This is consistent with the fact that zoospores orientate precisely on roots prior to encystment, such that the flagella are positioned next to the host surface and the germ-tube emerges towards the root, from a pre-determined point near the point of flagellar insertion (Mitchell & Deacon, 1986c; Paktitis *et al.*, 1986; Hardham & Gubler, 1990).

Mitchell & Deacon (1986b) have demonstrated that surface recognition events mediate selective accumulation of zoospores on insoluble substrata such as cellulose and chitin, and that this could be blocked by prior treatment of the substrata with alcian blue and WGA respectively. Zoospores of the three *Pythium* spp. used here also showed differential responses to cellulose and chitin (Table 3.10). Motile spores of *P. aphanidermatum* accumulated on neither cellulose nor chitin but encysted on chitin (confirming the work of Mitchell & Deacon, 1986b) whereas *P. dissotocum* accumulated and encysted on

both cellulose and chitin, and *P. catenulatum* accumulated on both cellulose and chitin but encysted only on cellulose. In accordance with the work of Mitchell & Deacon (1986b), zoospores did not show chemotaxis towards these insoluble materials but were retained in their vicinity after random contact, due to a reduction in the rate of swimming (negative orthokinesis) and an increase in the frequency of turning (positive klinokinesis).

The work in this chapter, on induction of encystment by purified samples of polysaccharides of diverse origin has shown for the first time, a high level of species-differential encystment by defined, compounds, supporting the findings of Mitchell & Deacon (1986b) for effects of chitin- and cellulose-containing materials. It must be emphasised that none of these polysaccharide samples is necessarily related to root surface components. Nevertheless, their differential effects demonstrate that the zoospores of different *Pythium* spp. probably have different types of surface receptors that are involved in encystment. Such differential effects may provide the key to elucidating the role of zoospore receptors in encystment *in vivo*. For example, Goldberg *et al.* (1989) found that *P. catenulatum* and *P. dissotocum* encysted differentially on roots of cotton and also showed differential encystment on various regions of seedling roots of these plants. Mitchell & Deacon (1986a) had earlier demonstrated that *P. aphanidermatum* and *P. ultimum* showed non-host specific encystment on roots of dicotyledonous and graminaceous plants whereas *P. graminicola* and *P. arrhenomanes* showed a high degree of graminaceous-specific encystment. The results here from the use of pure samples of polysaccharides will need to be related to *in vivo* in further work, and the active components of these polysaccharides will also need to be determined. Nevertheless, it is interesting that the responses of the zoospores to the mixed glucan correlated with the encystment responses of the *Pythium* spp. to cellulosic substrata (Table 3.10, 3.11). Also, one of the *Pythium* isolates (*P. dissotocum*) was induced to encyst by non-fucosylated xyloglucan whereas another isolate (*P. catenulatum*) encysted only in response to the fucosylated xyloglucan. Notable also is the fact that many of the component sugars did not alone induce encystment (see Chapter 5). This strongly suggests that encystment is caused by binding of residues at more than one site on the spore and by polymers that effect a cross-linking and perhaps a constraint of the zoospore surface receptors.

Up to this point, the discussion has focused on the roles of surface-receptors alone in encystment. But the evidence from the use of alginate-coated

roots strongly suggests that surface recognition alone is not responsible for encystment on roots. If it were so, then zoospores that were originally attracted to the root cap region (and later vacated this region) should have encysted there, because spores encysted in the root-hair zone which was encased by the same alginate layer. The essential difference is that the root cap region was only transitorily attractive to zoospores- after milling there and making frequent contact with the alginate surface they swam away- whereas the region behind the root cap (especially the zone of young root hairs) continued to attract zoospores for many minutes (see also Jones *et al.,* 1991). The implication is that encystment required both the presence of a "conducive" surface and sustained chemoattraction of the zoospores. Such an apparent synergistic effect was described by Royle & Thomas (1973) for encystment around stomata. In this case zoospores of *Pseudoperonospora humuli* were able to locate open stomata on both vine leaves and on vine leaf replicates, therefore suggesting a response to topographical signals (thigmotaxis). However responses to leaf replicas were weaker than those on true leaves which led Royle & Thomas (1973) to suggest that chemical signals curtail the motility of spores and thus act synergistically with contact stimuli. Synergistic effects may be a general phenomenon, but poorly modelled by *in vitro* studies to date, where perhaps abnormally high levels of encystment-inducing compounds have been used.

4. CHEMOTAXIS AND MOTILITY CHARACTERISTICS OF FUNGAL ZOOSPORES

4.1 Chemotaxis

4.1.1. Taxis to amino acids, sugars and ethanol

Zoospores of various fungi have been reported to show chemotaxis toward amino acids (Royle & Hickman, 1964b; Khew & Zentmyer, 1973; Jones *et al.,* 1991), sugars (Orpin & Bountiff, 1978; Jones *et al.,* 1991) and alcohols or other volatile metabolites (Allen & Newhook, 1973; Cameron & Carlile, 1978). However, there have been few comparative studies (*e.g.* Halsall, 1976) and none involving *Pythium* spp. so the aim of this experiment was to examine the effects of amino acids, sugars and ethanol on chemotaxis of zoospores of three *Pythium* spp.

Using the methods described in Section 2.3.1, zoospores of *P. aphanidermatum*, *P. catenulatum* and *P. dissotocum* all displayed chemotaxis to microcapillaries containing L-alanine, L-asparagine, L-aspartic acid, L-glutamine and L-glutamic acid but were indifferent to other L-amino acids (Table 4.1). Overall, the strongest responses were to L-glutamic acid and L-aspartic acid, and when different concentrations were tested for *P. aphanidermatum* these compounds elicited attraction at threshold source concentrations of 1mM and 4mM respectively (Table 4.2). D-amino acids and ethanol did not induce taxis of any of the *Pythium* spp. (Tables 4.3, 4.4).

The responses to sugars, tested at both 5 and 10mM source concentrations, were more variable (Table 4.5) than towards amino acids. Mannose attracted all three *Pythium* spp; glucose attracted only *P. catenulatum* and *P. dissotocum*; fructose attracted only *P. aphanidermatum* and *P. dissotocum*; sucrose and maltose attracted *P. aphanidermatum* alone and fucose attracted only *P. dissotocum*.

4.1.2. Effects of pre-incubation of zoospores in the presence of amino acids on chemotaxis

Both bacterial (Mesibov & Adler, 1972) and zoosporic (Orpin & Bountiff, 1978) chemotactic receptors have been characterised by the method of Adler (1969), whereby saturating background levels of specific compounds should

Table 4.1 Numbers of zoospores of three *Pythium* spp in test capillaries containing L-amino acids and matched buffer controls 20 min after they were inserted into zoospore suspensions†

Test attractant[+] (10mM)	*P. aphanidermatum*		*P. catenulatum*		*P. dissotocum*	
	Test	Control	Test	Control	Test	Control
Leu	9 ± 2.4	8 ± 2.0	9 ± 1.3	10 ± 1.7	7 ± 1.6	8 ± 1.0
Trp	6 ± 1.7	5 ± 1.1	9 ± 1.7	9 ± 1.8	6 ± 1.8	6 ± 1.3
His	6 ± 1.0	6 ± 0.9	9 ± 1.4	7 ± 1.3	7 ± 1.7	7 ± 1.5
Met	3 ± 1.1	3 ± 0.5	8 ± 1.8	7 ± 1.8	8 ± 1.8	6 ± 1.7
Asn	16 ± 2.7**	2 ± 0.4	42 ± 5.8**	10 ± 1.6	41 ± 8.2**	7 ± 1.9
Phe	2 ± 0.6	3 ± 1.3	8 ± 0.8	8 ± 0.8	10 ± 1.5	9 ± 2.1
Arg	7 ± 1.6	5 ± 1.1	10 ± 1.7	9 ± 0.9	4 ± 1.1	5 ± 1.1
Ser	5 ± 1.6	4 ± 0.9	7 ± 1.7	7 ± 1.1	7 ± 1.9	5 ± 1.2
Pro	7 ± 1.4	7 ± 1.2	8 ± 1.2	6 ± 0.9	11 ± 2.1	9 ± 1.6
Ile	7 ± 2.0	7 ± 1.2	5 ± 0.9	5 ± 1.0	6 ± 0.9	5 ± 1.5
OHPro	8 ± 2.3	7 ± 1.6	9 ± 1.8	8 ± 1.2	7 ± 1.5	9 ± 1.3
Ala	26 ± 3.7**	8 ± 1.8	28 ± 9.1*	10 ± 3.9	37 ± 6.2**	6 ± 1.4
Thr	5 ± 1.4	6 ± 1.1	6 ± 1.7	6 ± 1.8	7 ± 2.0	8 ± 1.4
Val	5 ± 1.4	4 ± 1.0	6 ± 1.0	6 ± 1.1	8 ± 1.8	6 ± 1.5
Lys	2 ± 0.7	3 ± 0.9	8 ± 1.6	7 ± 1.5	4 ± 1.3	3 ± 0.9
Asp	43 ± 4.1**	9 ± 1.1	52 ± 4.7**	10 ± 1.3	57 ± 4.5**	7 ± 1.2
Gln	23 ± 2.6**	7 ± 1.4	46 ± 7.3**	7 ± 1.4	46 ± 5.8**	8 ± 2.1
Glu	49 ± 7.1**	11 ± 1.8	43 ± 4.9**	11 ± 1.8	60 ± 5.6**	8 ± 1.3
Gly[++]	5 ± 1.3	4 ± 1.3	7 ± 1.1	7 ± 1.4	8 ± 2.4	7 ± 1.4

† Means ± s.e. for 9 replicates; significance of difference between test and control capillaries by paired-samples t-tests: *,0.02; **, 0.001.

[+] Leu, leucine; Trp, tryptophan; His, histidine; Met, methionine; Asn, asparagine; Phe, phenylalanine; Arg, arginine; Ser, serine; Pro, proline; Ile, isoleucine; OHPro, hydroxyproline; Ala, alanine; Thr, threonine; Val, valine; Lys, Lysine; Asp, aspartic acid; Gln, glutamine; Glu, glutamic acid, Gly, glycine.

[++] Glycine is included but has no L or D-isomers.

Table 4.2 Numbers of zoospores of *Pythium aphanidermatum* in test capillaries containing different concentrations of L-glutamate or L-aspartate and capillaries containing phosphate buffer, 20 min after they were inserted into a zoospore suspension; means ± s.e. for 12 replicates.

Test treatment (mM)	Test L-glutamate	Control (Buffer)	Test L-aspartate	Control (Buffer)
10	75 ± 4.4***	8 ± 0.8	68 ± 8.9***	12 ± 1.8
9	48 ± 3.9***	12 ± 2.0	49 ± 7.0***	11 ± 2.0
8	32 ± 4.8***	14 ± 2.1	64 ± 16.2**	14 ± 3.2
7	46 ± 10.2**	9 ± 2.0	53 ± 10.5***	12 ± 4.1
6	32 ± 6.3**	9 ± 1.7	26 ± 9.2	8 ± 2.5
5	11 ± 1.3**	5 ± 0.6	22 ± 7.6	11 ± 3.1
4	31 ± 5.9**	10 ± 1.2	15 ± 3.4*	9 ± 0.9
3	47 ± 7.8**	16 ± 2.1	6 ± 1.0	3 ± 0.7
2	30 ± 5.0**	12 ± 2.2	18 ± 5.6	9 ± 2.2
1	12 ± 1.4*	4 ± 0.8	9 ± 1.1	7 ± 0.7
0.9	5 ± 0.8	3 ± 0.8	4 ± 1.0	3 ± 0.7
0.7	4 ± 0.8	3 ± 0.4	3 ± 0.8	3 ± 0.9
0.5	3 ± 0.6	2 ± 0.4	4 ± 1.0	5 ± 0.6

*,**,*** Denotes significant difference between test and control data at P= 0.05, 0.01 and 0.001 respectively, based on paired-samples t-tests.

Table 4.3 Numbers of zoospores of three *Pythium* spp in test capillaries containing D-amino acids and matched buffer controls 20 min after they were inserted into zoospore suspensions†

Test attractant+	*P. aphanidermatum*		*P. catenulatum*		*P.dissotocum*	
(10mM)	Test	Control	Test	Control	Test	Control
Leu	10 ± 1.4	11 ± 1.2	7 ± 1.3	5 ± 1.1	4 ± 1.3	5 ± 1.0
Trp	7 ± 2.2	6 ± 1.1	7 ± 1.6	7 ± 1.4	8 ± 1.5	7 ± 1.4
His	11 ± 1.9	12 ± 1.5	10 ± 1.3	8 ± 1.0	11 ± 1.5	9 ± 1.4
Met	6 ± 1.6	6 ± 1.0	6 ± 1.5	5 ± 1.0	10 ± 1.3	9 ± 1.4
Asn	8 ± 1.5	6 ± 1.0	8 ± 1.8	7 ± 1.6	7 ± 1.1	6 ± 1.1
Phe	10 ± 1.5	9 ± 1.3	7 ± 1.4	7 ± 1.2	8 ± 0.7	9 ± 1.8
Arg	8 ± 1.9	9 ± 1.6	12 ± 0.9	10 ± 0.8	7 ± 1.5	6 ± 1.2
Ser	7 ± 1.1	6 ± 0.9	6 ± 0.9	5 ± 1.2	8 ± 1.6	7 ± 1.2
Pro	6 ± 1.6	7 ± 1.4	6 ± 1.6	5 ± 1.4	8 ± 1.6	7 ± 1.2
Ile	8 ± 1.4	9 ± 1.3	7 ± 2.0	5 ± 1.2	8 ± 1.8	5 ± 1.4
OHPro	7 ± 1.0	8 ± 1.5	7 ± 1.1	7 ± 1.3	9 ± 1.2	9 ± 1.3
Ala	8 ± 1.2	7 ± 1.2	14 ± 1.4	11 ± 0.8	12 ± 1.4	9 ± 0.8
Thr	9 ± 1.6	10 ± 0.9	8 ± 1.5	5 ± 1.2	8 ± 1.7	6 ± 1.3
Val	6 ± 1.8	7 ± 0.9	6 ± 1.6	6 ± 1.1	8 ± 1.2	9 ± 1.4
Lys	5 ± 1.1	5 ± 1.0	6 ± 1.4	5 ± 1.2	9 ± 2.0	7 ± 1.6
Asp	7 ± 2.1	7 ± 1.0	9 ± 1.7	8 ± 1.1	8 ± 1.7	9 ± 1.3
Gln	8 ± 0.9	6 ± 1.3	5 ± 0.9	6 ± 1.2	8 ± 1.9	7 ± 1.4
Glu	14 ± 2.0	13 ± 1.5	6 ± 1.6	4 ± 1.4	10 ± 1.8	11 ± 1.4
Gly++	11 ± 2.2	10 ± 1.4	11 ± 1.9	12 ± 1.5	13 ± 2.1	13 ± 1.6

† Means ± s.e. for 9 replicates; no significant difference was found between amino acid and matching buffer controls in any instance.

+ Leu, leucine; Trp, tryptophan; His, histidine; Met, methionine; Asn, asparagine; Phe, phenylalanine; Arg, arginine; Ser, serine; Pro, proline; Ile, isoleucine; OHPro, hydroxyproline; Ala, alanine; Thr, threonine; Val, valine; Lys, Lysine; Asp, aspartic acid; Gln, glutamine; Glu, glutamic acid, Gly, glycine.

++ Glycine is included but has no L or D-isomers.

Table 4.4 Numbers of zoospores of three *Pythium* spp in test capillaries containing ethanol (25mM) and control capillaries containing phosphate buffer, 20 min after they were inserted into a zoospore suspension; means ± s.e. for 9 replicates.

	Test	Control
P. aphanidermatum	8.0 ± 1.1	5.9 ± 1.4
P. catenulatum	10.2 ± 1.5	8.3 ± 1.4
P. dissotocum	6.3 ± 1.1	7.8 ± 1.4

Table 4.5 Numbers of zoospores of three *Pythium* spp in test capillaries containing sugars and control capillaries containing phosphate buffer, 20 min after they were inserted into zoospore suspensions†

Test attractant+		P. aphanidermatum		P. catenulatum		P. dissotocum	
(mM)		Test	Control	Test	Control	Test	Control
D (+) Mann	(5)	14 ± 1.8****	5 ± 1.2	34 ± 4.1****	7 ± 1.0	29 ± 3.8****	5 ± 1.5
	(10)	39 ± 6.8****	5 ± 1.3	9 ± 1.4*	4 ± 1.0	8 ± 0.6*	4± 0.5
L (+) Ara	(5)	3 ± 1.0	3 ± 0.9	6 ± 1.2	4 ± 1.1	6 ± 1.1	6 ± 0.9
	(10)	7 ± 1.4	5 ± 1.9	6 ± 1.2	7 ± 1.3	5 ± 0.7	4 ± 1.0
D (-) Ara	(5)	5 ± 1.2	4 ± 1.6	7 ± 1.0	7 ± 1.1	2 ± 0.6	2 ± 0.6
	(10)	6 ± 1.1	6 ± 1.8	5 ± 1.0	5 ± 0.9	10 ± 1.4	11 ± 1.2
D (+) Xyl	(5)	5 ± 0.8	4 ± 1.2	7 ± 1.2	8 ± 1.8	6 ± 0.9	5 ± 1.0
	(10)	11 ± 2.1	9 ± 1.6	7 ± 0.8	6 ± 1.1	8 ± 0.6	6 ± 0.9
L (-) Fuc	(5)	7 ± 1.5	6 ± 1.6	7 ± 1.2	6 ± 1.3	15 ± 2.0****	5 ± 1.3
	(10)	6 ± 1.2	4 ± 1.1	8 ± 1.3	5 ± 1.2	6 ± 0.9	4 ± 0.7
D (+) Gal	(5)	3 ± 1.1	3 ± 0.8	9 ± 1.7	7 ± 0.8	6 ± 0.8	6 ± 1.3
	(10)	10 ± 1.0	8 ± 1.6	8 ± 1.6	8 ± 1.0	11 ± 1.9	10 ± 1.1
D (+) Glc	(5)	9 ± 1.3	8 ± 1.4	8 ± 1.1	7 ± 1.7	10 ± 1.3***	5 ± 1.0
	(10)	9 ± 1.3	8 ± 2.2	8 ± 0.9**	5 ± 0.8	17 ± 2.0****	8 ± 1.2
Suc	(5)	13 ± 2.5***	5 ± 1.3	11 ± 1.8	10 ± 2.4	6 ± 1.0	5 ± 1.4
	(10)	30 ± 5.2****	7 ± 0.9	6 ± 1.1	5 ± 1.3	6 ± 1.1	5 ± 1.0
Frc	(5)	12 ± 2.0***	5 ± 0.7	6 ± 0.7	6 ± 1.1	18 ± 2.9***	4 ± 1.2
	(10)	34 ± 9.4****	5 ± 1.0	10 ± 1.4	10 ± 1.1	20 ± 4.0****	7 ± 1.1
Mal	(5)	12 ± 1.9***	6 ± 1.3	8 ± 1.0	7 ± 1.3	6 ± 1.7	5 ± 1.6
	(10)	21 ± 2.2****	6 ± 1.2	8 ± 1.7	7 ± 1.6	10 ± 1.4	8 ± 1.1

† Means ± s.e. for 9 replicates; significance of difference from matched buffer controls by paired-samples t-tests: *, 0.05; **, 0.02; ***, 0.01; ****, 0.001.

+ Man, mannose; Ara, arabinose; Xyl, xylose; Fuc, fucose; Gal, galactose; Glc, glucose; Suc, sucrose; Frc, fructose; Mal, maltose.

negate taxis to an attractant that shares the same chemoreceptor.

The same approach was used here in an attempt to block or reduce chemotaxis of zoospores to the attractant L-glutamic acid and, thereby to characterise the receptor specificity for the different *Pythium* spp. Various background amino acids were added to zoospore suspensions 90 sec before capillaries containing both L-glutamate and the background compound were inserted into them.

The results (Tables 4.6-4.8) demonstrate that the presence of background levels of L-alanine, L-glutamine and L-glutamic acid (which was used at a lower concentration to prevent encystment) significantly blocked or reduced chemotaxis of all three *Pythium* spp. towards L-glutamic acid (7mM). These three background compounds had previously been found to be chemoattractants of all three fungi. In addition, the chemoattractant L-asparagine and the non-chemoattractants L-threonine, L-tryptophan and L-methionine (Section 4.1.1.) negated or reduced taxis of *P. aphanidermatum* spores towards L-glutamic acid whereas L-histidine and glycine reduced or blocked chemotaxis of both *P. aphanidermatum* (Table 4.6) and *P. catenulatum* (Table 4.7). Similarly, the effects of backgrounds of D-amino acids were tested (Tables 4.9-4.11). In this case the D-forms of alanine, asparagine, glutamine, threonine, histidine, glycine and tryptophan blocked or reduced chemotaxis of *P. aphanidermatum* to L-glutamic acid in capillaries but chemotaxis of *P. catenulatum* and *P. dissotocum* were unaffected by background concentrations of any D-amino acids.

In tests with *P. aphanidermatum* only (Table 4.12), the effect of a background of L-glutamic acid (2mM) on attraction to other amino acids was assessed. The background was found to block chemotaxis towards L-glutamine, L-alanine and L-asparagine but had no significant effect on chemotaxis towards L-aspartic acid.

4.1.3. Observations on the behaviour of Pythium aphanidermatum in capillaries containing L-glutamic acid

Microcapillaries containing a range of concentrations of L-glutamate were placed in zoospore suspensions and the behaviour of the spores were recorded on video tapes. In all cases the zoospores enter the capillaries and then swim progressively further into them with time, before encysting. This distance,

Table 4.6 Numbers of zoospores of *Pythium aphanidermatum* in test capillaries (7mM L-glutamate) and control capillaries (phosphate buffer), after 20 min, in the presence or absence of various L-amino acids in the zoospore bathing medium; means ± s.e. for 9 replicates (15 for asparagine background)

Background L-amino acid+	Background compound present†		Background compound absent		Significance of difference of test capillaries in presence/ absence of background
	Test capillary	Control capillary	Test capillary	Control capillary	
Leu	77 ± 9.9***	7 ± 1.8	89 ± 8.0***	9 ± 1.4	n.s.d.
Trp	19 ± 1.3**	7 ± 1.1	79 ± 8.5***	5 ± 1.2	0.001
His	6 ± 1.0	7 ± 1.1	55 ± 6.0***	4 ± 1.0	0.001
Met	25 ± 2.5***	2 ± 0.5	53 ± 8.4***	4 ± 1.8	0.02
Asn	2 ± 0.7	1 ± 0.4	36 ± 6.8***	2 ± 0.3	0.001
Phe	39 ± 3.2***	3 ± 0.9	51 ± 7.9***	3 ± 0.6	n.s.d.
Arg	56 ± 6.4***	6 ± 1.5	62 ± 6.0***	5 ± 1.5	n.s.d.
Ser	59 ± 8.2***	9 ± 2.1	66 ± 10.4***	7 ± 1.7	n.s.d.
Pro	64 ± 8.8***	10 ± 1.6	80 ± 5.4***	11 ± 2.4	n.s.d.
Ile	76 ± 10.6***	7 ± 1.6	69 ± 11.4***	7 ± 2.2	n.s.d.
OHPro	55 ± 8.8***	9 ± 1.2	62 ± 8.2***	6 ± 1.5	n.s.d.
Ala	6 ± 0.8	4 ± 0.5	37 ± 5.4***	5 ± 1.6	0.001
Thr	17 ± 2.3*	7 ± 1.5	68 ± 7.2***	10 ± 1.5	0.001
Val	36 ± 3.9***	4 ± 1.0	41 ± 5.3***	6 ± 1.6	n.s.d.
Lys	26 ± 2.9***	2 ± 0.5	40 ± 4.2***	3 ± 0.7	n.s.d.
Asp	34 ± 5.7***	9 ± 1.1	50 ± 6.7***	9 ± 1.0	n.s.d.
Gln	8 ± 1.7	7 ± 2.3	43 ± 5.0***	7 ± 1.4	0.001
Glu	12 ± 2.5	9 ± 0.9	33 ± 4.1***	6 ± 1.1	0.001
Gly	4 ± 1.1	3 ± 0.5	49 ± 9.4***	4 ± 1.4	0.001

Significance of difference of test capillary from matched buffer control by paired-samples t-tests: *,0.05; **, 0.02; ***, 0.001.

† Background compound was also present in the test and control capillaries.

+ See Table 4.3; all background compounds were used at 7mM concentration, except glutamate (2mM) and aspartate (3mM) and in these tests 7mM and 3mM L-glutamate, respectively, was present in the test capillary.

Table 4.7 Numbers of zoospores of *Pythium catenulatum* in test capillaries (7mM L-glutamate) and control capillaries (phosphate buffer), after 20 min, in the presence or absence of various L-amino acids in the zoospore bathing medium; means ± s.e. for 9 replicates

Background L-amino acid+	Background compound present†		Background compound absent		Significance of difference of test capillaries in presence/ absence of background
	Test capillary	Control capillary	Test capillary	Control capillary	
Leu	78 ± 11.8***	6 ± 1.1	69 ± 8.7***	9 ± 1.5	n.s.d.
Trp	41 ± 7.9**	5 ± 1.6	27 ± 5.0**	6 ± 1.6	n.s.d.
His	16 ± 2.9**	6 ± 1.5	82 ± 10.5***	8 ± 1.7	0.001
Met	73 ± 11.3***	9 ± 1.4	81 ± 6.3***	17 ± 1.3	n.s.d.
Asn	75 ± 11.1***	8 ± 1.4	62 ± 8.3***	10 ± 0.9	n.s.d.
Phe	82 ± 10.2***	10 ± 0.8	74 ± 9.6***	13 ± 1.2	n.s.d.
Arg	41 ± 6.2***	5 ± 1.5	50 ± 6.5***	6 ± 1.3	n.s.d.
Ser	68 ± 12.8***	9 ± 1.4	76 ± 8.1***	6 ± 1.9	n.s.d.
Pro	62 ± 8.7***	6 ± 1.6	52 ± 5.2***	9 ± 1.9	n.s.d.
Ile	74 ± 8.4***	6 ± 1.5	79 ± 6.9***	6 ± 1.6	n.s.d.
OHPro	59 ± 7.3***	7 ± 1.6	76 ± 7.1***	6 ± 1.3	n.s.d.
Ala	25 ± 4.5***	7 ± 1.8	60 ± 11.2***	7 ± 1.4	0.001
Thr	65 ± 9.3***	9 ± 1.2	67 ± 8.1***	7 ± 1.6	n.s.d.
Val	80 ± 7.5***	10 ± 1.4	68 ± 5.3***	8 ± 1.2	n.s.d.
Lys	82 ± 8.0***	10 ± 1.5	77 ± 7.6***	9 ± 1.2	n.s.d.
Asp	42 ± 4.2***	11 ± 1.3	46 ± 3.6***	10 ± 1.1	n.s.d.
Gln	24 ± 3.3**	9 ± 1.4	102 ± 5.1***	10 ± 1.1	0.001
Glu	10 ± 1.8	8 ± 1.0	52 ± 5.2***	10 ± 1.1	0.001
Gly	14 ± 2.9*	8 ± 1.9	88 ± 7.0***	7 ± 1.5	0.001

Significance of difference of test capillary from matched buffer control by paired-samples t-tests: *,0.02; **, 0.01; ***, 0.001.

† Background compound was also present in the test and control capillaries.

+ See Table 4.3; all background compounds were used at 7mM concentration, except glutamate (2mM) and aspartate (3mM) and in these tests 7mM and 3mM L-glutamate, respectively, was present in the test capillary.

Table 4.8 Numbers of zoospores of *Pythium dissotocum* in test capillaries (7mM L-glutamate) and control capillaries (phosphate buffer), after 20 min, in the presence or absence of various L-amino acids in the zoospore bathing medium; means ± s.e. for 9 replicates

Background L-amino acid+	Background compound present†		Background compound absent		Significance of difference of test capillaries in presence/ absence of background
	Test capillary	Control capillary	Test capillary	Control capillary	
Leu	62 ± 8.6***	7 ± 1.9	69 ± 8.4***	7 ± 1.9	n.s.d.
Trp	71 ± 7.5***	7 ± 1.9	66 ± 7.5***	5 ± 1.3	n.s.d.
His	92 ± 9.1***	12 ± 2.1	76 ± 8.1***	12 ± 1.4	n.s.d.
Met	79 ± 7.6***	7 ± 2.3	66 ± 9.4***	7 ± 2.1	n.s.d.
Asn	65 ± 7.9***	9 ± 1.2	70 ± 7.9***	6 ± 1.9	n.s.d.
Phe	69 ± 7.9***	6 ± 1.5	56 ± 6.4***	6 ± 1.3	n.s.d.
Arg	51 ± 5.3***	5 ± 1.4	49 ± 5.4***	6 ± 1.7	n.s.d.
Ser	73 ± 10.2***	8 ± 1.5	81 ± 6.9***	5 ± 1.5	n.s.d.
Pro	85 ± 5.6***	7 ± 1.4	91 ± 7.4***	9 ± 1.9	n.s.d.
Ile	52 ± 4.9***	8 ± 1.6	44 ± 4.4***	7 ± 1.5	n.s.d.
OHPro	56 ± 4.8***	7 ± 1.7	34 ± 4.4***	6 ± 1.8	n.s.d.
Ala	24 ± 4.6**	8 ± 2.4	72 ± 6.6***	5 ± 1.8	0.001
Thr	77 ± 8.5***	9 ± 1.9	66 ± 7.1***	5 ± 1.7	n.s.d.
Val	50 ± 5.3***	5 ± 1.3	65 ± 7.0***	7 ± 1.7	n.s.d.
Lys	47 ± 6.2***	7 ± 1.0	40 ± 2.5***	5 ± 1.7	n.s.d.
Asp	45 ± 5.3***	6 ± 1.0	51 ± 4.9***	10 ± 0.9	n.s.d.
Gln	14 ± 3.0	7 ± 1.2	74 ± 6.0***	5 ± 1.7	0.001
Glu	13 ± 1.1	10 ± 1.0	49 ± 6.3***	13 ± 1.5	0.001
Gly	61 ± 6.1***	6 ± 1.2	64 ± 7.9***	6 ± 1.6	n.s.d.

Significance of difference of test capillary from matched buffer control by paired-samples t-tests: *,0.02; **, 0.01; ***, 0.001.

† Background compound was also present in the test and control capillaries.

+ See Table 4.3; all background compounds were used at 7mM concentration, except glutamate (2mM) and aspartate (3mM) and in these tests 7mM and 3mM L-glutamate, respectively, was present in the test capillary.

Table 4.9 Numbers of zoospores of *Pythium aphanidermatum* in test capillaries (7mM L-glutamate) and control capillaries (phosphate buffer), after 20 min, in the presence or absence of various D-amino acids in the zoospore bathing medium; means ± s.e. for 9 replicates

Background D-amino acid[+]	Background compound present[†]		Background compound absent		Significance of difference of test capillaries in presence/ absence of background
	Test capillary	Control capillary	Test capillary	Control capillary	
Leu	50 ± 6.5****	8 ± 1.3	63 ± 6.8****	8 ± 1.2	n.s.d.
Trp	16 ± 2.8**	7 ± 1.4	67 ± 6.1****	6 ± 1.0	0.001
His	20 ± 2.2*	9 ± 1.3	38 ± 5.7****	12 ± 1.2	0.01
Met	72 ± 5.2****	9 ± 1.4	75 ± 7.5****	9 ± 1.6	n.s.d.
Asn	8 ± 0.9*	5 ± 1.0	51 ± 6.1****	8 ± 1.4	0.001
Phe	91 ± 4.9****	11 ± 1.5	79 ± 4.9****	10 ± 1.3	n.s.d.
Arg	59 ± 6.5****	8 ± 1.2	72 ± 7.7****	10 ± 1.6	n.s.d.
Ser	47 ± 5.1****	8 ± 1.0	41 ± 4.7****	4 ± 1.2	n.s.d.
Pro	36 ± 4.6****	5 ± 1.7	42 ± 4.0****	5 ± 1.1	n.s.d.
Ile	51 ± 5.7****	8 ± 1.0	58 ± 4.1****	9 ± 0.8	n.s.d.
OHPro	38 ± 4.8****	8 ± 1.2	49 ± 6.1****	7 ± 0.9	n.s.d.
Ala	13 ± 1.6**	6 ± 1.2	69 ± 8.0****	9 ± 1.5	0.001
Thr	29 ± 3.4***	8 ± 1.2	74 ± 8.3****	6 ± 1.1	0.001
Val	50 ± 5.3****	7 ± 1.4	38 ± 5.7****	7 ± 1.9	n.s.d.
Lys	32 ± 4.6****	4 ± 0.8	41 ± 5.0****	4 ± 0.9	n.s.d.
Asp	47 ± 4.5****	6 ± 0.9	58 ± 7.0****	6 ± 1.2	n.s.d.
Gln	9 ± 2.4	7 ± 1.6	38 ± 7.6***	7 ± 1.2	0.02
Glu	63 ± 3.8****	12 ± 1.3	58 ± 5.4****	9 ± 1.5	n.s.d.
Gly	19 ± 2.2***	10 ± 1.6	31 ± 4.7***	10 ± 1.4	0.05

Significance of difference of test capillary from matched buffer control by paired-samples t-tests: *,0.05; **, 0.02; ***, 0.01; ****, 0.001.

† Background compound was also present in the test and control capillaries.

+ See Table 4.3.

Table 4.10 Numbers of zoospores of *Pythium catenulatum* in test capillaries (7mM L-glutamate) and control capillaries (phosphate buffer), after 20 min, in the presence or absence of various D-amino acids in the zoospore bathing medium; means ± s.e. for 9 replicates

Background D-amino acid[+]	Background compound present[†]		Background compound absent		Significance of difference of test capillaries in presence/ absence of background
	Test capillary	Control capillary	Test capillary	Control capillary	
Leu	68 ± 7.2**	8 ± 1.5	74 ± 6.0**	7 ± 1.4	n.s.d.
Trp	65 ± 5.8**	7 ± 1.5	85 ± 5.3**	7 ± 1.3	n.s.d.
His	46 ± 7.2*	11 ± 1.2	38 ± 4.6*	7 ± 1.5	n.s.d.
Met	69 ± 7.4**	6 ± 1.0	76 ± 4.7**	7 ± 1.1	n.s.d.
Asn	77 ± 5.6**	9 ± 1.2	91 ± 5.7**	9 ± 1.4	n.s.d.
Phe	62 ± 6.6**	3 ± 1.5	52 ± 6.0**	4 ± 1.0	n.s.d.
Arg	33 ± 4.1**	12 ± 1.4	31 ± 2.8**	9 ± 1.3	n.s.d.
Ser	58 ± 7.3**	8 ± 1.3	52 ± 6.8**	5 ± 1.2	n.s.d.
Pro	44 ± 4.8**	5 ± 1.0	48 ± 6.7**	4 ± 0.9	n.s.d.
Ile	51 ± 8.0**	5 ± 1.2	45 ± 8.3**	5 ± 1.2	n.s.d.
OHPro	80 ± 6.3**	6 ± 1.1	67 ± 6.1**	7 ± 0.9	n.s.d.
Ala	46 ± 6.6*	11 ± 1.8	54 ± 5.1**	11 ± 1.7	n.s.d.
Thr	67 ± 5.1**	7 ± 1.6	59 ± 5.3**	8 ± 1.7	n.s.d.
Val	69 ± 10.0**	3 ± 0.9	46 ± 6.2**	6 ± 1.3	n.s.d.
Lys	55 ± 8.0**	5 ± 1.4	37 ± 4.8**	7 ± 1.4	n.s.d.
Asp	50 ± 6.1**	10 ± 0.9	43 ± 6.7**	9 ± 1.6	n.s.d.
Gln	49 ± 7.8**	6 ± 1.4	41 ± 5.3**	6 ± 1.6	n.s.d.
Glu	74 ± 8.9**	7 ± 2.3	65 ± 8.0**	5 ± 1.1	n.s.d.
Gly	50 ± 4.7**	10 ± 0.9	58 ± 4.0**	8 ± 1.2	n.s.d.

Significance of difference of test capillary from matched buffer control by paired-samples t-tests: *, 0.01; **, 0.001.

† Background compound was also present in the test and control capillaries.

[+] See Table 4.3.

Table 4.11 Numbers of zoospores of *Pythium dissotocum* in test capillaries (7mM L-glutamate) and control capillaries (phosphate buffer), after 20 min, in the presence or absence of various D-amino acids in the zoospore bathing medium; means ± s.e. for 9 replicates

Background D-amino acid[+]	Background compound present[†]		Background compound absent		Significance of difference of test capillaries in presence/ absence of background
	Test capillary	Control capillary	Test capillary	Control capillary	
Leu	52 ± 5.2**	6 ± 1.4	49 ± 4.5**	6 ± 1.0	n.s.d.
Trp	55 ± 6.0**	6 ± 1.5	64 ± 5.2**	6 ± 0.9	n.s.d.
His	40 ± 6.0*	10 ± 1.5	49 ± 5.9**	8 ± 1.5	n.s.d.
Met	41 ± 5.4**	8 ± 1.7	38 ± 5.2**	7 ± 1.5	n.s.d.
Asn	77 ± 9.7*	4 ± 1.2	54 ± 5.9**	5 ± 1.5	n.s.d.
Phe	70 ± 4.5**	12 ± 1.5	64 ± 5.8**	10 ± 1.7	n.s.d.
Arg	75 ± 7.4**	6 ± 1.2	63 ± 6.1**	5 ± 1.5	n.s.d.
Ser	80 ± 4.8**	7 ± 2.3	70 ± 5.3**	8 ± 1.7	n.s.d.
Pro	44 ± 5.2**	5 ± 1.1	22 ± 4.2**	3 ± 1.0	n.s.d.
Ile	42 ± 6.9**	6 ± 1.9	53 ± 5.0**	5 ± 1.3	n.s.d.
OHPro	66 ± 7.6**	6 ± 1.2	75 ± 7.8**	7 ± 1.5	n.s.d.
Ala	39 ± 4.1**	10 ± 1.2	34 ± 4.6*	9 ± 1.8	n.s.d.
Thr	71 ± 6.6**	8 ± 1.7	54 ± 8.8**	6 ± 1.0	n.s.d.
Val	56 ± 7.3**	11 ± 1.9	63 ± 7.8**	13 ± 2.2	n.s.d.
Lys	48 ± 5.6**	8 ± 2.1	42 ± 6.5*	7 ± 1.8	n.s.d.
Asp	46 ± 5.3**	9 ± 1.8	42 ± 4.6**	12 ± 1.4	n.s.d.
Gln	35 ± 5.6**	7 ± 1.2	41 ± 7.0*	6 ± 1.3	n.s.d.
Glu	46 ± 4.6**	9 ± 1.6	51 ± 4.8**	8 ± 1.3	n.s.d.
Gly	64 ± 9.7**	12 ± 1.6	53 ± 4.6**	14 ± 1.4	n.s.d.

Significance of difference of test capillary from matched buffer control by paired-samples t-tests: *, 0.01; **, 0.001.

† Background compound was also present in the test and control capillaries.

+ See Table 4.3.

Table 4.12 Numbers of zoospores of *Pythium aphanidermatum* in test capillaries containing various L-amino acids (10mM) and control capillaries (phosphate buffer), after 20 min, in the presence or absence of L-glutamate (2mM) in the zoospore bathing medium; means ± s.e. for 9 replicates

Test attractant (10mM)[+]	Background glutamate present[†]		Background glutamate absent		Significance of difference of test capillaries in presence/ absence of background
	Test capillary	Control capillary	Test capillary	Control capillary	
L-Gln	4 ± 0.8	3 ± 0.7	26 ± 4.5**	4 ± 0.8	0.01
L-Ala	5 ± 0.9	4 ± 0.8	13 ± 1.8*	6 ± 0.9	0.01
L-Asn	5 ± 1.4	3 ± 0.7	21 ± 3.6**	4 ± 0.8	0.01
L-Asp	31 ± 3.5**	5 ± 0.9	39 ± 4.1**	6 ± 1.2	n.s.d.

Significance of difference between test capillary and matched buffer control by paired-samples t-test: *, 0.01; **, 0.001.

† Background compound was also present in the test and control capillaries.

+ Asn, asparagine; Ala, alanine; Asp, aspartic acid; Gln, glutamine

representing the "encystment front", increased progressively with time (Figure 4.1). In all cases regression analysis revealed a linear relationship between the distance of the front and time of immersion, the correlation coefficient never being less than 0.936 and always statistically significant at P= 0.001. It was also found that at any one time, the distance from the capillary mouth to the encystment front was inversely related to the concentration of the glutamate in the capillary (Figure 4.1), indicating that the spores encysted when they encountered a critical glutamate concentration.

It was commonly seen that at higher concentrations of glutamate spores were attracted to the capillary mouth then entered it before being repelled. Alternatively, spores swam past the encystment front (the position of the furthest spore in the tube) and then turned abruptly and retreated down the capillary. All spores displayed a series of jerking motions before encysting.

4.2. Characteristics of zoospore motility

4.2.1. Effects of cations and EGTA on motility characteristics of zoospores of Pythium aphanidermatum

Cations are known to influence the motile behaviour of a wide range of organisms including bacteria, (*e.g.* Ordal, 1977), *Chlamydomonas* (*e.g.* Harz & Hegemann, 1991), trout sperm (*e.g.* Boitano & Omoto, 1992) and fungal zoospores (*e.g.* Allen & Harvey, 1974; Thomas & Butler, 1989). However, most of the studies on zoospores have involved *Phytophthora* spp., *Achlya* spp. or *Chytridiomycetes* but not *Pythium* spp. The experiments here were designed to obtain equivalent information for *Pythium aphanidermatum*. Methods were as described in Section 2.5 and the motility features that it was intended to examine are shown in Figure 4.2.

Control spores in either distilled water or phosphate buffer, swam in a typical extended helix (Figure 4.3) with periodic abrupt changes of direction, typical of Oomycete zoospores. Their swimming speeds and other motility characteristics, measured by analysis of videotapes, are shown in *e.g.* Table 4.13. The addition of either monovalent cations (Na^+, K^+ and Li^+) at final concentrations up to 1mM, or trivalent cations (Fe^{3+}, Cr^{3+}) at 65-250μM had no significant effect on motility (Tables 4.13-4.16).

Figure 4.1 The relationship between time and the distance of the encystment front of zoospores of *P. aphanidermatum* in capillaries containing different millimolar concentrations of L-glutamate

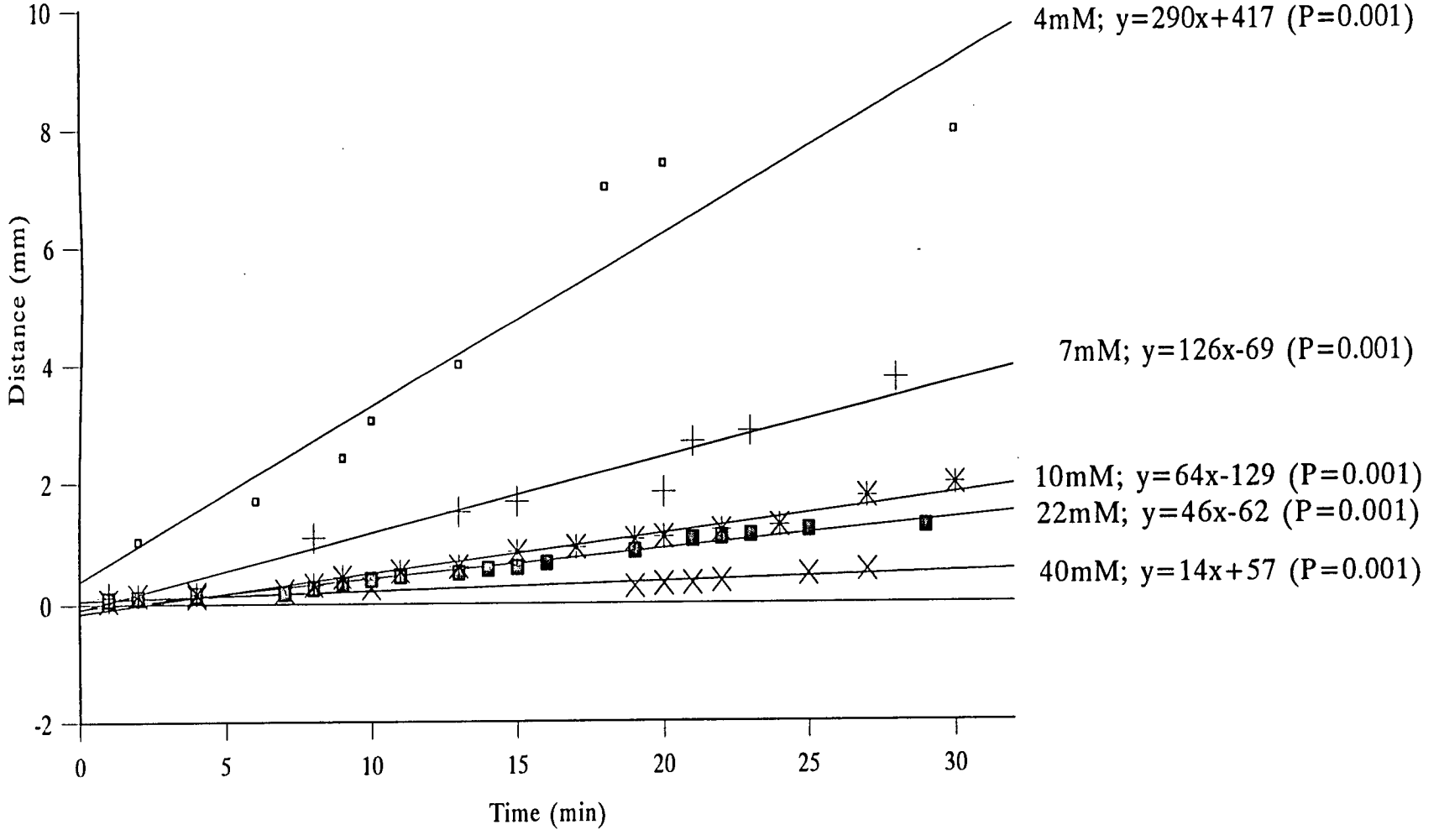
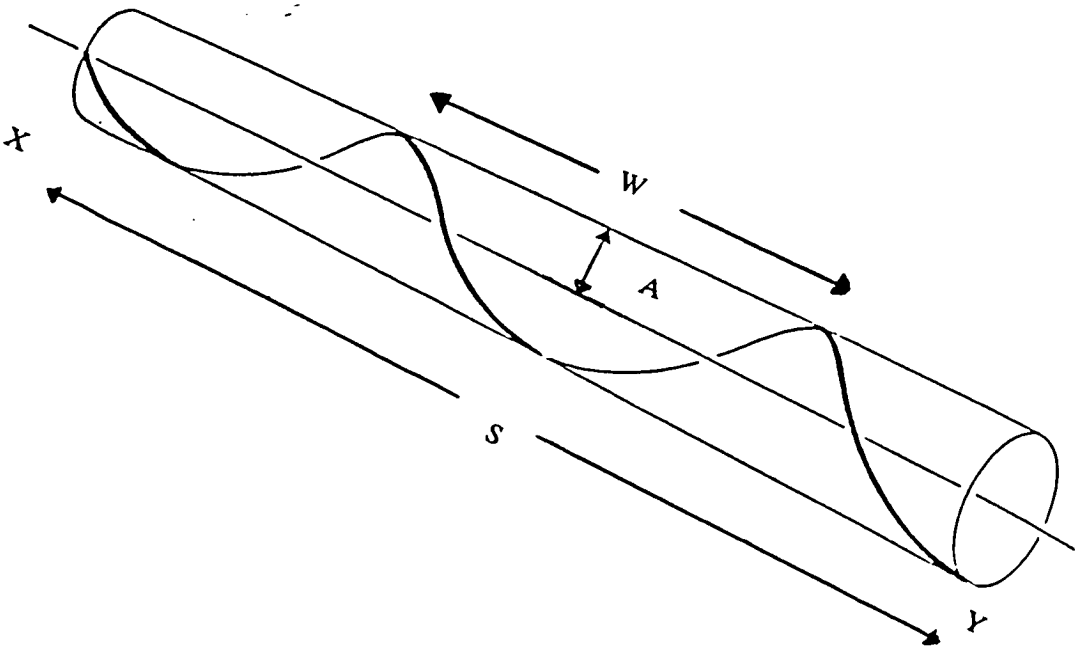


Figure 4.2 Diagrammatic representation of a zoospore of *P. aphanidermatum* showing the typical helical path of movement when a zoospore swims in water (adapted from Allen & Newhook, 1974)



speed (S) = distance between two points, X and Y, per unit time
amplitude (A)
wavelength (W)
velocity^2 = (2π amplitude)^2 + speed^2
frequency (Hz) = speed/velocity

Table 4.13 Effect of Li^+, in the presence or absence of EGTA, on motility characteristics of zoospores of *Pythium aphanidermatum*[+]

	Treatment and concentration (μM)							
Attributes of locomotion [‡]	Control (water)	Li^+ (125)	Li^+ (125) + EGTA (125)	Li^+ (250)	Li^+ (250) + EGTA (250)	Li^+ (500)	Li^+ (500) + EGTA (500)	Li^+ (1000)
Speed (μms^{-1})	97 ± 3.6	105 ± 15.8	98 ± 5.3	101 ± 8.5	95 ± 6.7	99 ± 6.4	88 ± 5.1	94 ± 6.9
Velocity (μms^{-1})	204 ± 20.0	210 ± 9.8	114 ± 5.7	223 ± 15.9	112 ± 6.8	198 ± 10.7	106 ± 4.4	207 ± 7.7
Frequency (Hz)	0.5 ± 0.03	0.5 ± 0.03	0.8 ± 0.03	0.5 ± 0.06	0.8 ± 0.03	0.5 ± 0.04	0.8 ± 0.04	0.5 ± 0.03
Amplitude (μm)	28 ± 2.2	29 ± 1.9	8 ± 1.0	31 ± 2.9	9 ± 1.2	27 ± 1.9	9 ± 1.2	29 ± 1.3
Wavelength (μm)	98 ± 3.2	99 ± 7.1	106 ± 5.1	106 ± 3.4	105 ± 7.7	124 ± 5.8	108 ± 5.5	113 ± 3.8
Time (sec) between changes of direction	1.6 ± 0.13	1.2 ± 0.18	∞	1.0 ± 0.10	∞	1.2 ± 0.18	∞	1.0 ± 0.10
Distance (μm) between changes of direction	134 ± 11.8	112 ± 10.9	∞	91 ± 8.7	∞	112 ± 10.9	∞	97 ± 8.8
Replicates	28	11	11	11	11	11	11	11

[+] Means ± s.e. for the number of replicate spores shown, 10 min after addition of ions or EGTA. The motility parameters within treatments were not necessarily measured for the same spores within the population.

[‡] See Figure 4.2.

Table 4.14 Effect of K^+, in the presence or absence of EGTA, on motility characteristics of zoospores of *Pythium aphanidermatum* [+]

	Treatment and concentration (μM)						
Attributes of [‡] locomotion	Control (water)	K^+ (125)	K^+ (250)	K^+ (250) + EGTA (250)	K^+ (500)	K^+ (1000)	K^+ (1000) + EGTA (500)
Speed (μms^{-1})	97 ± 3.6	100 ± 6.9	95 ± 6.8	92 ± 6.1	107 ± 6.5	99 ± 4.1	105 ± 5.2
Velocity (μms^{-1})	204 ± 20.0	205 ± 15.3	218 ± 11.1	161 ± 11.1	255 ± 15.4	229 ± 11.5	129 ± 10.1
Frequency (Hz)	0.5 ± 0.03	0.5 ± 0.04	0.4 ± 0.03	0.6 ± 0.03	0.4 ± 0.04	0.4 ± 0.03	0.8 ± 0.04
Amplitude (μm)	28 ± 2.2	28 ± 2.5	31 ± 2.0	21 ± 2.1	36 ± 3.2	30 ± 2.5	11 ± 1.1
Wavelength (μm)	98 ± 3.2	96 ± 3.5	97 ± 6.4	94 ± 5.6	103 ± 8.2	85 ± 5.6	94 ± 4.8
Time (sec) between changes of direction	1.6 ± 0.13	1.5 ± 0.17	1.3 ± 0.13	∞	1.1 ± 0.10	1.4 ± 0.13	∞
Distance (μm) between changes of direction	134 ± 11.8	140 ± 14.9	124 ± 11.8	∞	99 ± 10.4	111 ± 8.1	∞
Replicates	28	11	11	11	11	11	11

[+] Means ± s.e. for the number of replicate spores shown, 10 min after addition of ions or EGTA. The motility parameters within treatments were not necessarily assessed for the same spores within the population.

[‡] See Figure 4.2.

Table 4.15 Effect of Na^+, in the presence or absence of EGTA, on motility characteristics of zoospores of *Pythium aphanidermatum* [+]

	Treatment and concentration (μM)						
Attributes of [‡] locomotion	Control (water)	Na^+ (125)	Na^+ (250)	Na^+ (250) + EGTA (250)	Na^+ (500)	Na^+ (1000)	Na^+ (1000) + EGTA (500)
Speed (μms^{-1})	97 ± 3.6	100 ± 5.1	103 ± 6.8	86 ± 4.6	95 ± 6.4	102 ± 4.6	99 ± 4.7
Velocity (μms^{-1})	204 ± 20.0	223 ± 12.8	217 ± 12.6	113 ± 5.7	209 ± 12.3	211 ± 13.0	142 ± 7.7
Frequency (Hz)	0.5 ± 0.03	0.5 ± 0.04	0.5 ± 0.04	0.8 ± 0.03	0.5 ± 0.04	0.5 ± 0.05	0.7 ± 0.04
Amplitude (μm)	28 ± 2.2	33 ± 2.3	30 ± 2.4	11 ± 2.4	29 ± 2.4	28 ± 2.4	14 ± 1.9
Wavelength (μm)	98 ± 3.2	98 ± 3.9	106 ± 5.1	86 ± 3.2	101 ± 5.5	96 ± 5.2	97 ± 3.9
Time (sec) between changes of direction	1.6 ± 0.13	1.5 ± 0.17	1.3 ± 0.12	∞	1.4 ± 0.18	1.4 ± 0.14	∞
Distance (μm) between changes of direction	134 ± 11.8	140 ± 18.8	127 ± 12.5	∞	122 ± 17.7	134 ± 13.1	∞
Replicates	28	11	11	14	11	14	14

[+] Means ± s.e. for the number of replicate spores shown, 10 min after addition of ions or EGTA. The motility parameters within treatments were not necessarily measured for the same spores within the population.

[‡] See Figure 4.2.

Table 4.16 Effects of Fe^{3+} and Cr^{3+}, in the presence or absence of EGTA, on motility characteristics of zoospores of *Pythium aphanidermatum* [+]

	Treatment and concentration (μM)							
Attributes of [‡] locomotion	Control (water)	Fe^{3+} (65)	Fe^{3+} (125)	Fe^{3+} (125) + EGTA (125)	Fe^{3+} (250)	Fe^{3+} (250) + EGTA (250)	Cr^{3+} (125)	Cr^{3+} (250)
Speed (μms^{-1})	97 ± 3.6	98 ± 6.6	107 ± 4.0	99 ± 5.4	108 ± 5.3	99 ± 5.9	104 ± 7.5	103 ± 8.8
Velocity (μms^{-1})	204 ± 20.0	216 ± 12.6	217 ± 12.9	248 ± 16.6	212 ± 13.0	211 ± 10.7	208 ± 12.9	209 ± 13.7
Frequency (Hz)	0.5 ± 0.03	0.5 ± 0.04	0.5 ± 0.02	0.4 ± 0.04	0.5 ± 0.03	0.4 ± 0.04	0.5 ± 0.04	0.5 ± 0.05
Amplitude (μm)	28 ± 2.2	30 ± 2.3	30 ± 1.8	36 ± 3.0	29 ± 2.2	36 ± 3.0	28 ± 2.4	29 ± 2.5
Wavelength (μm)	98 ± 3.2	110 ± 2.8	106 ± 3.1	113 ± 5.5	103 ± 3.3	113 ± 5.5	112 ± 7.9	124 ± 5.9
Time (sec^{-1}) between changes of direction	1.6 ± 0.13	1.2 ± 0.04	1.3 ± 0.12	1.4 ± 0.12	1.3 ± 0.12	1.3 ± 0.12	0.9 ± 0.12	1.2 ± 0.08
Distance (μm) between changes of direction	134 ± 11.8	111 ± 8.0	120 ± 13.8	114 ± 14.6	127 ± 9.5	114 ± 14.6	78 ± 6.6	96 ± 8.9
Replicates	28	11	11	11	11	11	11	11

[+] Means ± s.e. for the number of replicate spores shown, 10 min after addition of ions or EGTA. The motility parameters within treatments were not necessarily measured for the same spores within the population.

[‡] See Figure 4.2.

In contrast, the divalent cations Ca^{2+}, Mg^{2+}, Sr^{2+}, Mn^{2+} and Ba^{2+} drastically altered the pattern of motility and caused spores to swim in a continuous circular motion (Tables 4.17-4.21). Because of the large number of tests required, not all ions were tested at all concentrations, but where this was done the response was found to be concentration dependent. For example (Table 4.21) Ba^{2+} at 65μM had no effect, 125 and 250μM caused some spores to swim in circles and 500μM Ba^{2+} caused all spores to swim in circles.

Of the tested cations Sr^{2+} seemed to be the least effective, because it caused only some of the spores to swim in tight circles whilst the remainder displaying normal motile behaviour (Table 4.19). Mg^{2+} also was unusual because it caused no effect at 500μM but induced circular swimming at 125μM (Table 4.18). Spores that displayed circular swimming patterns could not be assessed for most of the normal motility characteristics. Instead the speed of the circular motion, the radius of the circular path and the number of revolutions per minute were all recorded.

The presence of EGTA alone, in the absence of supplementary cations, completely suppressed random changes of direction and the zoospores swam in continuous straight paths (Figure 4.4). In addition, the amplitude of the swimming helix was markedly reduced and although the swimming speed of the spore (the rate of linear displacement) was unaltered the velocity (a function of both speed and amplitude) was significantly reduced (Table 4.17).

Cations were also tested in combination with EGTA, having been preincubated with it for 24h, and then added simultaneously. Combinations of monovalent cations (Li^+, Na^+ and K^+) with equimolar concentrations of EGTA caused the zoospores to swim in straight paths (Tables 4.13-4.15), as with EGTA alone (Table 4.17). Combinations of the divalent cations Ca^{2+} (500μm) and Mg^{2+} (250μm) with equimolar EGTA initially caused spores to swim in perpetual circles (indicative of motion elicited by Ca^{2+} and Mg^{2+}) but by 50 min all the spores had reverted to the EGTA pattern of motility and swam continuously in linear paths (Tables 4.17,4.18). In contrast, spores treated with Mg^{2+} and EGTA, both added at 125μM, caused spores to swim in circles but they later reverted to normal motility (Table 4.18). When Ca^{2+} was added at a concentration double that of EGTA a proportion of the spores swam continuously in circles whereas the remainder displayed normal motile behaviour (Table 4.17). Similarly, treatment with Mn^{2+} (500μm) or Ba^{2+} (125μm) and equimolar concentrations of EGTA induced spores to swim in circles but by 50 min all spores had reverted to normal motility (Tables 4.20,

Table 4.17 Effects of Ca^{2+} and EGTA on motility characteristics of zoospores of *Pythium aphanidermatum* 10 min after addition of ions and EGTA except where stated[+]

Attributes of locomotion[‡]	Treatment and concentration (μM)							
	Control (water)	EGTA (125)	Ca^{2+} (500)	Ca^{2+} (500) + EGTA (500)	Ca^{2+} (500) + EGTA (500) (50 min)[*]	Ca^{2+} (1000)	Ca^{2+} (1000) + EGTA (500)[†] a	b
Speed (μms^{-1})	97 ± 3.6	98 ± 3.0	100 ± 6.8	94 ± 7.1	106 ± 5.5	93 ± 7.9	-102 ± 4.8-	
Velocity (μms^{-1})	204 ± 20.0	145 ± 9.2	n.a.	n.a.	142 ± 4.5	n.a.	216 ± 13.1	n.a.
Frequency (Hz)	0.5 ± 0.03	0.7 ± 0.03	n.a.	n.a.	0.7 ± 0.04	n.a.	0.5 ± 0.04	n.a.
Amplitude (μm)	28 ± 2.2	15 ± 1.5	n.a.	n.a.	13 ± 1.4	n.a.	30 ± 2.4	n.a.
Wavelength (μm)	98 ± 3.2	93 ± 1.9	n.a.	n.a.	99 ± 4.5	n.a.	66 ± 4.8	n.a.
Time (sec^{-1}) between changes of direction	1.6 ± 0.13	∞	n.a.	n.a.	∞	n.a.	1.8 ± 0.27	n.a.
Distance (μm) between changes of direction	134 ± 11.8	∞	n.a.	n.a.	∞	n.a.	116 ± 17.8	n.a.
Radius (r)	n.a.	n.a.	30 ± 2.4	30 ± 2.0	n.a.	28 ± 2.5	n.a.	38 ± 2.8
Revs (min^{-1})	n.a.	n.a.	32 ± 2.5	30 ± 2.1	n.a.	32 ± 3.0	n.a.	26 ± 2.3
Replicates	28	25	14	14	14	14	- -14 - -	

[+] Means ± s.e. for the number of replicate spores shown, 10 min after addition of ions or EGTA. The motility parameters within treatments were not necessarily measured for the same spores within the population.[*] Motility reassessed after 50 min; [†] spores showed mixed behaviour, recorded separately as (a) and (b).
[‡] See Figure 4.2. Spores with circular swimming paths could not be assessed for most attributes. Instead the radius of the circular path and the number of revolutions per minute are shown. Speed was calculated as $2\pi r$ / time for one revolution.
n.a. not applicable

Table 4.18 Effect of Mg^{2+}, in the presence or absence of EGTA, on motility characteristics of zoospores of *Pythium aphanidermatum* 10 min after addition of ions and EGTA except where stated[+]

Attributes of locomotion[‡]	Treatment and concentration (μM)									
	Control (water)	Mg^{2+} (125)[†]		Mg^{2+} (125) + EGTA (125)	Mg^{2+} (125) + EGTA (125) (50min)[*]	Mg^{2+} (250)[†]		Mg^{2+} (250) + EGTA (250)	Mg^{2+} (250) + EGTA (250) (50 min)[*]	Mg^{2+} (500)
		a	b			a	b			
Speed (μms^{-1})	97 ± 3.6	- 101 ± 6.0 -		118 ± 7.0	99 ± 7.6	- 101 ± 7.5 -		113 ± 5.6	100 ± 6.3	109 ± 4.5
Velocity (μms^{-1})	204 ± 20.0	205 ± 10.6	-	-	214 ± 10.3	218 ± 6.2	-	-	117 ± 6.2	225 ± 11.1
Frequency (Hz)	0.5 ± 0.03	0.5 ± 0.05	-	-	0.5 ± 0.04	0.5 ± 0.03	-	-	0.8 ± 0.03	0.5 ± 0.03
Amplitude (Hz)	28 ± 2.2	28 ± 1.8	-	-	30 ± 2.2	30 ± 2.0	-	-	9 ± 1.1	31 ± 2.0
Wavelength (μm)	98 ± 3.2	99 ± 3.5	-	-	102 ± 3.8	103 ± 3.6	-	-	97 ± 3.3	100 ± 4.7
Time (sec^{-1}) between changes of direction	1.6 ± 0.13	1.3 ± 0.11	-	-	1.4 ± 0.13	1.9 ± 0.24	-	-	∞	3.2 ± 0.33
Distance (μm) between changes of direction	134 ± 11.8	129 ± 13.0	-	-	124 ± 11.4	156 ± 18.9	-	-	∞	136 ± 19.2
Radius (r)	n.a.	n.a.	30 ± 3.6	39 ± 4.6	n.a.	n.a.	31 ± 3.2	39 ± 5.4	n.a.	n.a.
Revs (min^{-1})	n.a.	n.a.	37 ± 4.8	28 ± 3.4	n.a.	n.a.	34 ± 3.6	28 ± 3.1	n.a.	n.a.
Replicates	28	- 11 -		11	11	- 11 -		11	11	11

[+] Means ± s.e. for the number of replicate spores shown, 10 min after addition of ions or EGTA. The motility parameters within treatments were not necessarily measured for the same spores within the population. [*] Motility reassessed after 50 min; [†] spores showed mixed behaviour, recorded separately as (a) and (b).

[‡] See Figure 4.2. Spores with circular swimming paths could not be assessed for most attributes. Instead the radius of the circular path and the number of revolutions per minute are shown. Speed was calculated as $2\pi r$/time for one revolution.

Table 4.19 Effects of Sr^{2+}, in the presence or absence of Ca^{2+} or EGTA, on motility characteristics of zoospores of *Pythium aphanidermatum*[+]

Attributes of locomotion[‡]	Treatment and concentration (μM)						
	Control (water)	Sr^{2+} (500)[†] a	Sr^{2+} (500)[†] b	Sr^{2+} (500) + EGTA (500)	Sr^{2+} (750) + EGTA (500)	Sr^{2+} (500) + Ca^{2+} (500) + EGTA (500)	Sr^{2+} (500) + Ca^{2+} (1000) + EGTA (500)
Speed (μms^{-1})	97 ± 3.6	85 ± 8.3		104 ± 5.1	101 ± 5.1	102 ± 4.0	102 ± 3.8
Velocity (μms^{-1})	204 ± 20.0	206 ± 9.8	n.a.	228 ± 11.9	285 ± 28.3	220 ± 8.1.	225 ± 11.6
Frequency (Hz)	0.5 ± 0.03	0.5 ± 0.03	n.a.	0.5 ± 0.03	0.4 ± 0.04	0.5 ± 0.02	0.5 ± 0.03
Amplitude (μm)	28 ± 2.2	28 ± 1.5	n.a.	32 ± 2.3	42 ± 4.4	31 ± 1.2	32 ± 2.1
Wavelength (μm)	98 ± 3.2	71 ± 4.9	n.a.	105 ± 10.2	109 ± 3.9	99 ± 2.6	100 ± 2.4
Time (sec^{-1}) between changes of direction	1.6 ± 0.13	1.5 ± 0.18	n.a.	1.6 ± 0.18	2.4 ± 0.34	1.2 ± 0.17	1.3 ± 0.21
Distance (μm) between changes of direction	134 ± 11.8	129 ± 16.6	n.a.	116 ± 17.6	222 ± 35.9	118 ± 18.9	105 ± 14.4
Radius (r)	n.a.	n.a.	33	n.a.	n.a.	n.a.	n.a.
Revs (min^{-1})	n.a.	n.a.	26 ± 3.0	n.a.	n.a.	n.a.	n.a.
Replicates	28	- - 14 - -		14	14	14	14

[+] Means ± s.e. for the number of replicate spores shown, 10 min after addition of ions or EGTA. The motility parameters within treatments were not necessarily measured for the same spores within the population.

[†] spores showed mixed behaviour, recorded separately as (a) and (b).

[‡] See Figure 4.2. Spores with circular swimming paths could not be assessed for most attributes. Instead the radius of the circular path and the number of revolutions per minute are shown. Speed was calculated as $2\pi r$/time for one revolution.

Table 4.20 Effect of Mn^{2+}, in the presence or absence of EGTA, on motility characteristics of zoospores of *Pythium aphanidermatum* 10 min after addition of ions and EGTA except where stated[+]

Attributes of ‡ locomotion	Treatment and concentration (μM)					
	Control (water)	Mn^{2+} (125)	Mn^{2+} (125) + EGTA (125)	Mn^{2+} (500)	Mn^{2+} (500) + EGTA (500)	Mn^{2+} (500) + EGTA (500) (50min)*
Speed (μms^{-1})	97 ± 3.6	92 ± 7.1	96 ± 6.1	114 ± 10.4	120 ± 6.4	107 ± 6.5
Velocity (μms^{-1})	204 ± 20.0	202 ± 13.9	106 ± 6.0	n.a.	n.a.	221 ± 14.8
Frequency (Hz)	0.5 ± 0.03	0.5 ± 0.05	0.9 ± 0.02	n.a.	n.a.	0.5 ± 0.04
Amplitude (μm)	28 ± 2.2	28 ± 2.5	7 ± 0.8	n.a.	n.a.	29 ± 2.7
Wavelength (μm)	98 ± 3.2	107 ± 4.4	109 ± 6.3	n.a.	n.a.	112 ± 16.2
Time (sec^{-1}) between changes of direction	1.6 ± 0.13	1.1 ± 0.08	∞	n.a.	n.a.	1.1 ± 0.1
Distance (μm) between changes of direction	134 ± 11.8	96 ± 8.5	∞	n.a.	n.a.	113 ± 10.6
Radius (r)	n.a.	n.a.	n.a.	36 ± 3.4	33 ± 4.9	n.a.
Revs (min^{-1})	n.a.	n.a.	n.a.	31 ± 3.5	35 ± 2.6	n.a.
Replicates	28	11	11	11	11	14

[+] Means ± s.e. for the number of replicate spores shown, 10 min after addition of ions or EGTA. The motility parameters within treatments were not necessarily measured for the same spores within the population. * Motility reassessed after 50min.

‡ See Figure 4.2. Spores with circular swimming paths could not be assessed for most attributes. Instead the radius of the circular path and number of revolutions per minute are shown. Speed was calculated as $2\pi r$/time for one revolution.

Table 4.21 Effect of Ba^{2+}, in the presence or absence of EGTA, on motility characteristics of zoospores of *Pythium aphanidermatum* 10 min after addition of ions and EGTA except where stated[+]

Attributes of locomotion[‡]	Treatment and concentration (μM)								
	Control (water)	Ba^{2+} (65)	Ba^{2+} (125)[†]		Ba^{2+} (125) + EGTA (125)	Ba^{2+} (125) + EGTA (125) (50min)[*]	Ba^{2+} (250)[†]		Ba^{2+} (500)
			a	b			a	b	
Speed (μms^{-1})	97 ± 3.6	95 ± 6.4	- 92 ± 7.6 -		116 ± 7.4	99 ± 6.3	- 112 ± 4.4 -		108 ± 10.0
Velocity (μms^{-1})	204 ± 20.0	208 ± 11.6	212 ± 15.5	n.a.	n.a.	187 ± 10.1	214 ± 13.2.	n.a.	n.a.
Frequency (Hz)	0.5 ± 0.03	0.5 ± 0.04	0.5 ± 0.03	n.a.	n.a.	0.5 ± 0.04.	0.5 ± 0.03	n.a.	n.a.
Amplitude (μm)	28 ± 2.2	29 ± 2.2	30 ± 2.5	n.a.	n.a.	25 ± 2.3	28 ± 2.4	n.a.	n.a.
Wavelength (μm)	98 ± 3.2	97 ± 3.4	110 ± 5.4	n.a.	n.a.	102 ± 4.1	100 ± 2.7	n.a.	n.a.
Time (sec^{-1}) between changes of direction	1.6 ± 0.13	1.2 ± 0.13	1.5 ± 0.18	n.a.	n.a.	1.0 ± 0.01	1.3 ± 0.18	n.a.	n.a.
Distance (μm) between changes of direction	134 ± 11.8	121 ± 13.8	152 ± 19.4	n.a.	n.a.	94 ± 10.7	145 ± 15.7	n.a.	n.a.
Radius (r)	n.a.	n.a.	n.a.	30 ± 2.8	33 ± 3.3	n.a.	n.a.	44 ± 3.8	30 ± 5.2
Revs (min^{-1})	n.a.	n.a.	n.a.	33 ± 3.3	34 ± 4.0	n.a.	n.a.	27 ± 4.8	35 ± 4.6
Replicates	28	11	- - 11 -.-		11	11	- -11 - -		11

[+] Means ± s.e. for the number of replicate spores shown, 10 min after addition of ions or EGTA. The motility parameters within treatments were not necessarily measured for the same spores within the population.

[*] Motility reassessed after 50 min; [†] spores showed mixed behaviour, recorded separately as (a) and (b).

[‡] See Figure 4.2. Spores with circular swimming paths could not be assessed for most attributes. Instead the radius of the circular path and the number of revolutions per minute are shown. Speed was calculated as $2\pi r$/time for one revolution.

4.21). But treatment with both EGTA and Mn^{2+} (125μM) caused spores to swim in a straight line- the "EGTA" mode of swimming.

Combinations of EGTA with Fe^{3+} or Sr^{2+} caused no change in the normal (control) pattern of motility of spores (Tables 4.16, 4.19). Similarly, the presence of Sr^{2+} in combination with EGTA and Ca^{2+} led to normal motility, suggesting that Sr^{2+} suppressed the effects that Ca^{2+} and EGTA alone would elicit on spores.

Attempts to analyse the motility characteristics of Cr^{3+} in the presence of EGTA were unsuccessful because the spores were induced to encyst. Similarly attempts to "rescue" spores and to restore their normal motile behaviour, by subsequent addition of cations to EGTA-treated spores and *vice-versa*, were unsuccessful. The post-treatments in these cases led immediately to the premature encystment of spores that were swimming in the perturbed (non-normal) modes.

4.2.2. Effects of cations or EGTA on chemotaxis of zoospores of Pythium aphanidermatum towards glutamic acid

Calcium has been implicated in the intracellular mechanisms leading to chemotaxis of both *Physarum* and eosinophils (Natsume *et al.,* 1992; Brundage *et al.,* 1991). In the previous experiment (Tables 4.17-4.21) a range of divalent cations, including Ca^{2+}, and the Ca^{2+} chelator EGTA (Schmid & Reilley, 1957), were found to alter the motile behaviour of zoospores. It was of interest, therefore, to analyse the effect of cations and EGTA, supplied in the background medium, on chemotaxis of zoospores of *Pythium aphanidermatum* to the chemoattractant L-glutamic acid in capillaries.

As shown in Table 4.22 Na^{+} and Fe^{3+} had no effect on chemotaxis consistent with their lack of effect on zoospore motility. In contrast the presence of either Ca^{2+} or EGTA in the suspending medium completely annulled chemotaxis to L-glutamic acid, consistent with the effects of these supplements in causing perturbation of swimming.

4.2.3. Effects of Ca^{2+} modulators on the motile behaviour of zoospores of Pythium aphanidermatum

Calcium channel blockers have been demonstrated to inhibit trout sperm motility (Cosson *et al.,* 1989) and to prevent phototaxis of *Chlamydomonas*

Table 4.22 Numbers of zoospores of *Pythium aphanidermatum* accumulating in test capillaries containing L-glutamate (7mM) or control capillaries (phosphate buffer) at 10 min after insertion into zoospore suspension supplemented or not supplemented with cations or EGTA*

Supplement (μM) to zoospore suspending medium	Supplement present			Supplement absent		
	Test capillary		Control capillary	Test capillary		Control capillary
Ca^{2+} (500)	10 ± 1.6	- nsd -	8 ± 1.0	54 ± 6.4	- 0.001 -	5 ± 1.0
Fe^{3+} (250)	33 ± 3.4	- 0.001 -	5 ± 1.0	38 ± 3.0	- 0.001 -	6 ± 0.9
Na^{+} (500)	67 ± 5.0	- 0.001 -	5 ± 0.9	74 ± 4.6	- 0.001 -	6 ± 1.0
EGTA (500)	6 ± 1.5	- nsd -	4 ± 1.1	52 ± 8.0	- 0.001 -	7 ± 1.2

* Means ± s.e. for 9 replicate tests. Significance of difference between test and control capillaries shown as nsd (no significant difference) or P = 0.001, based on paired- samples t-test.

(Harz & Hegemann, 1991). It was therefore of interest to examine the effects of these and other Ca^{2+} modulators on the motile behaviour of fungal zoospores. Initial tests (not presented) showed that a range of calcium modulators induced premature immobilisation of zoospores, which then either lysed or remained intact but appeared to be non-viable. However, at extremely low levels (Table 4.23) a range of modulators enabled the spores to continue swimming for some minutes, and the characteristic changes of motility that they caused could be analysed.

In contrast to the normal pattern of motility in distilled water controls, the Ca^{2+} modulators caused marked aberrations which fell into different categories. In all cases the motility patterns of zoospores were analysed by video microscopy which enabled tracings of the zoospore tracks to be obtained (Figures 4.3-4.10).

The Ca^{2+} channel blockers lanthanum and verapamil (Godfraind *et al.*, 1986) caused spores to spiral and swim in perpetual circles (Figures 4.5, 4.6). In neither case, however, were the swimming speeds altered in comparison to spores in the presence of water (Table 4.23). The calmodulin antagonists trifluoperazine (TFP) and dibucaine (Bereza *et al.*, 1982) caused spores to swim slowly (Table 4.23) in approximately circular or spiral paths (Figures 4.7, 4.8). But the arc described by the posterior of each spore was consistently wider than that described by the anterior such that the tracks resemble a skid or a "handbrake" turn of a vehicle. The ionophore A23187 (Pressman, 1976) and amiloride, which interferes with ion flux across membranes (Godfraind *et al.*, 1986), caused spores to swim in tight helices and show sharp jerking movements, resulting in continuous changes of direction (Figures 4.9, 4.10) over extremely small distances (less than 10μm in some cases), with a greatly reduced swimming speed (Table 4.23).

In contrast to the other Ca^{2+} modulators, A23187 was prepared in DMSO (0.05% v/v). However, DMSO alone was found to have no effect at this concentration (Table 4.23).

4.2.4. Effects of L-amino acids and ethanol on the motile behaviour of zoospores

Ethanol has been demonstrated to be a chemoattractant (Allen & Newhook, 1973) and, at uniform background concentrations, to suppress the spontaneous turning activity of zoospores of *P. cinnamomi* (Allen & Newhook, 1974). The

Table 4.23 Effects of calcium modulators on the motile speed of zoospores of *Pythium aphanidermatum* +

Treatment (final concentration)	Speed (μm sec^{-1})	Swimming pattern
Water	104 ± 4.7	normal
Trifluoperazine (5μM)	37 ± 6.3	circular motion with posterior drift
Dibucaine (40μM)	26 ± 3.2	circular motion with posterior drift
Verapamil (20μM)	88 ± 10.2	circular swimming
La^{3+} (150μM)	107 ± 6.0	circular swimming
Amiloride (20μM)	11 ± 1.9	jerky swimming
A23187 (3μM) + DMSO (0.05%)	16 ± 2.3	jerky swimming
DMSO (0.05%) †	113 ± 3.8	normal

+ Means ± s.e. for 9 replicate tests 10 min after addition of test compounds.

† DMSO (dimethylsulphoxide) was tested alone as a control for A23187 which was dissolved in DMSO.

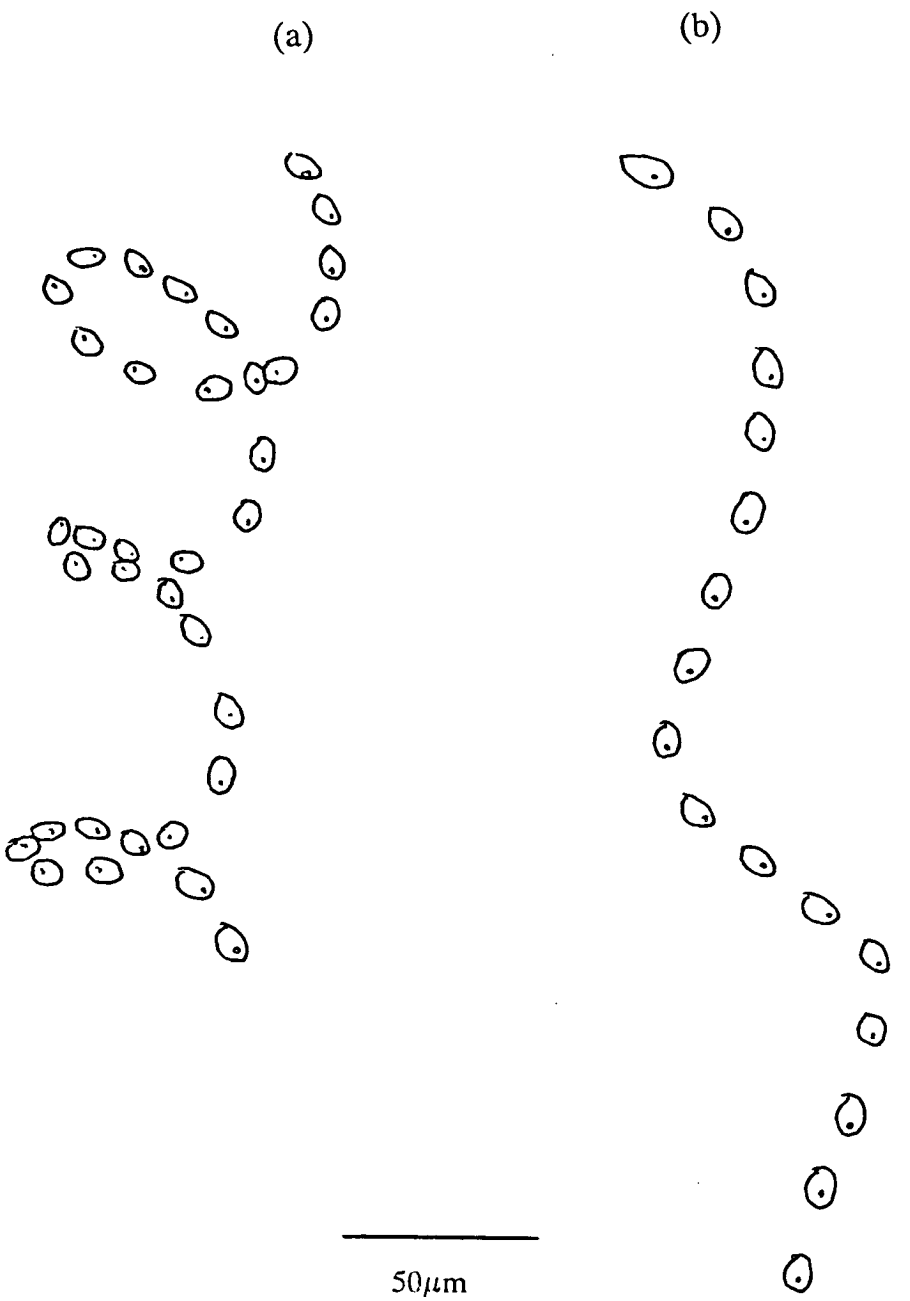


Figure 4.3 Tracings of single zoospores of *P. aphanidermatum* at (a) 0.1 sec intervals and (b) 0.2 sec intervals, exhibiting normal helical swimming in a background of distilled water. The front of each spore is marked by a dot.

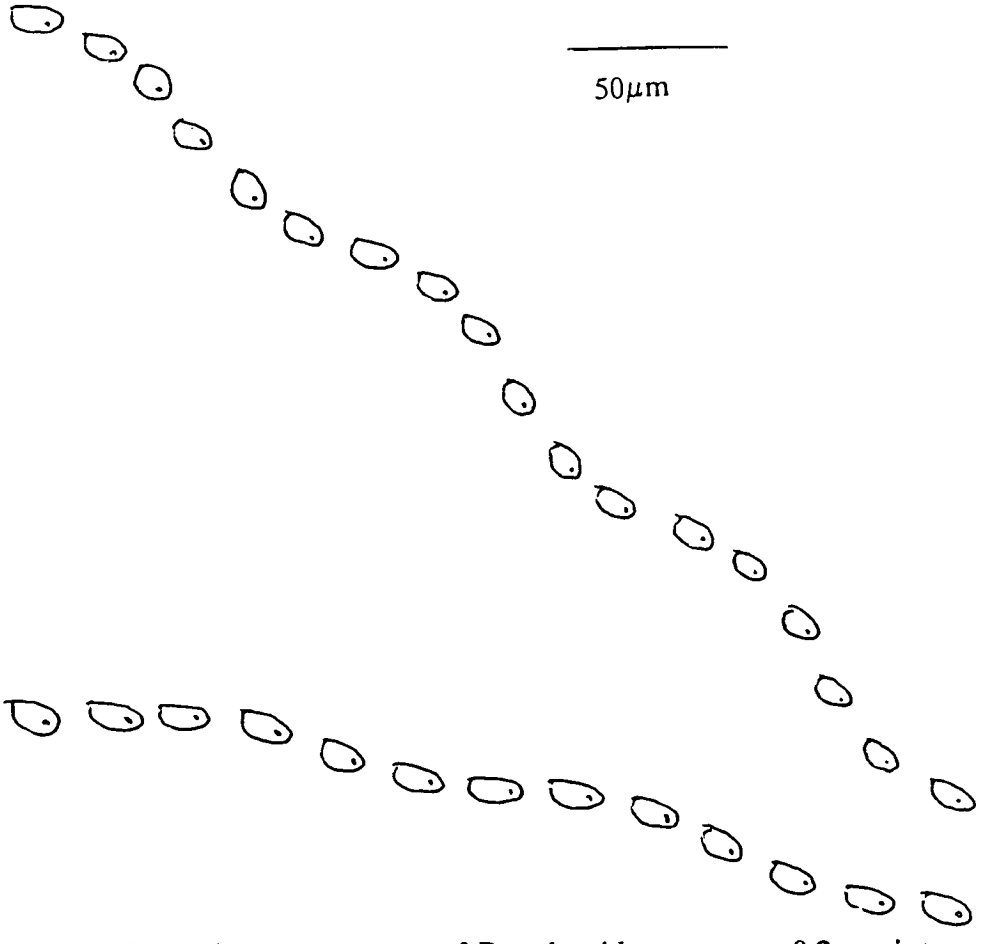


Figure 4.4 Tracings of two zoospores of *P. aphanidermatum* at 0.2 sec intervals in the presence of 125μM EGTA showing continuous "straight" swimming paths with a low helical amplitude.

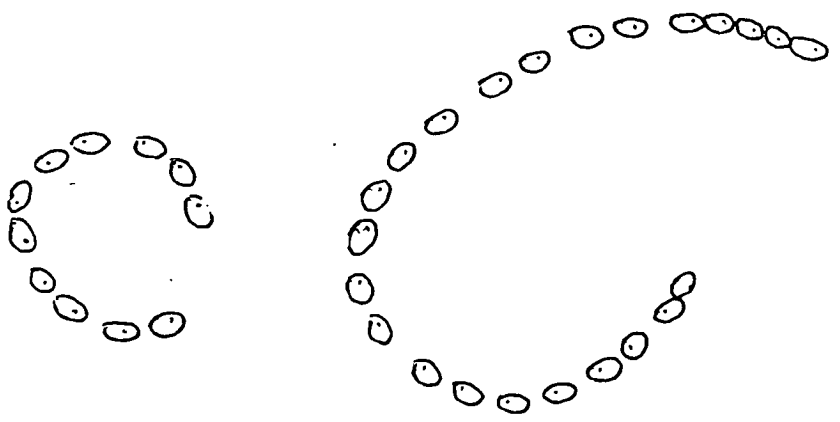


Figure 4.5 Tracings of two zoospores of *P. aphanidermatum* at 0.2 sec intervals in the presence of 20μM verapamil, showing continuous circular swimming.

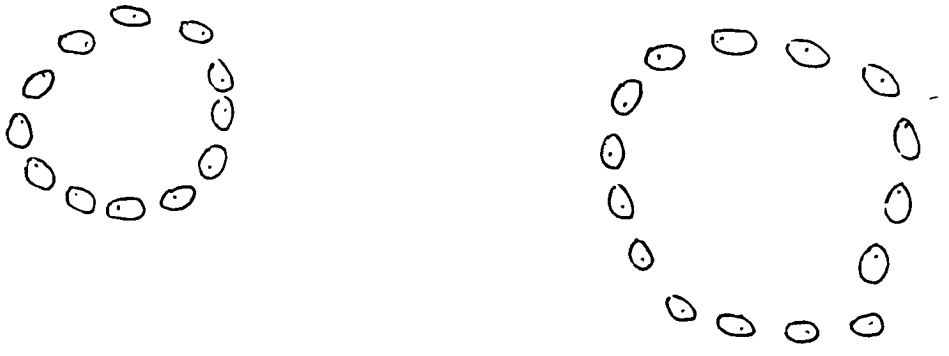


Figure 4.6 Tracings of two zoospores of *P. aphanidermatum* at 0.1 sec intervals in the presence of 150μM lanthanum, showing continuous circular swimming.

50μm

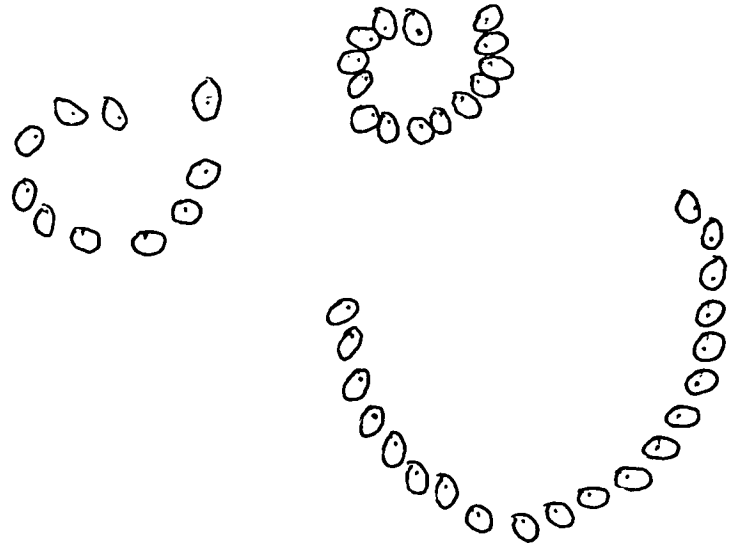


Figure 4.7 Tracings of three zoospores of *P. aphanidermatum* at 0.1 sec intervals in the presence of 5μM trifluoperazine, showing circular or spiral motion with a posterior "drift".

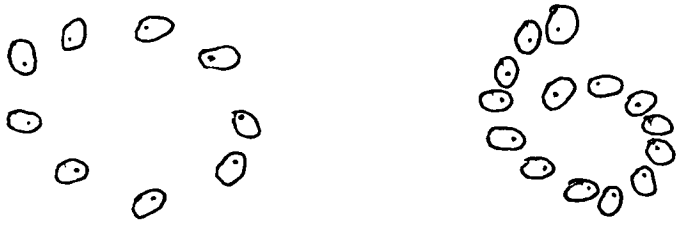


Figure 4.8 Tracings of two zoospores of *P. aphanidermatum* at 0.3 sec intervals in the presence of 40μM dibucaine, showing circular or spiral motion with a posterior "drift".

50μm

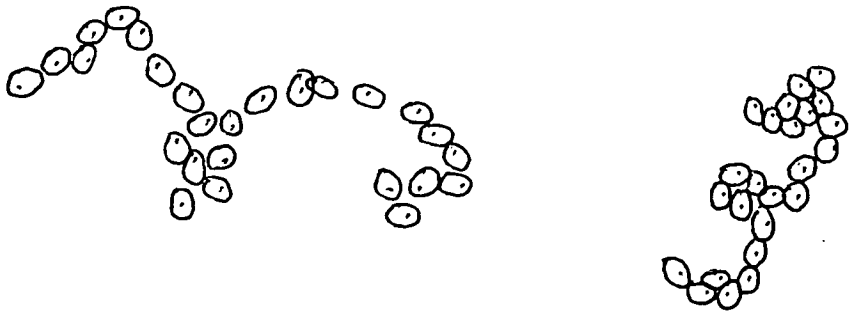


Figure 4.9 Tracings of two zoospores of *P. aphanidermatum* at 0.1 sec intervals in the presence of 20μM amiloride,showing "jerky" swimming with frequent changes of direction.

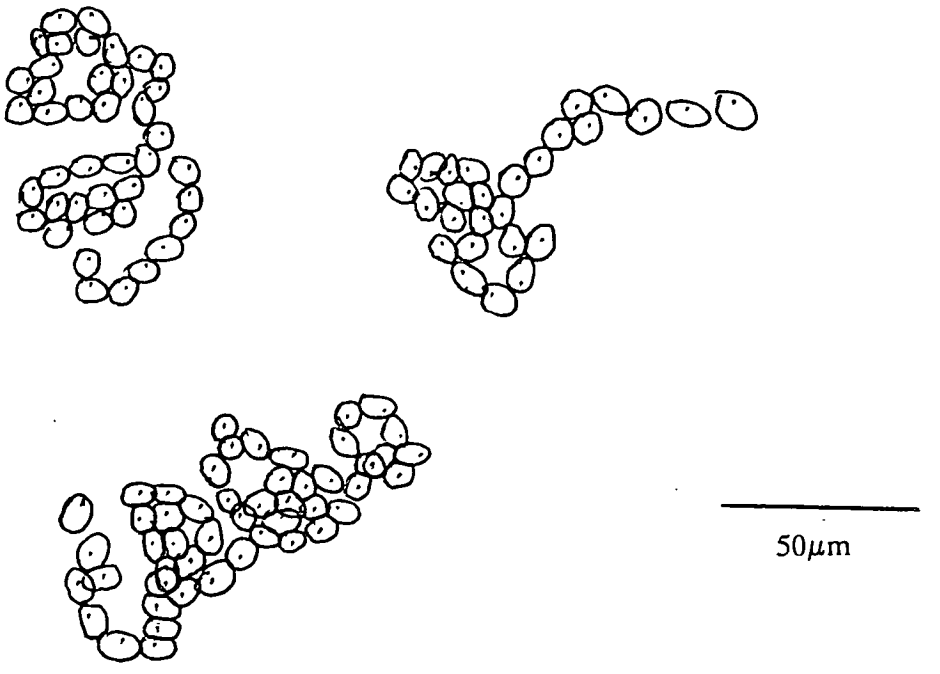


Figure 4.10 Tracings of three zoospores of *P. aphanidermatum* at 0.1 sec intervals in the presence of 3μM A23187, showing "jerky" swimming with frequent changes of direction.

Table 4.24 Effects of L-amino acids and glycine supplied to the zoospore bathing medium (7mM final concentration) on the motile behaviour of *Pythium catenulatum* [+]

Attributes of [+] locomotion	Treatment: Buffer	Glutamine	Glutamate	Glycine	Lysine
Speed (μms^{-1})	101 ± 5.1	106 ± 3.8	108 ± 5.4	89 ± 8.1	114 ± 6.4
Velocity (μms^{-1})	124 ± 4.9	128 ± 2.9	128 ± 3.6	120 ± 5.3	114 ± 6.4
Frequency (Hz)	0.8 ± 0.03	0.8 ± 0.02	0.8 ± 0.03	0.8 ± 0.02	0.8 ± 0.03
Amplitude (μm)	11 ± 1.6	11 ± 1.7	10 ± 1.6	10 ± 1.8	11 ± 1.4
Wavelength (μm)	117 ± 5.0	110 ± 4.4	121 ± 4.5	123 ± 4.1	115 ± 5.4
Time (sec^{-1}) between changes of direction	3.6 ± 0.53	2.2 ± 0.53	2.3 ± 0.47	3.3 ± 0.39	2.6 ± 0.49
Distance (μm) between changes of direction	333 ± 48.1	219 ± 32.8	220 ± 37.8	285 ± 26.4	191 ± 29.2

[+] Means ± s.e. for 12 replicate tests 10 min after addition of amino acids; motility parameters within treatments analysed for the same number of replicates but not necessarily for the same spores. For details see Figure 4.2.

Table 4.25 Effects of L-amino acids supplied to the zoospore bathing medium (7mM final concentration) on the motile behaviour of *Pythium dissotocum* [+]

	Treatment				
Attributes of locomotion	Buffer	Glutamine	Glutamate	Alanine	Lysine
Speed (μms^{-1})	99 ± 7.0	96 ± 1.9	101 ± 5.2	106 ± 6.3	109 ± 4.6
Velocity (μms^{-1})	122 ± 6.1	119 ± 3.2	126 ± 3.9	132 ± 4.4	134 ± 4.3
Frequency (Hz)	0.8 ± 0.04	0.8 ± 0.02	0.8 ± 0.03	0.8 ± 0.03	0.8 ± 0.02
Amplitude (μm)	11 ± 1.8	11 ± 2.0	12 ± 1.6	12 ± 1.9	12 ± 2.2
Wavelength (μm)	116 ± 5.2	109 ± 3.6	120 ± 4.2	113 ± 4.6	103 ± 5.4
Time (sec^{-1}) between changes of direction	2.4 ± 0.40	1.9 ± 0.20	1.6 ± 0.21	2.3 ± 0.26	2.2 ± 0.21
Distance (μm) between changes of direction	234 ± 41.0	186 ± 21.4	161 ± 25.0.	235 ± 26.2	218 ± 22.1

[+] Means ± s.e. for 12 replicate tests 10 min after addition of amino acids; motility parameters within treatments analysed for the same number of replicates but not necessarily for the same spores. For details see Figure 4.2.

Table 4.26 Effect of ethanol (25mM) supplied to the zoospore suspending medium on the motile behaviour of zoospores of *Pythium* spp.

Attributes of locomotion[+]	*P. catenulatum*		*P. dissotocum*	
	Control	Ethanol	Control	Ethanol
Speed (μms^{-1})	117 ± 3.9	118 ± 4.1	123 ± 4.2	125 ± 2.7
Velocity (μms^{-1})	137 ± 3.1	130 ± 4.9	144 ± 3.5	148 ± 3.5
Frequency (Hz)	0.9 ± 0.02	0.8 ± 0.02	0.8 ± 0.02	0.8 ± 0.02
Amplitude (μm)	11 ± 1.6	10 ± 2.0	11 ± 2.9	12 ± 1.5
Wavelength (μm)	123 ± 4.7	120 ± 3.3	124 ± 4.4	126 ± 4.2
Time (sec^{-1}) between changes of direction	2.2 ± 0.25	2.0 ± 0.27	1.8 ± 0.24	1.9 ± 0.28
Distance (μm) between changes of direction	245 ± 27.8	225 ± 35.3	194 ± 30.5	213 ± 33.1

[+] Means ± s.e. for 11 replicate tests 10 min after addition of ethanol; motility parameters within treatments analysed for the same number of replicates but not necessarily for the same spores. For details see Figure 4.2.

aim of this experiment was to test whether similar effects of ethanol, and attractant amino acids for comparison, could be obtained for *Pythium* spp. If so this might explain how some background amino acids had been able to reduce or block chemotaxis to a gradient of L-glutamate (Section 4.1.2). Thus the amino acids chosen for use as background compounds were the chemoattractant amino acids L-glutamine and L-glutamate (Section 4.1) both of which negated taxis to L-glutamate (Section 4.1.2). In addition, the attractant L-alanine (Section 4.1) and the non attractant glycine (Section 4.1) were used because both had blocked chemotaxis towards L-glutamate (Section 4.1.2). L-lysine, a non-attractant which also did not inhibit chemotaxis to L-glutamate (Section 4.1.2) was used for comparison. *P. catenulatum* and *P. dissotocum* were used in these tests, and in all cases their motility characteristics were wholly unaffected by the presence of either ethanol (25mM) or any of the amino acids (Tables 4.24-4.26).

4.3. Discussion

The results in this section demonstrate that amino acids and sugars are chemoattractants of *Pythium* zoospores. In addition, the motile behaviour of fungal zoospores, which determines co-ordinated movement and chemotaxis, could be manipulated by addition of cations or Ca^{2+} modulators to the zoospore suspension.

Zoospores of the three *Pythium* spp. tested showed chemotaxis to the same range of L-amino acids, only L-alanine, L-asparagine, L-glutamine, L-aspartic acid and L-glutamic acid (Table 4.1). This largely agrees with the work of Jones *et al.* (1991) on *P. aphanidermatum* except that L-phenylalanine also was then found to elicit weak chemotaxis. Similarly, Royle & Hickman (1964b) showed attraction of *P. aphanidermatum* zoospores to both glutamate and aspartate, and Halsall (1976) demonstrated that aspartic acid, glutamic acid, asparagine and glutamine, but not alanine, in the range 0.05-10mM, would elicit chemotaxis of zoospores of *Phytophthora* spp. Khew & Zentmyer (1973), found chemotaxis of *Phytophthora* spp. to glutamic and aspartic acid but also to arginine and methionine, which were not found to be attractive to the *Pythium* spp. here (Table 4.1).

There is thus a broad measure of agreement on at least some of the

amino acids that commonly elicit taxis by *Pythium* and *Phytophthora* spp. Nevertheless, different reports seldom give completely consistent results, even for the same species. For example, with *P. aphanidermatum* Royle & Hickman (1964b) reported strong attraction to glutamate and aspartate whereas Chang-Ho & Hickman (1970) working with a different isolate found strong attraction to glutamine and serine and weak attraction to glutamate, proline and histidine. Jones *et al.* (1991) and the work here, based on a third isolate, showed attraction only to glutamate, glutamine, aspartate, asparagine and alanine. Such differences might be due to the use of different test conditions or of different isolates. Halsall (1976) has shown, for *P. cinnamomi*, that isolates within a species, from different geographical origins, can show differences in the amino acids to which they respond. This is the likely explanation for the different findings on *P. aphanidermatum*, mentioned above, but it can be tested only in direct comparative studies of different isolates.

None of the three *Pythium* spp. tested showed chemotaxis to any D-amino acid, suggesting that attraction was stereospecific (Table 4.3). Contrastingly, zoospores of the nematode-parasitic fungus *Catenaria anguillulae* were found to show attraction towards both D and L-forms of serine (Jansson & Thiman, 1992). It cannot be discounted that the concentrations of D-amino acids tested here were too weak to elicit a response, because *Escherichia coli* is known to be attracted to D-isomers at a 100-fold higher threshold concentration than the corresponding L-amino acids (Mesibov & Adler, 1972).

Whereas all three *Pythium* spp. responded to the same amino acids, they differed in responses to sugars. Mannose attracted all three species whereas glucose, fucose, fructose, sucrose and maltose attracted one or two of the *Pythium* spp (Table 4.5). This finding for *P. aphanidermatum* agrees with the work of *Jones et al.* (1991) who found chemotaxis to fructose, mannose, maltose and sucrose by the same isolate. However, Royle & Hickman (1964b) found no attraction of *P. aphanidermatum* spores towards maltose, again perhaps because they used a different isolate from that here. Interestingly, in some cases stronger attraction was observed with lower concentrations of some sugars. This was similarly observed with *Phytophthora* zoospores (Halsall, 1976) whereby lower levels of amino acids were better at attracting zoospores, and the spores were observed to move down a concentration gradient to accumulate at a most favourable concentration.

Zoospores of *Phytophthora* spp. are also reported to show chemotaxis to alcohols such as ethanol (Allen & Newhook, 1974; Cameron &

Carlile, 1978) which is expected to be produced by roots during waterlogging of soil (Bolton & Erickson, 1970). However, neither *P. aphanidermatum*, as also reported by Jones *et al.* (1991), nor *P. catenulatum* or *P. dissotocum* showed attraction towards ethanol (Table 4.4).

In general, the responses obtained with amino acids, especially in the case of L-glutamic acid and L-aspartic acid, were stronger than those obtained with sugars. Similarly, Royle & Hickman (1964b) and Chang-Ho & Hickman (1970), working with *P. aphanidermatum*, demonstrated that most of the chemotactic activity in fractionated root exudates was in the amino acid fraction. In contrast, Rai & Strobel (1966) showed that the sugar and organic acid fractions of sugar beet roots were the most attractive towards zoospores of *Aphanomyces cochliodes*. The rumen chytrid *Neocallimasix frontalis* showed strong attraction towards sugars but not at all to amino acids (Orpin & Bountiff, 1978), consistent with the ecology and nutrition of this fungus.

It is notable that the threshold concentrations of L-glutamic and L-aspartic acid that elicited chemotaxis of zoospores of *P. aphanidermatum* in the conditions of this study- 1 and 4mM respectively (Table 4.2)- are relatively high compared to those for *E. coli*, which is attracted to glutamic acid and aspartic acid at levels down to $5x\ 10^{-6}$ M and $6x\ 10^{-8}$ M respectively (Adler, 1975). Also *N. frontalis* responds to fructose at $5x\ 10^{-6}$ M (Orpin & Bountiff, 1978). Nevertheless it is known that phycomycete zoospores, in general, are less sensitive to low concentrations of attractants than are bacteria (Chet & Mitchell, 1976). Evidently they are also less sensitive than is *N. frontalis*.

In order for micro-organisms to show chemotaxis, they must detect compounds such as amino acids and sugars by chemoreceptors, which in the case of zoospores are thought to be present on their membrane (Carlile, 1966). Bacterial chemosensors are well characterised-especially those detecting amino acids (Mesibov & Adler, 1972). In an attempt to characterise the zoospore receptors that elicit chemotaxis the method of Adler (1969) was adopted to investigate the putative receptor for L-glutamic acid. L-glutamic acid was chosen for this because, with *P. aphanidermatum*, it was the most highly and consistently attractive compound. The method, involving the ability of saturating background levels of compounds to block taxis to compounds sharing the same receptor, has been used successfully to deduce the presence of four different sugar receptors on the zoospores of *N. frontalis* (Orpin & Bountiff, 1978).

The results for the three *Pythium* spp. summarised in Table 4.27, however, were difficult to explain. Background concentrations of the

Table 4.27 Ability of the listed amino acids (7mM background unless stated) significantly to block (B) or reduce (R) taxis of zoospores to L-glutamic acid in capillary tubes; 0 = no effect.

	Effect of L-amino acids (with D-amino acids where effective in parentheses)		
Background amino acid†	*P. aphanidermatum*	*P. catenulatum*	*P. dissotocum*
Asn	B (R)	0	0
Glu*	B	B	B
Gln	B (B)	R	B
Ala	B (R)	R	R
Asp*	0	0	0
Thr/Trp	R (R)	0	0
His/Gly	B (R)	R	0
Met	R	0	0
Phe/Val/Lys/ Leu/Ile/Ser/ OHPro	0	0	0

† Asn, asparagine; Glu, glutamate; Gln, glutamine; Ala, alanine; Asp, aspartic acid; Thr, threonine, Trp; tryptophan; His, histidine; Gly, glycine; Met, methionine; Phe, phenylalanine; Val, valine; Lys, lysine; Leu, leucine; Ile, isoleucine; Ser, serine; OHPro, hydroxyproline.

* Glu used at 2mM background, with 7mM glu in capillary; asp used at 3mM background, with 3mM glu in capillary.

chemoattractants L-alanine, L-glutamine and L-glutamic acid, in addition to L-asparagine in the case of *P. aphanidermatum*, significantly reduced or blocked chemotaxis towards L-glutamic acid. In contrast, L-aspartic acid, which is also a chemoattractant, had no significant effect on chemotaxis of any of the three *Pythium* spp. towards L-glutamic acid. Furthermore, in reciprocal tests involving *P. aphanidermatum* (Table 4.12) background concentrations of L-glutamate were found to block chemotaxis towards L-alanine, L-asparagine, L-glutamine but not to L-aspartic acid. These results could be taken to suggest that the receptor detecting glutamic acid in *P. aphanidermatum* also senses L-asparagine, L-alanine and L-glutamine whereas a separate receptor senses L-aspartic acid. But for *P. catenulatum* and *P. dissotocum* there may also be a separate chemosensor detecting L-asparagine. Khew & Zentmyer (1973), using the same experimental design, similarly proposed that zoospores of *Phytophthora* have separate chemoreceptors for glutamic and aspartic acid. In contrast, in *E. coli* the aspartate receptor detects both L-glutamic and L-aspartic acid whereas the serine receptor detects L-serine and L-alanine and L-asparagine is detected by both receptors (Mesibov & Adler, 1972). Such simple explanations for *Pythium* spp. are complicated, however, by the finding that some non-attractant amino acids also blocked or reduced taxis to L-glutamate, as did some D-forms of attractant and non-attractant amino acids (none of which was an attractant in the D-form).

Thus, the non-attractants L-histidine and glycine, reduced chemotaxis of *P. aphanidermatum* and *P. catenulatum* zoospores towards L-glutamic acid. In addition both the L- and D-forms of threonine, histidine and tryptophan blocked chemotaxis of *P. aphanidermatum* spores towards glutamic acid but only L-histidine had an equivalent effect for *P. catenulatum* whereas *P. dissotocum* was wholly unaffected (Table 4.27). These results suggest that background concentrations of non-attractive amino acids can interfere with chemotaxis non-specifically. General interference of chemotaxis by background concentrations of amino acids has also been reported for zoospores of *Phytophthora* (Khew & Zentmyer, 1973) whereas in *E. coli* aspartic acid inhibits taxis towards threonine even although both are detected by different receptors (Mesibov & Adler, 1972). The mechanism whereby non-attractant amino acids interfere with chemotaxis remains unclear. The number of instances in which this was found for the *Pythium* spp., and the fact that few consistent patterns were found for the three *Pythium* spp., served to discourage further work on this

phenomenon. A possible more fruitful approach in further studies might employ mutants auxotrophic for specific amino acids.

When zoospores were observed to show chemotaxis towards glutamic acid diffusing from a microcapillary, they swam into the tube and encysted at a given point within it. When the distance of this point from the capillary mouth was plotted against time (Figure 4.1) a linear relationship was obtained. This suggests that, due to diffusion of the tube contents from the capillary mouth the concentration of glutamic acid required to induce encystment was encountered further down the tube with time. Bacteria also are known to migrate into capillaries filled with an attractant, as a band which progresses at a constant speed, producing a linear relationship with time (Adler & Dahl, 1967). It was also noted that the distance that zoospores travelled down a capillary, before encysting, became less with increasing concentrations of glutamic acid. In the case of high concentrations of glutamate, spores were initially attracted and then repelled, without encysting, before swimming back down the capillary. It is likely that such repulsion of *P. aphanidermatum* spores is a threshold response, being elicited at a critical concentration of glutamic acid, as observed for negative chemotaxis of *Phytophthora* (Allen & Harvey, 1974; Cameron & Carlile, 1980) whereby spores were observed to retreat linearly with time on encountering a critical, potentially toxic cation concentration.

A likely explanation of these results is that zoospores of *P. aphanidermatum* sense optimum concentrations of L-glutamate that elicit chemotaxis and encystment. Similarly, *Phytophthora* zoospores at high concentrations of glutamine or asparagine showed a reduced chemotactic response and formed a halo around the source of attractant suggesting that spores moved up to but not beyond a favourable concentration that was not potentially chaotropic (Halsall, 1976). It is possible that *in vivo* there are differing concentrations of nutrients released by roots, some of these nutrients being able to elicit chemotaxis and encystment at different concentrations than do others. Indeed, Chi & Sabo (1978) have reported that zoospores of *Phytophthora megasperma* detect a gradient of alfalfa root diffusate but move away from the maximum concentration at the source and travel to the optimum concentration in the gradient.

For zoospores to show chemotaxis to roots, or attractants, diffusing from a capillary, they must couple their chemosensory perception with changes in motility. In bacteria Ca^{2+} is reported to regulate chemotaxis by determining

the patterns of motility (Ordal, 1977). Similarly Ca^{2+} and other divalent cations were found to alter the motile behaviour and chemotactic ability of *P. aphanidermatum* zoospores. Zoospores in the presence of water or phosphate buffer show patterns of wave-like motility interspersed with frequent random changes of direction (Figure 4.3). However, addition of the divalent cations Ca^{2+}, Mg^{2+}, Sr^{2+}, Mn^{2+} or Ba^{2+} induced spores to swim in perpetual circles (Tables 4.17-4.21). Similarly, the initiation of trout sperm motility involves a circular swimming mode which can be induced by addition of Ca^{2+} or Mg^{2+} to the media (Boitano & Omoto, 1992). The cells then swim in an approximately linear mode. However after the sperm come to rest, after a naturally short (20-60 sec) period of motility, they again revert to circular swimming if in the presence of external Ca^{2+}. The responses of *P. aphanidermatum* to divalent cations were in some cases dependent on concentration, as for Mn^{2+} (Table 4.20), whereas some cations were capable of eliciting two different modes of motility within the same population, as in the case of Sr^{2+} (Table 4.19).

In contrast to these effects of divalent cations, monovalent (Table 4.13-4.15) and trivalent (Table 4.16) cations caused no change in the motile behaviour of spores compared to water controls. This contrasts with the work of Thomas & Butler (1989) who demonstrated that K^+ reduced the motile speed and induced spores of *Achlya heterosexualis* to swim in circles whereas this could be counteracted by subsequent addition of Ca^{2+}.

The calcium-chelation agent EGTA (Schmid & Reilley, 1957), alone (Table 4.17) or in combination with monovalent cations (Tables 4.13-4.15), caused a different type of swimming perturbation whereby random changes of direction were completely suppressed and the amplitude and velocity of the swimming helix were markedly reduced (Figure 4.4). Unfortunately, all attempts to reverse such effects of Ca^{2+} and EGTA by subsequent use of other treatments were unsuccessful, in contrast to the work of Thomas & Butler (1989) on *Achlya* spores, because any subsequent treatment of spores with perturbed swimming modes led to premature encystment. However, eventually the spores could resume their normal motion as seen after 50 min for spores treated with Mg^{2+} and EGTA (each at 125μM). They were also able to change from one mode to another, as when spores were treated simultaneously with Ca^{2+} or Mg^{2+} and equimolar concentrations of EGTA (Tables 4.17, 4.18). In these cases they were induced initially to swim in perpetual circles, presumably in response to the divalent cations, whereas by 50 min spores swam in straight lines, with no random changes of direction, typical of motility elicited by EGTA

(Table 4.17). Ca^{2+} similarly causes *Bacillus subtilis* to tumble, in a random orientation, whereas EGTA causes the bacteria to swim in a more direct line (Ordal, 1977), mirroring the effects of these supplements on turning versus straight swimming of *Pythium* zoospores. The results of further treatments applied to *Pythium* spores showed that such responses were under complex control. For example treatment with Ba^{2+} (125μm) or Mn^{2+} (500μm), caused spores to swim in circles even if EGTA was added at the same time (as with Ca^{2+} or Mg^{2+}) but by 50 min they had reverted to normal patterns of motility (Tables 4.20, 4.21). The presence of Fe^{3+} (which had no effect alone) negated the effects of EGTA, causing spores to show normal modes of motility (Table 4.16). Yet another pattern was seen with Sr^{2+} which, when added alone, caused spores to circle, but which suppressed any change in motility when added in combination with Ca^{2+} and EGTA (Table 4.19). Despite such complications, which have yet to be explained, it is encouraging that, as in the work of Thomas & Butler (1989), altered patterns of motility can be elicited by the addition or sequestration of cations and that such changed patterns are maintained for many minutes.

In addition to cations and EGTA, the motile behaviour of *Pythium* zoospores could be regulated by the addition of drugs modulating Ca^{2+} levels. Lanthanum and verapamil, which are Ca^{2+} channel blockers (Godfraind *et al.* 1986), caused spores to swim in circles (Figures 4.5, 4.6) thus resembling the effects of divalent cations. The Ca^{2+} ionophore A23187 (Pressman, 1976) and amiloride greatly reduced the motile speed (Table 4.23) and elicited an enhanced frequency of directional change which retained spores in an almost unchanged position. However, in several cases the zoospore tracks followed still displayed an approximate helical swimming pattern (Figures 4.9, 4.10). Similarly, A23187 has been reported to affect the motility of bacteria, causing *B. subtilis* to perpetually tumble (Ordal, 1977). A23187 is a Ca^{2+} ionophore (Pressman, 1976) and amiloride can inhibit Na^{+}/Ca^{2+} flux across cell membranes (Godfraind *et al.,* 1986) so both would be expected to alter the internal-to-external Ca^{2+} ratio, which perhaps is the basis of their common effect on zoospores. Indeed, it is likely that amiloride acted mainly by affecting Ca^{2+} levels and not that of Na^{+} because this ion was found to have no effect on zoospore motility (Table 4.15).

In contrast trifluoperazine (TFP) and dibucaine, which are antagonists of the Ca^{2+}-binding protein calmodulin (Bereza *et al.,* 1982), caused a significant reduction of zoospore swimming speed (Table 4.23) in addition to

eliciting a posterior drift of the spores (Figures 4.7, 4.8) as though the posterior flagellum was exerting an inordinate force compared to the anterior flagellum which did not appear to be operating normally. Thomas & Butler (1989) found that TFP, at 5μm, arrested motility of spores of *A. heterosexualis* but with *Pythium* it enabled spores to continue swimming for at least 10 min- albeit with a perturbed mode. The effects of TFP and dibucaine on the anterior flagellum of *Pythium* spores might be correlated with the observation of Gubler *et al.* (1990), that calmodulin was localised at the base of the anterior flagellum of *Phytophthora* zoospores, as evidenced by intense immunofluorescent staining.

In the work with *Pythium* above it was possible to detect five distinct swimming modes- the normal mode, EGTA mode, perpetual circular mode, "Ca^{2+} flux inhibitor mode" and calmodulin inhibitor mode. Unfortunately, it was not possible to visualise the flagella of the motile spores using the video system employed. But from other studies it may be deduced that all the treatments influenced flagellar activity in one way or another.

Analysis of *Phytophthora* zoospores suggests that the anterior flagellum provides almost 90% of the forward thrust and that the posterior flagellum acts mostly as a rudder moving from a trailing position to an orientation perpendicular to the direction of swimming during a spontaneous turn (Holwill, 1982; Carlile, 1983). If this is also the case for *Pythium* zoospores, then it can be deduced that the majority of treatments had no effect on the anterior flagellum as swimming speed, compared to water controls, was largely unaffected. Instead, the majority of treatments changed the orientation of swimming thus implicating an effect on the posterior flagellum. The perpetual straight swimming elicited by EGTA (Figure 4.4) may have been caused by permanent extension of the posterior flagellum. In contrast, circular swimming induced by divalent cations and Ca^{2+} channel blockers (Figures 4.5, 4.6) could have been due to permanent deflection of the posterior flagellum. Trout sperm flagella are frequently observed to be cane-shaped and bent upon cessation of motility if this is preceded by circular swimming induced by Ca^{2+}, but remain straight when the cells come to rest after straight swimming (Boitano & Omoto, 1992).

In other studies involving zoospores the effects of cations and other compounds in eliciting changes in motility have been explained in terms of changes in membrane polarisation (Allen & Harvey, 1974; Cameron & Carlile, 1980). It is reported that the plasma membrane of *P. cinnamomi* hyperpolarises during chemotaxis towards ethanol, such that the depolarisation which

accompanies flagellar re-orientation during spontaneous changes of direction is eliminated (Allen & Newhook, 1974). During the "avoidance response" (negative chemotaxis) to high concentrations of cations (Allen & Harvey, 1974; Cameron & Carlile, 1980) it is proposed that the cations act to reduce the negative charge of the zoospore surface. This in turn changes the transmembrane potential, altering flagella activity, perhaps by collapsing the electrostatic charge maintaining the rigid conformation of the flagella and thereby causing the zoospore to turn (Allen & Harvey, 1974). Similarly, changes in transmembrane potential affect the motile behaviour of bacteria (Szmelcman & Adler, 1976) and *Paramecium* (Van Houten, 1979) by altering flagellar and ciliary activity respectively.

Calcium currents are reported to be involved in signal transduction in other flagellate or ciliated cells. For example, the taxis of *Chlamydomonas* to light can be inhibited by the Ca^{2+} channel blocker verapamil (Harz & Hegemann, 1991). Similarly, spatio-temporal gradients of intracellular Ca^{2+} are known to correlate with changes of motility and with chemotaxis of both *Physarum* (Natsume *et al.*, 1992) and eosinophils (Brundage *et al.*, 1991). Divalent cations can also have direct effects on the axonemal components of the flagella as seen by their effects on motility and sliding disintegration of demembranated macrocilia of the ctenophore *Beroe* (Tamm, 1989). The distinction between the effects of cations on the "avoidance response" of zoospores and the effects noted in this study on zoospore motility is that the avoidance reaction occurs in response to all cations- it is non-specific. In contrast, as noted earlier, all the changes of motility seen with *Pythium* zoospores in this study were induced specifically by Ca^{2+} (or other divalent cations) or by substances that modulate Ca^{2+} levels, and always at cation levels lower than those that elicit avoidance reactions. This strongly suggests a specific role of Ca^{2+} (or other substitute divalent cations) in determining motility patterns in general.

With regard to chemotaxis of zoospores it has been postulated that fungal spores have two or more spatially separated chemoreceptors which provide information on the direction of the attractant by recording different intensities of stimulation (Carlile, 1966). During chemotaxis the zoospore turns in the direction of those receptors experiencing the greater stimulation until the receptors are stimulated equally with respect to the longitudinal axis of the zoospore. This then enables the spore to swim directly towards the attractant.

An alternative hypothesis is that zoospores sense changes in concentrations of chemoattractants by rapidly adjusting (sensory adaptation) to an existing level, such that they can still respond to an increased level. Such sensory adaptation mechanisms are well characterised for bacteria, in which they involve changes in degree of methylation of proteins linked to the chemoreceptors, altering the affinity of the receptor for binding to the attractant (Ames *et al.,* 1988; Koshland *et al.,* 1988; Bischoff & Ordal, 1992).

Increasing evidence suggests that there is a strong link between modes of motile behaviour, chemotaxis and transmembrane signalling in cilia and flagella, which in some cases may be governed by divalent cations (Bloodgood, 1991). To explain this relationship Carlile (1980) has proposed that a stimulus is perceived by a primary receptor which may in turn transmit the message to a secondary receptor which results in a change of membrane potential. The internal Ca^{2+} concentration of the cell then changes which alters the motor activity of cilia or flagella leading to a directional change and a tactic response. In the *Pythium* spores examined it is likely that the stimuli eliciting taxis *in vivo* will include sugars and amino acids rather than Ca^{2+} *per se* (Tables 4.1, 4.5). Ca^{2+} would then serve as part of the signal transduction sequence that links changes in motile behaviour to the binding of an attractant to an external receptor. In other words, the treatments that caused different patterns of motility (Ca^{2+}, EGTA, Ca^{2+} modulators) would have acted by by-passing or overloading the normal Ca^{2+}-mediated process, locking the zoospores into different perpetual swimming modes. This would account for the observation that spores in perturbed swimming modes failed to display a tactic response towards L-glutamate (Table 4.22).

Interestingly, neither attractant nor non-attractant amino acids were found to affect the motile behaviour of *Pythium* zoospores (Table 4.24, 4.25). This is not unexpected if zoospores show rapid sensory adaptation to a given level of attractant (Carlile, 1983). But it leaves unexplained the finding that various backgrounds of amino acids blocked taxis to L-glutamate (Tables 4.6-4.8).

In future the role of cations and attractants that affect chemotaxis and zoospore motility could be more easily interpreted, microscopically, by studying their effects on flagellar conformation and action. However, to date this has proved unsuccessful because zoospores with perturbed swimming modes are easily disrupted in the shallow liquid films needed for observation of flagellar movements.

5. EFFECTS OF NUTRIENTS, CATIONS AND CA^{2+} MODULATORS ON INDUCTION OF ENCYSTMENT AND GERMINATION OF *PYTHIUM* SPP.

5.1 Effects of nutrients on encystment

In contrast to chemotaxis, zoosporic encystment has been shown to exhibit host- and substrate-specificity (Mitchell & Deacon, 1986 a,b). More generally, zoospores can be induced to encyst by nutrients present in root exudates (Royle & Hickman, 1964a), including amino acids (Dill & Fuller, 1971) and sugars (Longman & Callow, 1987). However, in this respect there has been no direct comparative studies on the behaviour of different fungi.

The aim here was to compare the effects of amino acids and sugars on encystment of three different *Pythium* spp. Methods were as described in Section 2.3.2.

From a wide range of amino acids tested (Tables 5.1, 5.2) only L-glutamic acid and L-aspartic acid were found to elicit encystment, and did so for all three fungi (*P. aphanidermatum, P. catenulatum* and *P. dissotocum*). The other L-amino acids (Table 5.1) and all D-amino acids (Table 5.2) were wholly ineffective at concentrations up to 40mM.

In experiments designed to determine the threshold concentrations of L-glutamate and L-aspartate required to induce encystment of *P. aphanidermatum*, significant levels of encystment, compared to phosphate buffer controls, were not recorded below 5 and 6mM respectively (Table 5.3).

None of the sugars tested significantly induced encystment by any of the three *Pythium* spp. (Table 5.4).

5.2 Attempts to block L-glutamate induced encystment by incubation with other amino acids

Encystment of zoospores, which is stimulus-induced (Irving *et al.,* 1984), may be receptor-mediated (Hardham & Suzaki, 1986; Estrada-Garcia *et al.,* 1990b; Kelleher *et al.,* 1991), in which case it might be possible to block the encystment response to specific amino acids, using backgrounds of other compounds that are recognised by the receptors. The aim of this experiment was to test this possibility for *P. aphanidermatum* using L-glutamate as the encystment inducing agent and pre-incubating the zoospores in the presence of non-inducing amino acids. In all tests, however, (Table 5.5) the addition of L-glutamate (7mM final

Table 5.1 Effects of L-amino acids (40mM final concentration) on induction of encystment of zoospores of three *Pythium* spp. ; values shown are means ± s.e. for 9 replicates, with matched buffer controls, based on counts of 100 spores for each replicate after 1h.

Treatment[+]	P. aphanidermatum		P. catenulatum		P. dissotocum	
	Test	Control	Test	Control	Test	Control
Leu	11 ± 1.5	9 ± 1.3	7 ± 1.1	7 ± 1.0	10 ± 1.8	11 ± 1.5
Trp	10 ± 1.0	8 ± 1.4	9 ± 1.4	7 ± 1.0	13 ± 1.8	11 ± 1.5
His	10 ± 1.1	10 ± 1.6	6 ± 1.2	7 ± 1.0	11 ± 1.4	11 ± 1.5
Met	10 ± 1.5	9 ± 1.1	5 ± 0.9	7 ± 1.0	13 ± 1.7	11 ± 1.5
Asn	8 ± 1.0	9 ± 1.1	7 ± 1.1	6 ± 1.0	9 ± 1.3	11 ± 1.5
Phe	9 ± 0.8	11 ± 1.1	6 ± 0.9	7 ± 1.0	9 ± 0.9	8 ± 0.9
Arg	8 ± 1.4	9 ± 1.2	10 ± 1.3	10 ± 1.2	14 ± 2.2	12 ± 1.6
Ser	14 ± 2.3	13 ± 1.3	9 ± 0.9	10 ± 1.3	14 ± 1.6	12 ± 1.6
Pro	8 ± 1.5	10 ± 1.2	11 ± 1.4	10 ± 1.3	9 ± 1.7	11 ± 1.5
Ile	15 ± 1.6	13 ± 1.3	10 ± 1.0	10 ± 1.3	15 ± 1.5	12 ± 1.6
OHPro	11 ± 1.2	13 ± 1.3	12 ± 1.2	10 ± 1.3	10 ± 1.9	12 ± 1.6
Ala	9 ± 1.5	9 ± 1.1	8 ± 1.6	10 ± 1.3	11 ± 1.3	12 ± 1.6
Thr	9 ± 1.3	8 ± 1.5	9 ± 1.4	10 ± 1.3	12 ± 1.6	12 ± 1.6
Val	10 ± 1.5	10 ± 1.6	10 ± 0.9	10 ± 1.3	8 ± 1.5	8 ± 1.0
Lys	11 ± 1.5	11 ± 1.1	13 ± 1.5	10 ± 1.4	12 ± 1.3	12 ± 1.6
Asp	100 ± 0*	10 ± 1.3	98 ± 0.9*	10 ± 1.4	92 ± 4.0*	8 ± 1.0
Gln	11 ± 1.8	11 ± 1.1	9 ± 1.2	10 ± 1.4	6 ± 1.9	8 ± 1.0
Glu	100 ± 0*	10 ± 1.3	98 ± 0.8*	10 ± 1.4	95 ± 1.8*	8 ± 1.0
Gly[++]	9 ± 1.5	10 ± 1.6	10 ± 1.1	10 ± 1.4	7 ± 1.1	8 ± 1.0

* Values differ significantly ($\underline{P}$= 0.001) from matched controls by paired-samples t-tests.

[+] Leu, leucine; Trp, tryptophan; His, histidine; Met, methionine; Asn, asparagine; Phe, phenylalanine; Arg, arginine; Ser, serine; Pro, proline; Ile, isoleucine; OHPro, hydroxyproline; Ala, alanine; Thr, threonine; Val, valine; Lys, Lysine; Asp, aspartic acid; Gln, glutamine; Glu, glutamic acid, Gly, glycine.

[++] Glycine is included but has no L or D-isomers.

Table 5.2 Effects of D-amino acids (40mM final concentration) on induction of encystment of zoospores of three *Pythium* spp. ; values shown are means ± s.e. for 9 replicates, with matched buffer controls, based on counts of 100 spores for each replicate after 1h *

Treatment+	P. aphanidermatum		P. catenulatum		P.dissotocum	
	Test	Control	Test	Control	Test	Control
Leu	8 ± 1.0	11 ± 1.0	5 ± 1.1	6 ± 0.8	11 ± 1.5	10 ± 1.4
Trp	9 ± 1.0	9 ± 1.1	6 ± 0.9	6 ± 0.8	9 ± 0.6	10 ± 1.4
His	8 ± 0.8	8 ± 1.0	7 ± 1.0	6 ± 0.8	11 ± 1.0	10 ± 1.4
Met	8 ± 1.0	9 ± 1.1	6 ± 1.0	6 ± 0.8	8 ± 1.0	10 ± 1.4
Asn	10 ± 0.7	9 ± 1.0	7 ± 0.8	6 ± 0.8	9 ± 1.0	10 ± 1.4
Phe	11 ± 1.1	13 ± 1.3	9 ± 0.9	10 ± 0.8	11 ± 1.2	8 ± 1.0
Arg	10 ± 2.1	11 ± 1.0	8 ± 1.2	8 ± 1.2	5 ± 0.9	7 ± 0.9
Ser	11 ± 1.7	9 ± 1.1	13 ± 1.2	9 ± 0.9	7 ± 1.1	7 ± 0.9
Pro	8 ± 1.1	11 ± 1.0	6 ± 1.2	6 ± 0.8	9 ± 1.1	10 ± 1.4
Ile	9 ± 1.4	9 ± 1.1	10 ± 0.9	9 ± 0.9	5 ± 1.0	7 ± 0.9
OHPro	10 ± 1.0	9 ± 1.1	8 ± 1.2	9 ± 0.9	8 ± 1.0	7 ± 0.9
Ala	12 ± 1.5	13 ± 1.3	10 ± 1.1	9 ± 0.9	6 ± 1.0	7 ± 0.9
Thr	7 ± 0.9	9 ± 1.1	7 ± 0.8	9 ± 0.9	6 ± 1.0	7 ± 0.9
Val	14 ± 1.6	16 ± 2.0	11 ± 1.3	10 ± 0.8	8 ± 1.1	8 ± 1.0
Lys	11 ± 1.5	11 ± 1.1	13 ± 1.5	10 ± 1.4	12 ± 1.3	12 ± 1.6
Asp	13 ± 1.3	16 ± 1.9	10 ± 1.2	10 ± 0.8	9 ± 0.8	8 ± 1.0
Gln	14 ± 1.8	13 ± 1.3	12 ± 1.2	10 ± 0.8	9 ± 0.9	8 ± 1.0
Glu	15 ± 2.0	16 ± 1.9	10 ± 1.2	10 ± 0.8	10 ± 0.9	8 ± 1.0
Gly++	9 ± 1.1	8 ± 1.0	10 ± 0.8	10 ± 0.8	9 ± 0.9	8 ± 1.0

* No significant difference was found between amino acid and matching buffer controls in any instance.

+ See Table 5.1

++ Glycine is included but has no L or D-isomers.

Table 5.3 Effects of different concentrations of L-glutamic acid or L-aspartic acid on induction of encystment by zoospores of *Pythium aphanidermatum*

Concentration of amino acid (mM)	Percentage encystment[+]
L-Glutamate	
0	21 ± 3.6
1	21 ± 3.0
2	27 ± 2.1
3	25 ± 4.1
4	24 ± 2.8
5	42 ± 4.4*
6	96 ± 2.8**
7	98 ± 0.2**
8	100 ± 0**
9	100 ± 0**
10	100 ± 0**
L-Aspartate	
0	22 ± 2.1
1	22 ± 2.6
2	22 ± 2.7
3	28 ± 5.9
4	32 ± 5.6
5	37 ± 6.8
6	77 ± 7.5**
7	93 ± 1.4**
8	98 ± 1.4**
9	100 ± 0**
10	100 ± 0**

[+] Means ± s.e. for 9 replicates, based on 100 spores for each replicate; significance of difference from matched buffer controls shown as * ($\underline{P}$ = 0.05) and ** (0.001) based on paired-samples t-tests.

Table 5.4 Effects of sugars (25mM final concentration) on induction of encystment of zoospores of three *Pythium* spp. ; values shown are means ± s.e. for 6 replicates, with matched buffer controls, based on counts of 100 spores for each replicate after 1h.*

Treatment+	P. aphanidermatum		P. catenulatum		P.dissotocum	
	Test	Control	Test	Control	Test	Control
D (+) Mann	9 ± 1.0	11 ± 1.0	16 ± 1.1	15 ± 1.2	13 ± 1.9	12 ± 1.9
L (+) Ara	13 ± 1.8	11 ± 1.0	12 ± 1.7	15 ± 1.2	11 ± 2.6	12 ± 1.9
D (-) Ara	12 ± 1.4	11 ± 1.0	14 ± 1.8	12 ± 1.9	13 ± 1.8	10 ± 1.5
D (+) Xyl	9 ± 1.4	11 ± 1.0	13 ± 1.6	15 ± 1.2	12 ± 2.4	12 ± 1.9
L (-) Fuc	9 ± 1.2	8 ± 1.1	14 ± 1.9	12 ± 1.9	13 ± 1.1	10 ± 1.5
D (+) Gal	8 ± 1.8	8 ± 1.1	13 ± 1.5	12 ± 1.9	10 ± 1.3	10 ± 1.5
D (+) Glc	9 ± 1.3	8 ± 1.1	15 ± 2.2	12 ± 0.9	19 ± 2.5	16 ± 2.1
Suc	7 ± 1.0	8 ± 1.3	14 ± 2.4	12 ± 0.9	15 ± 1.1	16 ± 2.1
Frc	9 ± 0.8	8 ± 1.1	14 ± 1.3	12 ± 0.9	15 ± 1.3	16 ± 2.1
Mal	10 ± 1.4	8 ± 1.1	13 ± 1.9	12 ± 0.9	17 ± 1.6	16 ± 2.1

* No significant difference was found between sugar and matching buffer controls in any instance.

+ Man, mannose; Ara, arabinose; Xyl, xylose; Fuc, fucose; Gal, galactose, Glc, glucose; Suc, sucrose; Frc, fructose; Mal, maltose.

Table 5.5 Effects of L-glutamate (7mM) alone or when zoospores were suspended in a background medium containing other L-amino acids on induction of encystment by *Pythium aphanidermatum* +

Per cent encystment in presence of background amino acids alone	Per cent encystment by L-glutamate (7mM) in presence of background amino acid
range 11 ± 1.7 to 18 ± 2.4	range 99 ± 0.9 to 100 ± 0

+ Means ± s.e. for 9 replicates, based on 100 zoospores in each replicate; the tested amino acids were as listed in Table 3.1. All background amino acids were used at 40mM concentration, except for L-aspartate (3mM) and L-glutamate (2mM).

concentration) induced encystment irrespective of the presence of other amino acids in the zoospore bathing medium. This was true even for backgrounds of L-glutamate (2mM) and L-aspartate (3mM) that were present at sub-effective levels to cause encystment.

5.3 Effects of nutrients on germination

A wide range of amino acids and sugars were tested for their effects on germination of zoospore cysts of three different *Pythium* spp. (Table 5.6-5.8). The cysts had been produced by vortexing the zoospore suspensions (method-Section 2.3.3). In all cases L-glutamate, L-aspartate and L-asparagine significantly elevated germination levels above buffer controls, L-alanine stimulated germination of only *P. catenulatum* and *P. dissotocum*, L-glutamine triggered germination only of *P. catenulatum* and L-methionine only of *P. dissotocum*. In contrast, L-isoleucine significantly reduced germination of *P. aphanidermatum* cysts and L-histidine inhibited germination of *P. catenulatum* and *P. dissotocum* cysts (Table 5.6). D-amino acids (Table 5.7) had no effect on germination for any fungus. In further tests, D+glucose and sucrose enhanced germination of cysts of all three fungi; maltose was effective for *P. aphanidermatum* and *P. dissotocum*, and fructose elicited germination of *P. catenulatum* and *P. dissotocum* (Table 5.8). Other sugars tested had no effect except that D+galactose weakly inhibited germination of *P. dissotocum* (Table 5.8).

5.4 Effects of amino acids and sugars on germ-tube growth

The relatively narrow range of amino acids and sugars that induced germination in the experiments above was of interest because zoospores and zoospore cysts of *P. palmivora* are reported to be unable to take up significant amounts of radiolabelled amino acids (specifically glutamate) or sugars (specifically glucose) until after the germ-tube has grown to some length (Penington *et al.*, 1989). Thus the triggering effect might represent a receptor mediated event rather than a direct nutritional stimulus. In order to explore this possibility, an attempt was made to correlate the effect of amino acids and sugars on germination with effects on germ-tube growth (Table 5.9). Zoospore cysts were added to glass slides immediately after agitation in a vortex (Section 2.3.3). This ensured that a high proportion of the cysts were able to adhere to the glass and

Table 5.6 Effects of L-amino acids (10mM final concentration) on germination of zoospore cysts of three *Pythium* spp. ; values shown are means ± s.e. for 6 replicates, with matched buffer controls, based on counts of 100 spores for each replicate after 16h†

Treatment[+]	P. aphanidermatum		P. catenulatum		P.dissotocum	
	Test	Control	Test	Control	Test	Control
Leu	26 ± 2.0	29 ± 2.0	27 ± 3.0	29 ± 1.6	31 ± 3.5	31 ± 2.0
Trp	62 ± 4.0	58 ± 3.0	55 ± 5.6	51 ± 5.0	45 ± 5.0	53 ± 4.0
His	26 ± 3.0	30 ± 2.8	12 ± 2.5**	50 ± 4.1	37 ± 3.5*	53 ± 4.0
Met	30 ± 2.3	25 ± 2.9	60 ± 4.2	53 ± 3.6	77 ± 3.0*	58 ± 4.4
Asn	61 ± 4.1***	25 ± 2.9	78 ± 3.4**	55 ± 3.8	76 ± 3.2***	47 ± 4.2
Phe	29 ± 3.6	29 ± 1.9	47 ± 5.3	50 ± 4.8	54 ± 4.3	56 ± 5.0
Arg	33 ± 2.4	29 ± 1.9	27 ± 2.9	29 ± 1.6	32 ± 5.4	31 ± 2.0
Ser	31 ± 2.4	30 ± 2.8	67 ± 5.9	62 ± 6.5	58 ± 6.5	53 ± 3.0
Pro	54 ± 6.0	58 ± 3.0	50 ± 7.3	50 ± 4.8	49 ± 3.8	56 ± 5.0
Ile	39 ± 3.8**	58 ± 3.0	32 ± 2.6	29 ± 1.6	33 ± 4.4	31 ± 2.0
OHPro	28 ± 2.5	30 ± 2.8	54 ± 6.5	50 ± 4.8	48 ± 3.8	56 ± 5.0
Ala	36 ± 4.2	30 ± 2.8	77 ± 3.1**	55 ± 3.8	77 ± 2.3***	47 ± 4.2
Thr	56 ± 7.4	57 ± 3.1	51 ± 4.3	50 ± 4.8	56 ± 6.6	56 ± 5.0
Val	28 ± 3.4	25 ± 2.9	50 ± 4.1	51 ± 5.0	49 ± 4.6	46 ± 4.1
Lys	29 ± 3.6	25 ± 2.9	50 ± 5.3	53 ± 3.6	50 ± 2.7	58 ± 4.4
Asp	57 ± 4.1***	29 ± 1.9	68 ± 6.8**	29 ± 1.6	66 ± 4.8**	31 ± 2.0
Gln	32 ± 4.8	25 ± 2.9	85 ± 2.8**	53 ± 3.6	43 ± 2.9	58 ± 4.4
Glu	59 ± 5.1***	29 ± 1.9	64 ± 3.1***	29 ± 1.6	69 ± 4.2***	31 ± 2.0
Gly[++]	27 ± 2.3	29 ± 1.9	50 ± 2.4	51 ± 5.0	54 ± 3.5	46 ± 4.1

† Values differ significantly from matched controls shown: * (P= 0.05), ** (0.01) and *** (0.001) based on paired-samples t-tests.

[+] Leu, leucine; Trp, tryptophan; His, histidine; Met, methionine; Asn, asparagine; Phe, phenylalanine; Arg, arginine; Ser, serine; Pro, proline; Ile, isoleucine; OHPro, hydroxyproline; Ala, alanine; Thr, threonine; Val, valine; Lys, Lysine; Asp, aspartic acid; Gln, glutamine; Glu, glutamic acid, Gly, glycine.

[++] Glycine is included but has no L or D-isomers.

Table 5.7 Effects of D-amino acids (10mM final concentration) on germination of zoospores of three *Pythium* spp. ; values shown are means ± s.e. for 6 replicates, with matched buffer controls, based on counts of 100 spores for each replicate after 16h†

Treatment+	P. aphanidermatum		P. catenulatum		P.dissotocum	
	Test	Control	Test	Control	Test	Control
Leu	22 ± 2.2	28 ± 2.4	31 ± 3.4	33 ± 4.0	23 ± 2.3	25 ± 2.3
Trp	29 ± 3.1	31 ± 1.9	24 ± 2.0	29 ± 2.4	37 ± 2.2	32 ± 2.6
His	24 ± 3.8	28 ± 1.4	34 ± 4.5	38 ± 2.6	36 ± 4.6	33 ± 2.1
Met	25 ± 1.9	31 ± 1.9	26 ± 1.4	29 ± 2.4	34 ± 1.9	32 ± 2.6
Asn	31 ± 3.6	28 ± 1.4	36 ± 2.4	38 ± 2.6	34 ± 2.6	33 ± 2.1
Phe	30 ± 3.4	28 ± 2.4	41 ± 3.6	33 ± 4.0	24 ± 3.0	24 ± 2.3
Arg	27 ± 4.2	28 ± 2.4	34 ± 2.6	33 ± 4.0	22 ± 1.6	24 ± 2.3
Ser	27 ± 3.3	28 ± 1.4	33 ± 3.0	38 ± 2.6	33 ± 2.0	33 ± 2.0
Pro	27 ± 2.9	31 ± 1.9	27 ± 2.4	28 ± 2.4	32 ± 3.5	32 ± 2.6
Ile	37 ± 4.1	31 ± 1.9	32 ± 3.0	28 ± 2.4	36 ± 3.6	32 ± 2.6
OHPro	28 ± 5.1	28 ± 2.4	34 ± 4.3	33 ± 4.0	24 ± 2.6	24 ± 2.3
Ala	25 ± 2.7	28 ± 1.4	40 ± 3.3	38 ± 2.6	30 ± 2.9	33 ± 2.1
Thr	33 ± 2.2	31 ± 1.9	30 ± 2.8	28 ± 2.4	29 ± 2.9	32 ± 2.6
Val	30 ± 2.2	28 ± 1.4	35 ± 3.4	38 ± 2.6	30 ± 3.6	33 ± 2.1
Lys	33 ± 2.7	31 ± 1.9	32 ± 2.7	28 ± 2.4	27 ± 2.1	32 ± 2.6
Asp	30 ± 3.2	28 ± 2.4	28 ± 2.9	33 ± 4.0	30 ± 2.9	24 ± 2.3
Gln	25 ± 2.8	28 ± 1.4	31 ± 5.2	38 ± 2.6	30 ± 3.4	33 ± 2.1
Glu	30 ± 2.2	28 ± 2.4	36 ± 3.3	33 ± 4.0	26 ± 1.4	24 ± 2.3
Gly++	26 ± 2.2	28 ± 2.4	30 ± 2.7	33 ± 4.0	26 ± 2.2	24 ± 2.3

† No significant difference was found between amino acid and matching buffer controls in any instance.

+ See Table 5.6.

++ Glycine is included but has no L or D-isomers.

Table 5.8 Effects of sugars (25mM final concentration) on germination of zoospore cysts of three *Pythium* spp. ; values shown are means ± s.e. for 6 replicates, with matched buffer controls, based on counts of 100 spores for each replicate after 16h†

Treatment[+]	P. aphanidermatum		P. catenulatum		P.dissotocum	
	Test	Control	Test	Control	Test	Control
D (+) Mann	51 ± 4.5	43 ± 4.8	75 ± 3.7	64 ± 3.7	43 ± 2.0	44 ± 4.3
L (+) Ara	49 ± 3.5	43 ± 4.8	65 ± 6.5	64 ± 3.7	53 ± 2.4	44 ± 4.3
D (-) Ara	38 ± 8.9	43 ± 4.8	61 ± 3.5	64 ± 3.7	50 ± 4.3	44 ± 4.3
D (+) Xyl	39 ± 7.0	43 ± 4.8	58 ± 3.6	64 ± 3.7	44 ± 2.3	44 ± 4.3
L (-) Fuc	53 ± 9.8	43 ± 4.8	58 ± 4.0	64 ± 3.7	51 ± 2.9	44 ± 4.3
D (+) Gal	38 ± 5.2	42 ± 5.2	42 ± 4.7	46 ± 3.6	30 ± 1.1*	41 ± 2.8
D (+) Glc	60 ± 4.6***	42 ± 5.2	82 ± 3.6***	46 ± 3.6	82 ± 4.1****	41 ± 2.8
Suc	74 ± 5.2**	42 ± 5.2	78 ± 5.5***	46 ± 3.6	88 ± 3.5****	41 ± 2.8
Frc	58 ± 3.7	42 ± 5.2	77 ± 7.0*	46 ± 3.6	84 ± 2.5***	41 ± 2.8
Mal	66 ± 4.7***	42 ± 5.2	63 ± 5.4	46 ± 3.6	83 ± 3.8****	41 ± 2.8

† Values differ significantly from matched controls: * (P= 0.05), ** (0.02), *** (0.01) and **** (0.001) based on paired-samples t-tests.

[+] Man, mannose; Ara, arabinose; Xyl, xylose; Fuc, fucose; Gal, galactose, Glc, glucose; Suc, sucrose; Frc, fructose; Mal, maltose.

Table 5.9 Effects of sugars and amino acids on germ-tube growth of germlings of three *Pythium* spp; values shown are means ± s.e. for 2 replicates, 20 cysts per replicate, with matched buffer controls after 16h

	Mean germ-tube length (μm)†		
Treatment[+] (10 mM)	*P. aphanidermatum*	*P. catenulatum*	*P. dissotocum*
Buffer	37 ± 1.6	37 ± 2.7	41 ± 2.5
L-Ala	58 ± 4.7*	34 ± 2.4	47 ± 2.9
D-Ala	39 ± 2.6	39 ± 2.5	26 ± 1.6**
L-Glu	116 ± 10.3**	46 ± 2.9	124 ± 8.4**
D-Glu	38 ± 1.9	40 ± 3.0	29 ± 1.4**
L-Asn	151 ± 9.8**	105 ± 14.7**	162 ± 14.6**
D-Asn	36 ± 2.1	35 ± 2.2	43 ± 2.1
L-Gln	131 ± 10.4**	81 ± 5.5**	87 ± 12.9*
D-Gln	48 ± 3.7	32 ± 1.5	32 ± 1.8
L-Lys	51 ± 3.7*	40 ± 2.5	30 ± 1.3
D+Glc	147 ± 11.5**	129 ± 8.0**	159 ± 19.7**
Suc	76 ± 4.9*	50 ± 2.7**	72 ± 3.6**
Xyl	32 ± 1.5	32 ± 2.0	46 ± 2.5
Frc	90 ± 8.7**	n.d.	72 ± 4.1**

† Significance of difference from buffer controls by paired-samples t-tests: *, 0.01; **, 0.001.

[+] Ala, alanine; Glu, glutamate; Gln, glutamine; Asn, asparagine; Lys, lysine; Glc, glucose; Suc, sucrose; Xyl, xylose; Frc, fructose, n.d.; not determined.

were stimulated to germinate (Section 5.6). The spores were rinsed, after 10 min, to remove non-adhering spores to which amino acids or sugars (10mM concentration) were then added.

The amino acids L-asparagine and L-glutamine significantly increased germ-tube lengths of germinating cysts of all three *Pythium* spp., compared to buffer controls, as did the sugars glucose, sucrose and (where tested) fructose. L-glutamate increased the germ-tube length of only *P. aphanidermatum* and *P. dissotocum* and L-alanine and L-lysine enhanced germ-tube growth of only *P. aphanidermatum* cysts. Fructose increased germ-tube growth of both *P. aphanidermatum* and *P. dissotocum* but was not tested for *P. catenulatum*. The other tested compounds either had no effect on any fungus (D-glutamine, D-asparagine or xylose) or occasionally (D-glutamate and D-alanine) significantly decreased germ-tube growth. It is notable, therefore, that not all compounds that triggered germination were able to stimulate germ-tube growth, and *vice-versa*.

5.5 Effects of cations on encystment

Various cations have been found to elicit encystment of *Phytophthora* spp. (Byrt *et al.*, 1982a; Grant *et al.*, 1986), so an attempt was now made to extend these studies to *Pythium* spp. (Tables 5.10-5.12).

The divalent cations Ca^{2+}, Mg^{2+}, Sr^{2+}, Mn^{2+} and Ba^{2+} elicited significant encystment of all three fungi compared to water controls (Table 5.11). Of them, Mn^{2+} and Ba^{2+} had the strongest effects, consistently causing encystment even at 1mM concentration, but Ba^{2+} at higher concentrations also caused cell lysis. Similarly, Fe^{3+} and Cr^{3+} elicited encystment of all fungi at 1mM concentrations (Table 5.12), but in most cases at this and higher concentrations they also induced cell lysis. In contrast, L^{+} and K^{+} triggered encystment only at the highest tested concentrations whereas Na^{+} had no effect at all (Table 5.10).

5.6 Effects of EGTA and Ca^{2+} on adhesion and germination of zoopores of *P. aphanidermatum*

Zoospores of *P. palmivora* are reported to release up to 40% of their intracellular Ca^{2+} within 2 min of the induction of encystment (Irving *et al.*, 1984). They also begin to absorb exogenously supplied radiolabelled Ca^{2+}

Table 5.10 Effects of monovalent cations on encystment of *Pythium* zoospores[+]

Treatment	conc.(mM)	*P. aphanidermatum*	*P. catenulatum*	*P. dissotocum*
Li^+	0	15 ± 1.8	16 ± 2.2	24 ± 2.7
	1	11 ± 2.0	22 ± 2.8	28 ± 3.6
	5	16 ± 2.4	18 ± 1.8	26 ± 4.2
	7	13 ± 1.9	24 ± 4.0	32 ± 5.6
	10	15 ± 2.4	63 ± 9.2**	45 ± 6.1*
	25	73 ± 6.8****	88 ± 5.4****	54 ± 8.8**
Na^+	0	14 ± 2.0	20 ± 2.1	22 ± 2.2
	1	13 ± 2.0	20 ± 1.6	22 ± 2.9
	5	14 ± 1.7	22 ± 2.2	22 ± 2.1
	7	15 ± 2.1	21 ± 2.6	22 ± 2.7
	10	16 ± 2.2	18 ± 2.3	19 ± 3.1
	25	13 ± 2.1	25 ± 2.4	19 ± 2.9
K^+	0	12 ± 1.8	21 ± 2.1	18 ± 1.5
	1	11 ± 1.7	16 ± 1.6	18 ± 2.3
	5	10 ± 1.8	17 ± 2.0	15 ± 2.6
	7	13 ± 2.1	19 ± 1.8	16 ± 1.7
	10	16 ± 2.0	26 ± 2.6	29 ± 4.5*
	25	27 ± 5.9*	44 ± 4.1***	40 ± 2.8****

[+] All cations were supplied as chloride salts at the stated final molar concentrations.
Data are means ± s.e. for 7 replicates, after 1h, based on counts of 100 spores in each replicate. Significance of difference from distilled water controls by paired-samples t-tests: *, 0.05; **, 0.02; ***, 0.01; ****, 0.001.

Table 5.11 Effects of divalent cations on encystment of *Pythium* zoospores[+]

Treatment	conc.(mM)	*P. aphanidermatum*	*P. catenulatum*	*P. dissotocum*
Ca^{2+}	0	13 ± 1.6	25 ± 4.2	21 ± 1.6
	1	16 ± 2.6	19 ± 2.2	16 ± 1.5
	5	14 ± 2.3	31 ± 3.8	20 ± 2.2
	7	31 ± 4.9**	33 ± 3.8	32 ± 4.7
	10	73 ± 3.9****	51 ± 6.6***	42 ± 4.4***
	25	60 ± 9.2***	70 ± 6.2****	74 ± 6.3****
Mg^{2+}	0	11 ± 2.4	21 ± 2.8	22 ± 2.5
	1	11 ± 1.3	28 ± 7.9	20 ± 2.5
	5	10 ± 2.1	48 ± 4.9***	30 ± 6.8
	7	12 ± 1.6	63 ± 6.2***	49 ± 7.4*
	10	23 ± 2.9	63 ± 4.2***	64 ± 4.8****
	25	34 ± 4.5***	82 ± 4.1****	72 ± 6.1****
Sr^{2+}	0	15 ± 2.6	21 ± 2.4	18 ± 2.1
	1	14 ± 2.0	18 ± 1.9	16 ± 1.3
	5	32 ± 3.8***	21 ± 2.2	26 ± 3.5
	7	58 ± 6.2****	67 ± 6.6****	63 ± 5.5****
	10	60 ± 7.8****	67 ± 6.6****	63 ± 5.5****
	25	70 ± 6.6****	62 ± 6.7****	72 ± 6.8****
Mn^{2+}	0	15 ± 1.8	16 ± 2.2	24 ± 2.7
	1	69 ± 5.8****	50 ± 8.3****	59 ± 6.0****
	5	72 ± 7.5****	72 ± 6.8****	73 ± 5.3****
	7	76 ± 6.3****	86 ± 10.2**	82 ± 7.1****
	10	92 ± 5.5****	85 ± 5.0****	97 ± 1.8****
	25	100 ± 0****	97 ± 2.0****	100 ± 0****
Ba^{2+}	0	16 ± 2.7	16 ± 2.9	15 ± 3.3
	1	98 ± 1.1****	96 ± 2.0****	100 ± 0****
	5	100 ± 0****	89 ± 4.9****	100 ± 0****
	7 †	93 ± 3.6****	88 ± 8.1****	98 ± 1.7****
	10†	99 ± 1.0****	92 ± 6.5****	40 ± 9.6***
	25†	75 ± 6.7**	21 ± 10.2	35 ± 12.1*

[+] All cations were supplied as chloride salts at the stated final molar concentrations. Data are means ± s.e. for 7 replicates, after 1h, based on counts of 100 spores in each replicate. Significance of difference from distilled water controls by paired-samples t-tests: *, 0.05; **, 0.02; ***, 0.01; ****, 0.001.

† All spores not shown as encysted in these treatments had lysed.

Table 5.12 Effects of trivalent cations on encystment of *Pythium* zoospores[+]

Treatment[†]	conc.(mM)	*P. aphanidermatum*	*P. catenulatum*	*P. dissotocum*
Fe^{3+}	0	16 ± 2.7	16 ± 2.9	15 ± 3.3
	1	96 ± 3.0****	99 ± 1.4****	97 ± 1.8****
	5	88 ± 6.6****	89 ± 4.5****	79 ± 8.7****
	7	62 ± 11.9**	44 ± 11.0*	57 ± 7.4***
	10	38 ± 10.0	20 ± 4.8	13 ± 2.9
	25	18 ± 4.2	14 ± 2.4	19 ± 3.1
Cr^{3+}	0	15 ± 1.8	16 ± 2.2	24 ± 2.7
	1	100 ± 0****	98 ± 1.4****	98 ± 0.9****
	5	98 ± 1.5****	100 ± 0****	96 ± 2.2****
	7	95 ± 2.5****	91 ± 5.6****	76 ± 10.7***
	10	88 ± 5.7****	64 ± 7.4****	74 ± 9.5***
	25	88 ± 8.5 ****	24 ± 5.2	76 ± 12.2***

[+] All cations were supplied as chloride salts at the stated final molar concentrations. Data are means ± s.e. for 7 replicates, after 1h, based on counts of 100 spores in each replicate. Significance of difference from distilled water controls by paired-samples t-tests: *, 0.05; **, 0.02; ***, 0.01; ****, 0.001.
[†] All spores treated with cations and not shown as encysted had lysed.

within this time (Irving *et al.*, 1984). This release and subsequent uptake of Ca^{2+} has been implicated in adhesion (Gubler *et al.*, 1989). In view of this a possible relationship between adhesion and germination was investigated for *P. aphanidermatum*.

Cysts were induced by agitation for 70 sec in an Eppendorf tube (Section 2.3.3) and then either immediately added to glass cavity slides (t_0) or held in suspension in the tubes for 10 min before being added to slides (t_{10}). Coupled with these treatments, some cysts received supplements of Ca^{2+} or EGTA, designed respectively to enhance or decrease the availability of Ca^{2+} (Table 5.13).

No difference in the mean number of spores was recorded for the slides of different treatments precluding the possibility that spores had adhered to the walls of the Eppendorf tubes between t_0 and t_{10}. Thus it was assumed that equivalent populations of cells were present in all cases. It was found that after 1.5h the t_0 cells had germinated significantly better than t_{10} spores. Germination of t_0 spores was not increased by addition of Ca^{2+} but was suppressed by addition of EGTA if this was added immediately after the cells were added to slides, although not if EGTA was added after a 10 min delay during which the spores were present on the slides. In contrast, germination of t_{10} spores was significantly enhanced by addition of Ca^{2+} and, in the absence of extracellular Ca^{2+}, were not suppressed below spores similarly treated with EGTA.

After germination had been assessed, the slides were rinsed with water to remove non-adhered cells and then reassessed for the numbers of spores that remained and the numbers of these that had germinated (Table 5.13). A high proportion of the t_0 (control) spores had adhered, compared with a significantly lower proportion of t_{10} spores. The degree of adhesion of t_0 spores was not enhanced by addition of Ca^{2+}. Conversely the adhesion of t_0 spores had been significantly reduced by immediate addition of EGTA, but not if EGTA was added 10 min after the spores were placed on slides.

A strong relationship between adhesion and germination was found, in that similar numbers of spores germinated and adhered within each treatment. This was reinforced by the uniformly high percentage germination (60-70%) of cells retained after washing in all treatments. Also of potential relevance, a maximal level of competence (approx. 60%) to adhere or germinate was noted in the experiment as a whole.

In a supplementary experiment to that above, cysts were transferred to slides, without further treatment, at 1 min intervals after induction of

Table 5.13 Relationship between adhesion and germination of cysts of *Pythium aphanidermatum*, after 1.5h, when zoospores were pre-encysted by agitation and then transferred to glass slides immediately (t_0) or after 10 min (t_{10}) and then treated with $CaCl_2$ or EGTA (7mM final concentration)[+]

	T_0	T_{10}	$T_0 + Ca^{2+}$	$T_{10} + Ca^{2+}$	T_0 + EGTA	T_0 + EGTA after 10min	T_{10} +EGTA
No. of spores	188 ± 29.6	171 ± 28.7	185 ± 16.0	150 ± 18.7	143 ± 12.6	162 ± 17.7	147 ± 14.9
Germination (%)	59 ± 7.0 --b--	25 ± 5.0	59 ± 6.0	54 ± 4.7	10 ± 1.1 --b--	57 ± 5.3	20 ± 4.1
Adhesion (%)	59 ± 6.6 --b--	30 ± 6.7	61 ± 4.9	62 ± 5.4	21 ± 4.1 --c--	53 ± 4.9	21 ± 2.6
Germination (%) after washing	69 ± 5.3	51 ± 4.8	65 ± 7.3	63 ± 4.1	48 ± 7.5	70 ± 5.2	51 ± 4.8
Germinated cysts retained after washing (%)	70 ± 5.0	61 ± 7.6	67 ± 6.5	75 ± 5.0	75 ± 5.6	67 ± 6.5	61 ± 5.6
Non-germinated cysts retained after washing (%)	47 ± 7.1 --a--	23 ± 8.4	47 ± 3.3	45 ± 8.0	11 ± 3.0 --b--	38 ± 6.3	12 ± 2.0

[+] Means ± s.e. for 9 replicates; values linked by a, b or c differ from one another at P= 0.05 (a), 0.01 (b) and 0.001 (c) by paired-samples t-test.

encystment. This revealed (Table 5.14) that the maximum ability to germinate was retained during a delay of up to 4 min in transfer of cysts to the slides. A delay of 7 min or more reduced germination to a low basal level with intermediate values obtained for a delay of 5 and 6 min. However the addition of EGTA to cysts at the times of transfer to slides caused marked suppression of germination of cysts transferred within 5 min and no significant effect on spores transferred to slides after 6-7 min.

The relationship between adhesion and germination was further investigated by physically scraping adhered spores from slides and then assessing their subsequent germination (Table 5.15). The method was to add spores to slides immediately (t_0) or 10 min after induction of encystment (t_{10}). Then spores, from both treatments, were scraped off slides, at t_{10}, and added to clean glass cavity slides or slides coated with the adhesive polylysine. After 1.5h, the number of adhered and germinated spores were assessed for spores which were scraped off and transferred to other cavity slides, with or without polylysine, and also for spores remaining on the original slides which were not removed by scraping.

Spores added to slides immediately after encystment (t_0) displayed high levels of germination and remained attached after washing. Cells treated similarly but physically detached after 10 min germinated well but did not readhere in large numbers. In contrast, spores added to slides 10 min after induction of encystment (t_{10}) adhered and germinated poorly. In all cases, prior coating of slides with the adhesive polylysine did not significantly increase adhesion (or germination) despite the expectation that it would do so.

5.7 Effects of ions and EGTA on germination of suspended cysts

All the experiments above demonstrated, firstly, that spores adhere naturally to glass slides if placed in contact with these immediately after encystment; or within the first 4-5 min, but not if added at later times. It also seemed that calcium was responsible for this, because "older" cysts would adhere if supplied with exogenous calcium and conversely, "young" cysts did not adhere if EGTA was present. Germination also correlated with adhesion, being affected in the same way by the same treatments. But the "trigger" to germinate was required only in the initial few minutes because adhesion or germination was unaffected by addition of EGTA at 10 min after cysts were added to slides and germination

Table 5.14 Effects of EGTA on germination of cysts of *Pythium aphanidermatum*, when zoospores were pre-encysted by agitation and transferred to glass slides at different times (0-10 min) and then treated immediately with EGTA (7mM final concentration) or distilled water.

Time (min) of transfer	Germination[+] Distilled water	EGTA
0	69 ± 5.8**	14 ± 2.7
1	71 ± 5.3**	14 ± 2.9
2	71 ± 3.9**	18 ± 3.6
3	70 ± 3.0**	18 ± 2.8
4	70 ± 3.0**	25 ± 3.2
5	50 ± 5.9*	33 ± 4.1
6	37 ± 5.8	35 ± 5.7
7	25 ± 2.4	26 ± 4.8
8	30 ± 2.7	31 ± 3.8
9	26 ± 2.1	26 ± 2.8
10	28 ± 3.1	31 ± 3.7

[+] Means ± s.e. for 9 replicates based on 100 spores in each replicate, after 2h.
Significance of difference from matching EGTA treatments by paired-samples t-tests: *, 0.02; **, 0.001.

Table 5.15 Germination of spores of *Pythium aphanidermatum* after detachment from glass slides†

Spore age and treatment‡	Germination (%)	Adhesion (%)
t_0		
Undisturbed	62 ± 7.0	65 ± 6.2
Detached, transferred to new slides	57 ± 5.2	27 ± 3.2**
Detached, transferred to poly-lysine coated slides	51 ± 9.6	33 ± 6.9*
t_{10}		
Undisturbed	25 ± 4.9++	29 ± 3.1+++
Detached, transferred to new slides	23 ± 3.3++++	29 ± 4.2
Detached, transferred to poly-lysine coated slides	28 ± 3.8+	32 ± 4.9

† Means ± s.e. for 9 replicates, based on counts of 100 spores for each replicate, after 1.5h. Significance of difference from undisturbed treatments by paired-samples t-test: *, 0.05; **, 0.001. Significance of difference of t_{10} spores from matching t_0 treatments by paired-samples t-test: +, 0.05; ++, 0.02; +++, 0.01; ++++, 0.001.

‡ Spores encysted by agitation were added immediately (t_0) or after 10 min (t_{10}) to glass slides. Some of the adherent cells were scraped from the slides and reincubated on fresh slides with or without poly-lysine coating.

was unaffected by physical removal of attached cysts at 10 min. In order to further investigate these features, experiments were done on cysts that were continuously maintained in suspension. Cysts were produced as before by agitation in Eppendorf tubes but then incubated in these tubes with different supplements (Tables 5.16-5.24).

All three *Pythium* spp. showed significant germination responses to Ca^{2+}, Mg^{2+} and Sr^{2+} supplements (Tables 5.16-5.18) but no response to Li^{+}, Na^{+}, K^{+} (Tables 5.19-5.21), or Mn^{2+} (Tables 5.16-5.18), whereas Fe^{3+}, Cr^{3+} (Tables 5.22-5.24) and Ba^{2+} (Tables 5.16-5.18) caused spores to lyse and inhibited cyst germination. No divalent cation showed a significant germination response below 7mM but all were effective at 10mM. In general, the intensity and speed of the response increased with increasing cation concentration up to 25mM (as seen by comparing germination after 1.5h and 16h respectively). Also Sr^{2+} was on the whole less effective, than Ca^{2+} or Mg^{2+}, especially at intermediate concentrations.

In a similar experiment the effect of cations on encystment and germination of originally motile zoospores was analysed (Tables 5.25-5.30), *i.e.* the treatments were applied directly to the motile zoospore suspension.

After 1.5h all the cations except Na^{+} had caused at least some encystment compared to that in water controls, and for all three fungi. However, assessments of germination at this time proved difficult due to residual motility of some spores or lysing of others in some treatments. When this is taken into account, and the percentage of encysted spores that germinate (G/E) is analysed, then only Ca^{2+}, Mg^{2+} and Sr^{2+}, for all three fungi (Tables 5.25-5.27), and Mn^{2+} in the case of *P. dissotocum* and *P. catenulatum* (Tables 5.26, 5.27) enhanced cyst germination compared to that in distilled water controls. Cr^{3+} inhibited germination of both *P. catenulatum* and *P. aphanidermatum* (Tables 5.28, 5.29) and Ba^{2+} suppressed germination of *P. dissotocum* (Table 5.27). By 16h most spores had encysted and comparisons became clearer. Overall, after 16h, Ca^{2+}, Mg^{2+} and Sr^{2+} significantly enhanced germination (Tables 5.25-5.27) for all three fungi whereas Na^{+} and K^{+} at higher concentrations elicited a weak response for *P. aphanidermatum* only (Table 5.28). Contrastingly, Mn^{2+} and Li^{+} had no effect on germination but Fe^{3+}, Cr^{3+} and Ba^{2+} significantly inhibited the final number of germinated spores and the proportion of encysted spores that germinated (Tables 5.25-5.30).

Table 5.16 Effects of divalent cations on germination of suspended zoospore cysts of *Pythium aphanidermatum*+

Treatment	conc.(mM)	Germination (%)	
		1.5h	16h
Ca^{2+}	0	38 ± 5.2	44 ± 4.1
	1	36 ± 4.0	42 ± 4.9
	5	47 ± 4.9	51 ± 6.2
	7	72 ± 5.0**	74 ± 5.0**
	10	72 ± 6.1**	77 ± 4.8**
	25	71 ± 4.3***	74 ± 3.8***
Mg^{2+}	0	30 ± 3.4	36 ± 3.1
	1	29 ± 3.4	34 ± 3.2
	5	36 ± 5.8	40 ± 5.7
	7	48 ± 5.8*	56 ± 4.9**
	10	67 ± 7.6**	70 ± 6.7**
	25	71 ± 3.8***	76 ± 2.4***
Sr^{2+}	0	39 ± 3.7	43 ± 5.0
	1	38 ± 2.8	43 ± 5.0
	5	36 ± 5.8	40 ± 5.7
	7	50 ± 5.3	58 ± 5.4*
	10	60 ± 6.7*	65 ± 5.7*
	25	72 ± 3.2***	80 ± 2.8**
Mn^{2+}	0	32 ± 2.2	37 ± 2.0
	1	30 ± 3.2	37 ± 2.3
	5	37 ± 2.6	41 ± 3.0
	7	30 ± 2.2	35 ± 1.8
	10	33 ± 2.9	37 ± 3.1
	25	35 ± 3.5	41 ± 3.2
Ba^{2+}	0	27 ± 4.2	37 ± 2.3
	1	7 ± 1.2**	9 ± 1.3***
	5	8 ± 1.3**	9 ± 1.3***
	7	6 ± 1.5**	7 ± 1.4***
	10	6 ± 1.2**	8 ± 1.1***
	25	5 ± 1.6**	6 ± 1.4***

+ All cations were supplied as chloride salts at the stated final molar concentrations. Data are means ± s.e. for 7 replicates, after 1.5h and reassessed after 16h, based on counts of 100 spores in each replicate. Significance of difference from distilled water controls by paired-samples t-tests: *, 0.05; **, 0.01; ***, 0.001.

Table 5.17 Effects of divalent cations on germination of suspended zoospore cysts of *Pythium catenulatum*+

Treatment	conc.(mM)	Germination (%)	
		1.5h	16h
Ca^{2+}	0	18 ± 1.6	24 ± 2.1
	1	16 ± 2.6	23 ± 3.8
	5	20 ± 1.6	30 ± 3.1
	7	29 ± 3.9*	40 ± 5.4*
	10	51 ± 6.8***	57 ± 5.9***
	25	53 ± 4.2****	59 ± 4.1****
Mg^{2+}	0	20 ± 2.6	28 ± 2.4
	1	20 ± 2.6	24 ± 2.4
	5	20 ± 2.5	26 ± 2.4
	7	30 ± 2.8*	43 ± 4.4
	10	49 ± 6.2***	57 ± 5.4***
	25	59 ± 6.2***	65 ± 4.6****
Sr^{2+}	0	22 ± 1.9	30 ± 3.2
	1	22 ± 3.1	32 ± 2.1
	5	19 ± 2.4	25 ± 2.1
	7	19 ± 2.3	29 ± 2.8
	10	34 ± 4.5**	44 ± 4.2**
	25	54 ± 4.4****	60 ± 4.6***
Mn^{2+}	0	34 ± 4.3	40 ± 3.6
	1	30 ± 2.9	36 ± 2.8
	5	42 ± 6.0	47 ± 5.4
	7	33 ± 4.1	40 ± 3.4
	10	30 ± 4.0	35 ± 3.8
	25	32 ± 2.7	38 ± 3.8
Ba^{2+}	0	31 ± 3.4	35 ± 3.8
	1	16 ± 3.6**	21 ± 4.2*
	5	8 ± 1.8***	11 ± 1.2***
	7	8 ± 2.4**	11 ± 1.7***
	10	10 ± 1.8****	12 ± 2.0***
	25	4 ± 1.4****	6 ± 1.1****

+ All cations were supplied as chloride salts at the stated final molar concentrations. Data are means ± s.e. for 7 replicates, after 1.5h and reassessed after 16h, based on counts of 100 spores in each replicate. Significance of difference from distilled water controls by paired-samples t-tests: *, 0.05; **, 0.02; ***, 0.01; ****, 0.001.ï

Table 5.18 Effects of divalent cations on germination of suspended zoospore cysts of *Pythium dissotocum*+

Treatment	conc.(mM)	Germination (%)	
		1.5h	16h
Ca^{2+}	0	23 ± 2.3	37± 2.9
	1	28 ± 3.9	35 ± 3.3
	5	25 ± 2.8	33 ± 2.0
	7	35 ± 3.2***	45 ± 3.8**
	10	72 ± 4.2****	76 ± 3.6****
	25	63 ± 3.3****	70 ± 2.9****
Mg^{2+}	0	28 ± 2.4	32 ± 3.0
	1	30 ± 3.5	38 ± 3.8
	5	28 ± 2.4	34 ± 2.0
	7	37 ± 3.6*	44 ± 2.8*
	10	57 ± 4.6****	66 ± 4.9****
	25	50 ± 6.0***	56 ± 5.8***
Sr^{2+}	0	23 ± 2.9	29 ± 3.0
	1	21 ± 3.6	28 ± 3.4
	5	22 ± 2.7	27 ± 2.4
	7	27 ± 2.5	32 ± 2.6
	10	53 ± 4.4****	61 ± 4.5****
	25	50 ± 6.0***	56 ± 5.8***
Mn^{2+}	0	30 ± 3.6	34 ± 3.7
	1	34 ± 6.5	39 ± 5.4
	5	29 ± 3.2	37 ± 4.1
	7	30 ± 4.1	39 ± 3.3
	10	26 ± 4.1	31 ± 4.1
	25	27 ± 3.5	34 ± 4.2
Ba^{2+}	0	36 ± 5.7	41 ± 5.7
	1	21 ± 3.6	26 ± 3.5
	5	19 ± 2.4**	24 ± 3.3*
	7	9 ± 2.7****	10 ± 2.6****
	10	3 ± 0.7****	4 ± 0.8****
	25	2 ± 0.9****	3 ± 0.8****

+ All cations were supplied as chloride salts at the stated final molar concentrations. Data are means ± s.e. for 7 replicates, after 1.5h and reassessed after 16h, based on counts of 100 spores in each replicate. Significance of difference from distilled water controls by paired-samples t-tests: *, 0.05; **, 0.02; ***, 0.01; ****, 0.001.

Table 5.19 Effects of monovalent cations on germination of suspended zoospore cysts of *Pythium aphanidermatum* +

Treatment	conc.(mM)	Germination (%)	
		1.5h	16h
Li^+	0	31 ± 2.9	39 ± 4.2
	1	29 ± 2.9	41 ± 3.5
	5	34 ± 2.8	42 ± 4.2
	7	28 ± 2.5	36 ± 2.0
	10	33 ± 3.0	38 ± 3.5
	25	33 ± 3.6	38 ± 3.6
Na^+	0	38 ± 3.1	43 ± 3.8
	1	36 ± 5.4	41 ± 4.6
	5	41 ± 5.6	44 ± 5.5
	7	41 ± 3.1	48 ± 1.7
	10	37 ± 5.8	43 ± 5.5
	25	36 ± 5.9	43 ± 5.5
K^+	0	36 ± 5.5	42 ± 6.2
	1	36 ± 6.8	41 ± 5.6
	5	43 ± 6.1	47 ± 5.9
	7	40 ± 4.8	43 ± 5.1
	10	40 ± 6.8	43 ± 6.2
	25	40 ± 7.4	46 ± 6.8

+ All cations were supplied as chloride salts at the stated final molar concentrations.

Data are means ± s.e. for 7 replicates, after 1.5h and reassessed after 16h, based on counts of 100 spores in each replicate. No significance of difference was found between cation treatments and matching distilled water controls in any instance by paired-samples t-tests.

Table 5.20 Effects of monovalent cations on germination of suspended zoospore cysts of *Pythium catenulatum*[+]

Treatment	conc.(mM)	Germination (%)	
		1.5h	16h
Li^+	0	36 ± 4.0	41 ± 4.7
	1	37 ± 5.8	47 ± 6.3
	5	40 ± 5.3	45 ± 4.6
	7	35 ± 3.7	41 ± 3.6
	10	32 ± 5.4	37 ± 4.9
	25	40 ± 4.0	46 ± 3.4
Na^+	0	22 ± 3.0	34 ± 3.3
	1	26 ± 3.7	32 ± 3.2
	5	20 ± 4.7	30 ± 3.7
	7	24 ± 2.9	32 ± 2.7
	10	22 ± 3.6	30 ± 3.7
	25	26 ± 3.4	29 ± 2.7
K^+	0	21 ± 2.8	32 ± 2.9
	1	19 ± 3.4	29 ± 3.8
	5	21 ± 3.9	28 ± 3.4
	7	26 ± 3.2	33 ± 3.2
	10	22 ± 1.9	30 ± 4.0
	25	24 ± 2.5	31 ± 3.3

[+] All cations were supplied as chloride salts at the stated final molar concentrations.

Data are means ± s.e. for 7 replicates, after 1.5h and reassessed after 16h, based on counts of 100 spores in each replicate. No significance of difference was found between cation treatments and matching distilled water controls in any instance by paired-samples t-tests.

Table 5.21 Effects of monovalent cations on germination of suspended zoospore cysts of *Pythium dissotocum*[+]

Treatment	conc.(mM)	Germination (%)	
		1.5h	16h
Li^+	0	37 ± 2.8	43 ± 2.1
	1	42 ± 3.5	46 ± 2.9
	5	36 ± 3.7	42 ± 3.7
	7	38 ± 3.3	44 ± 2.6
	10	37 ± 3.1	41 ± 3.8
	25	33 ± 3.7	38 ± 4.2
Na^+	0	23 ± 1.6	29 ± 2.5
	1	22 ± 2.0	31 ± 1.6
	5	22 ± 2.8	31 ± 2.1
	7	23 ± 1.9	30 ± 2.3
	10	19 ± 2.1	24 ± 3.0
	25	22 ± 2.1	28 ± 1.8
K^+	0	23 ± 2.2	29 ± 2.8
	1	23 ± 2.5	31 ± 3.0
	5	23 ± 3.3	29 ± 3.6
	7	20 ± 2.6	29 ± 3.2
	10	22 ± 3.1	28 ± 2.3
	25	23 ± 2.1	26 ± 2.6

[+] All cations were supplied as chloride salts at the stated final molar concentrations.

Data are means ± s.e. for 7 replicates, after 1.5h and reassessed after 16h, based on counts of 100 spores in each replicate. No significance of difference was found between cation treatments and matching distilled water controls in any instance by paired-samples t-tests.

Table 5.22 Effects of trivalent cations on germination of suspended zoospore cysts of *Pythium aphanidermatum* +

Treatment	conc.(mM)	Germination (%)	
		1.5h	16h
Fe^{3+}	0	29 ± 3.9	36 ± 4.0
	1	6 ± 1.0*	7 ± 1.2*
	5	3 ± 0.7*	5 ± 1.0*
	7	3 ± 0.6*	2 ± 0.5*
	10	1 ± 0.4*	2 ± 0.5*
	25	1 ± 0.6*	2 ± 0.7*
Cr^{3+}	0	29 ± 3.0	39 ± 3.0
	1	9 ± 1.6*	12 ± 1.6*
	5	9 ± 1.6*	10 ± 1.4*
	7	9 ± 1.5*	10 ± 1.6*
	10	2 ± 0.5*	3 ± 0.4*
	25	0*	2 ± 0.6*

+ All cations were supplied as chloride salts at the stated final molar concentrations.

Data are means ± s.e. for 7 replicates, after 1.5h and reassessed after 16h, based on counts of 100 spores in each replicate. Significance of difference from distilled water controls by paired-samples t-tests: *, 0.001.

Table 5.23 Effects of trivalent cations on germination of suspended zoospore cysts of *Pythium catenulatum* +

Treatment	conc.(mM)	Germination (%)	
		1.5h	16h
Fe^{3+}	0	34 ± 5.5	37 ± 5.1
	1	2 ± 0.8*	7 ± 1.2*
	5	1 ± 0.6*	1 ± 0.6*
	7	1 ± 0.6*	1 ± 0.7*
	10	1 ± 0.3*	1 ± 0.3*
	25	1 ± 0.5*	2 ± 0.5*
Cr^{3+}	0	27 ± 3.4	29 ± 2.2
	1	1 ± 0.6*	2 ± 0.6*
	5	1 ± 0.5*	1 ± 0.4*
	7	1 ± 0.2*	1 ± 0.3*
	10	0*	0*
	25	0*	1 ± 0.4*

+ All cations were supplied as chloride salts at the stated final molar concentrations.

Data are means ± s.e. for 7 replicates, after 1.5h and reassessed after 16h, based on counts of 100 spores in each replicate. Significance of difference from distilled water controls by paired-samples t-tests: *, 0.001.

Table 5.24 Effects of trivalent cations on germination of suspended zoospore cysts of *Pythium dissotocum*+

Treatment	conc.(mM)	Germination (%)	
		1.5h	16h
Fe^{3+}	0	25 ± 2.4	33 ± 3.2
	1	1 ± 0.6***	2 ± 0.6***
	5	1 ± 0.3***	1 ± 0.4***
	7	0***	0***
	10	1 ± 0.3***	1 ± 0.3***
	25	1 ± 0.5***	1 ± 0.5***
Cr^{3+}	0	23 ± 2.0	29 ± 2.9
	1	12 ± 1.8**	14 ± 1.8*
	5	8 ± 1.5**	11 ± 1.9***
	7	10 ± 1.9*	12 ± 1.0***
	10	10 ± 2.0**	12 ± 2.0**
	25	4 ± 1.2***	5 ± 1.2***

+ All cations were supplied as chloride salts at the stated final molar concentrations.

Data are means ± s.e. for 7 replicates, after 1.5h and reassessed after 16h, based on counts of 100 spores in each replicate. Significance of difference from distilled water controls by paired-samples t-tests: *, 0.02; **, 0.01; ***, 0.001.

Table 5.25 Effects of divalent cations on percentage encystment and germination of motile zoospores of *Pythium aphanidermatum*

Treatment and conc. (mM)[+]		1.5h			16h[†]	
		Encyst (E)	Germinate (G)	G/E	Germinate (G)	G/E
Ca^{2+}	0	15 ± 1.2	6 ± 0.5	43 ± 5.2	16 ± 2.2	16 ± 2.2
	1	17 ± 1.8	8 ± 1.0	48 ± 4.9	16 ± 2.2	16 ± 2.2
	5	37 ± 3.7****	24 ± 2.6****	64 ± 5.3**	30 ± 4.8*	30 ± 4.8*
	7	41 ± 5.2****	26 ± 3.4****	62 ± 4.5***	50 ± 4.9****	50 ± 4.9****
	10	56 ± 3.8****	37 ± 3.1****	66 ± 4.0***	49 ± 3.5****	49 ± 3.5****
	25	76 ± 4.6****	55 ± 7.6****	72 ± 3.6****	68 ± 5.3****	68 ± 5.3****
Mg^{2+}	0	16 ± 1.6	3 ± 0.4	18 ± 2.1	16 ± 1.3	16 ± 1.3
	1	15 ± 1.3	3 ± 0.4	19 ± 2.8	16 ± 1.8	16 ± 1.8
	5	23 ± 3.0*	6 ± 0.8	23 ± 2.8	17 ± 3.0	17 ± 3.0
	7	31 ± 3.4****	16 ± 2.5****	50 ± 2.7****	27 ± 3.3*	27 ± 3.3*
	10	56 ± 5.0****	26 ± 3.4****	46 ± 4.4***	40 ± 4.4***	40 ± 4.4***
	25	76 ± 3.8****	31 ± 3.3****	41 ± 5.2****	46 ± 5.0****	46 ± 5.0****
Sr^{2+}	0	24 ± 2.9	4 ± 0.6	18 ± 3.0	17 ± 1.7	17 ± 1.7
	1	27 ± 4.4	5 ± 0.9	17 ± 2.3	14 ± 1.3	14 ± 1.3
	5	89 ± 3.5****	13 ± 1.8***	14 ± 1.9	19± 1.4	19 ± 1.4
	7	90 ± 4.0****	43 ± 7.0***	47 ± 6.6***	55 ± 7.2****	55 ± 7.2****
	10	96 ± 2.6****	45 ± 6.7****	47 ± 6.8***	59 ± 6.0****	59 ± 6.0****
	25	100 ± 0****	46 ± 5.9***	46 ± 5.9***	62 ± 4.2****	62 ± 4.2****
Ba^{2+}	0	8 ± 1.6	<1 ± 0.4	6 ± 3.2	18 ± 2.5	18 ± 2.5
	1	100 ± 0****	6 ± 1.4**	6 ± 1.4	8 ± 1.1*	8 ± 1.1*
	5	99 ± 0.7****	1 ± 0.5	1 ± 0.4	2 ± 1.0***	2 ± 1.0***
	7	92 ± 2.8****	1 ± 0.4	1 ± 0.4	2 ± 0.5****	3 ± 0.6****
	10	56 ± 5.5****	1 ± 0.3	2 ± 0.8	2 ± 0.5***	2 ± 0.5***
	25	58 ± 9.6****	1 ± 0.3	2 ± 0.8	2 ± 0.3****	4 ± 1.3***
Mn^{2+}	0	11 ± 2.2	1 ± 0.3	10 ± 2.9	14 ± 2.5	14 ± 2.5
	1	43 ± 6.3***	4 ± 1.7	9 ± 2.0	12 ± 2.0	12 ± 2.0
	5	49 ± 5.3****	3 ± 1.1	6 ± 1.0	17 ± 2.0	17 ± 2.0
	7	72 ± 6.3****	11 ± 1.9***	14 ± 2.2	16 ± 2.3	16 ± 2.3
	10	85 ± 4.4****	13 ± 2.5***	16 ± 2.6	18 ± 2.7	18 ± 2.7
	25	89 ± 4.0****	10 ± 1.4****	11 ± 1.4	13 ± 1.7	13 ± 1.7

[+] All cations were supplied as chloride salts at the stated final molar concentrations. Data are means ± s.e. for 7 replicates, after 1.5h and reassessed after 16h, based on counts of 100 spores in each replicate. Significance of difference from distilled water controls by paired-samples t-tests: *, 0.05; **, 0.02; ***, 0.01; ****, 0.001. [†] In most cases, irrespective of treatment, all spores had encysted by 16h.

Table 5.26 Effects of divalent cations on percentage encystment and germination of motile zoospores of *Pythium catenulatum*

Treatment and conc. (mM)[+]		1.5h			16h†	
		Encyst (E)	Germinate (G)	G/E	Germinate (G)	G/E
Ca^{2+}	0	9 ± 1.2	<1 ± 0.2	8 ± 2.5	10 ± 1.6	10 ± 1.6
	1	11 ± 1.3	1 ± 0.2	12 ± 3.7	9 ± 1.5	9 ± 1.6
	5	12 ± 1.2	1 ± 0.3	9 ± 2.5	12 ± 2.0	12 ± 2.0
	7	31 ± 5.2***	14 ± 2.8****	43 ± 3.0****	28 ± 4.5***	28 ± 4.5***
	10	56 ± 4.6****	28 ± 3.6****	49 ± 4.8****	39 ± 4.3****	39 ± 4.3****
	25	72 ± 6.1****	39 ± 3.9****	54 ± 3.4****	48 ± 3.5****	49 ± 3.5****
Mg^{2+}	0	12 ± 0.9	1 ± 0.3	8 ± 2.2	13 ± 2.2	13 ± 2.2
	1	13 ± 1.5	<1 ± 0.2	7 ± 1.7	11 ± 2.0	11 ± 2.0
	5	16 ± 1.7	2 ± 0.6	11 ± 2.6	12 ± 1.7	12 ± 1.7
	7	45 ± 3.0****	20 ± 1.5****	45 ± 2.4****	40 ± 5.7***	40 ± 6.0***
	10	58 ± 3.8****	27 ± 2.6****	48 ± 3.8****	37 ± 3.4***	38 ± 3.4***
	25	59 ± 4.9****	29 ± 3.6****	47 ± 3.8****	46 ± 5.6****	46 ± 5.6****
Sr^{2+}	0	11 ± 1.0	1 ± 0.2	11 ± 2.2	10 ± 1.8	10 ± 1.8
	1	14 ± 1.6	1 ± 0.3	10 ± 2.4	12 ± 1.8	12 ± 1.8
	5	28 ± 4.5*	4 ± 1.5	13 ± 3.5	11 ± 1.0	11 ± 1.0
	7	60 ± 4.4****	13 ± 1.6****	22 ± 2.0*	16 ± 1.8	16 ± 1.8
	10	71 ± 2.7****	28 ± 2.5****	39 ± 2.4****	38 ± 3.8****	38 ± 3.8****
	25	78 ± 5.2****	34 ± 2.4****	44 ± 1.4****	47 ± 4.3****	47 ± 4.3****
Ba^{2+}	0	9 ± 3.2	<1 ± 0.3	7 ± 3.4	23 ± 2.3	23 ± 2.3
	1	98 ± 1.8****	2 ± 0.4*	2 ± 0.4	4 ± 1.7***	4 ± 1.7***
	5	99 ± 0.7****	1 ± 0.5	2 ± 0.4	2 ± 1.0***	2 ± 1.0***
	7	90 ± 4.4****	2 ± 0.7	2 ± 0.7	2 ± 0.6****	2 ± 0.6****
	10	92 ± 4.5****	2 ± 0.5	2 ± 0.7	1 ± 0.3***	2 ± 0.6****
	25	74 ± 9.3****	1 ± 0.3	2 ± 0.7	2 ± 0.5****	5 ± 2.0***
Mn^{2+}	0	13 ± 1.6	1 ± 0.4	10 ± 2.7	23 ± 1.8	23 ± 1.8
	1	46 ± 5.6***	10 ± 2.1*	22 ± 3.6*	12 ± 2.0	12 ± 2.0
	5	48 ± 5.6***	18 ± 2.4***	38 ± 2.0****	21 ± 3.0	21 ± 3.0
	7	65 ± 4.6****	16 ± 1.3****	26 ± 2.1**	24 ± 2.4	24 ± 2.4
	10	73 ± 5.4****	17 ± 2.9****	23 ± 3.1*	27 ± 2.7	27 ± 2.7
	25	75 ± 9.4****	20 ± 1.4****	24 ± 1.5***	23 ± 1.8	23 ± 1.8

[+] All cations were supplied as chloride salts at the stated final molar concentrations. Data are means ± s.e. for 7 replicates, after 1.5h and reassessed after 16h, based on counts of 100 spores in each replicate. Significance of difference from distilled water controls by paired-samples t-tests: *, 0.05; **, 0.02; ***, 0.01; ****, 0.001. † In most cases, irrespective of treatment, all spores had encysted by 16h.

Table 5.27 Effects of divalent cations on percentage encystment and germination of motile zoospores of *Pythium dissotocum*

Treatment and conc. (mM)[+]		1.5h			16h†	
		Encyst (E)	Germinate (G)	G/E	Germinate (G)	G/E
Ca^{2+}	0	13 ± 1.4	<1 ± 0.2	8 ± 2.4	14 ± 1.7	14 ± 1.7
	1	13 ± 1.4	1 ± 0.2	6 ± 1.4	14 ± 2.3	14 ± 2.3
	5	14 ± 2.3	1 ± 0.4	9 ± 2.4	16 ± 1.5	16 ± 1.5
	7	49 ± 5.8****	24 ± 3.9****	50 ± 4.2****	38 ± 5.6****	38 ± 5.6****
	10	75 ± 3.3****	38 ± 3.0****	50 ± 3.3****	48 ± 4.8****	48 ± 4.8****
	25	74 ± 5.4****	35 ± 4.5****	49 ± 2.2****	41 ± 3.8****	41 ± 3.8****
Mg^{2+}	0	13 ± 1.0	1 ± 0.4	10 ± 3.5	14 ± 1.9	14 ± 1.9
	1	13 ± 1.0	1 ± 0.3	10 ± 2.9	14 ± 1.6	14 ± 1.6
	5	14 ± 2.0	<1 ± 0.3	5 ± 1.6	17 ± 1.8	17 ± 1.8
	7	59 ± 4.1****	26 ± 2.1****	44 ± 2.6****	36 ± 2.2****	36 ± 2.2****
	10	59 ± 5.2****	27 ± 3.4****	46 ± 2.8****	38 ± 4.9****	38 ± 4.9****
	25	66 ± 5.2****	30 ± 4.5****	45 ± 5.4***	42 ± 5.7***	42 ± 5.7***
Sr^{2+}	0	14 ± 1.2	2 ± 0.4	12 ± 2.2	12 ± 1.2	12 ± 1.2
	1	15 ± 1.9	2 ± 0.4	13 ± 1.7	11 ± 1.0	11 ± 1.0
	5	29 ± 2.5****	3 ± 0.5*	9 ± 1.4	12 ± 0.8	12 ± 0.8
	7	55 ± 5.4****	10 ± 1.6****	19 ± 2.6***	14 ± 1.5	14 ± 1.5
	10	71 ± 7.6****	29 ± 4.4****	42 ± 6.9***	35 ± 4.2****	35 ± 4.2****
	25	86 ± 3.9****	38 ± 5.3****	43 ± 4.4****	44 ± 4.8****	44 ± 4.8****
Ba^{2+}	0	13 ± 1.6	1 ± 0.4	9 ± 2.8	20 ± 2.3	20 ± 2.3
	1	100 ± 0****	5 ± 2.0	5 ± 2.0	5 ± 2.0****	5 ± 2.0****
	5	100 ± 0****	7 ± 1.6**	7 ± 1.7	7 ± 1.7***	7 ± 1.7***
	7	94 ± 2.6****	1 ± 0.4	1 ± 0.5*	1 ± 0.5****	1 ± 0.6****
	10	74 ± 3.7****	1 ± 0.3	1 ± 0.5*	1 ± 0.3****	2 ± 0.5****
	25	63 ± 6.5****	<1 ± 0.3	1 ± 0.5*	1 ± 0.4****	1 ± 0.8***
Mn^{2+}	0	12 ± 2.4	<1 ± 0.5	4 ± 2.5	19 ± 2.6	19 ± 2.6
	1	40 ± 4.4***	8 ± 1.5**	20 ± 3.2*	22 ± 2.0	22 ± 2.0
	5	45 ± 5.4***	10 ± 2.3**	21 ± 3.9*	22 ± 2.9	22 ± 2.9
	7	45 ± 5.1***	6 ± 1.9	12 ± 3.6	18 ± 1.9	18 ± 1.9
	10	68 ± 5.5****	15 ± 1.0****	23 ± 3.4***	16 ± 2.3	16 ± 2.3
	25	83 ± 6.3****	15 ± 2.3****	19 ± 2.7***	19 ± 2.6	19 ± 2.6

[+] All cations were supplied as chloride salts at the stated final molar concentrations. Data are means ± s.e. for 7 replicates, after 1.5h and reassessed after 16h, based on counts of 100 spores in each replicate. Significance of difference from distilled water controls by paired-samples t-tests: *, 0.05; **, 0.02; ***, 0.01; ****, 0.001. † In most cases, irrespective of treatment, all spores had encysted by 16h.

Table 5.28 Effects of monovalent and trivalent cations on percentage encystment and germination of motile zoospores of *Pythium aphanidermatum*

Treatment and conc. (mM)[+]		1.5h			16h†	
		Encyst (E)	Germinate (G)	G/E	Germinate (G)	G/E
Na^+	0	8 ± 1.2	<1 ± 0.3	6 ± 3.4	8 ± 1.2	8 ± 1.2
	1	7 ± 1.2	<1 ± 0.3	10 ± 7.5	11 ± 1.2	11 ± 1.2
	5	7 ± 1.6	<1 ± 0.3	5 ± 2.7	10 ± 1.6	10 ± 1.6
	7	9 ± 1.6	<1 ± 0.3	8 ± 3.8	12 ± 1.1	12 ± 1.1
	10	8 ± 1.3	<1 ± 0.4	9 ± 4.5	13 ± 1.3*	13 ± 1.4*
	25	9 ± 1.5	1 ± 0.3	10 ± 4.3	12 ± 1.5	12 ± 1.5
K^+	0	9 ± 1.3	1 ± 0.4	11 ± 1.3	10 ± 1.3	10 ± 1.3
	1	10 ± 2.3	<1 ± 0.2	9 ± 1.5	11 ± 1.5	11 ± 1.5
	5	12 ± 1.5	<1 ± 0.2	8 ± 1.4	11 ± 1.7	11 ± 1.7
	7	43 ± 8.6***	2 ± 0.4	6 ± 0.6	13 ± 1.8	13 ± 1.8
	10	54 ± 7.3****	2 ± 0.4**	5 ± 1.1	8 ± 1.0	8 ± 1.0
	25	80 ± 7.5****	8 ± 1.7****	10 ± 4.4	15 ± 1.5*	15 ± 1.5*
Li^+	0	14 ± 1.5	1 ± 0.4	9 ± 2.6	24 ± 2.9	24 ± 2.9
	1	15 ± 2.4	1 ± 0.5	7 ± 3.1	20 ± 3.2	20 ± 3.2
	5	18 ± 2.5	2 ± 0.5	11 ± 2.7	22± 2.7	22 ± 2.7
	7	31 ± 3.5**	3 ± 0.6	12 ± 3.3	28 ± 3.0	28 ± 3.0
	10	52 ± 5.8***	3 ± 0.9	5 ± 1.8	23 ± 4.2	23 ± 4.2
	25	77 ± 7.3***	6 ± 1.4*	7 ± 1.3	20 ± 2.4	20 ± 2.4
Fe^{3+}	0	10 ± 2.1	<1 ± 0.3	8 ± 3.3	26 ± 3.1	26 ± 3.1
	1	11 ± 3.4	<1 ± 0.3	1 ± 0.4	4 ± 0.8****	4 ± 0.8****
	5‡	70 ± 5.6****	1 ± 0.6	2 ± 0.8	4 ± 0.5****	5 ± 0.5***
	7‡	50 ± 5.4****	<1 ± 0.3	1 ± 0.7	3 ± 0.5****	5 ± 1.2****
	10‡	39 ± 5.1***	<1 ± 0.3	2 ± 0.7	3 ± 0.6****	5 ± 1.2****
	25‡	37 ± 8.3**	<1 ± 0.3	1 ± 0.4	3 ± 0.5****	6 ± 1.2****
Cr^{3+}	0	19 ± 2.7	2 ± 0.4	8 ± 1.8	15 ± 2.9	16 ± 2.9
	1‡	97 ± 1.3***	2 ± 0.5	2 ± 0.5*	3 ± 0.8***	3 ± 0.9***
	5‡	98 ± 0.9****	2 ± 0.8	2 ± 0.8*	3 ± 0.5**	3 ± 0.6**
	7‡	89 ± 3.8****	1 ± 0.5	2 ± 0.6**	3 ± 0.8***	3 ± 0.9***
	10‡	71 ± 9.4***	1 ± 0.5	2 ± 1.0*	2 ± 0.5***	4 ± 1.2***
	25‡	75 ± 9.6****	1 ± 0.6	1 ± 0.8***	3 ± 0.8***	4 ± 1.0***

[+] All cations were supplied as chloride salts at the stated final molar concentrations. Data are means ± s.e. for 7 replicates, after 1.5h and reassessed after 16h, based on counts of 100 spores in each replicate. Significance of difference from distilled water controls by paired-samples t-tests: *, 0.05; **, 0.02; ***, 0.01; ****, 0.001. † In most cases, irrespective of treatment, all spores had encysted by 16h.

‡ Spores not encysted in these treatments had lysed.

Table 5.29 Effects of monovalent and trivalent cations on percentage encystment and germination of motile zoospores of *Pythium catenulatum*

Treatment and conc. (mM)[+]		1.5h			16h†	
		Encyst (E)	Germinate (G)	G/E	Germinate (G)	G/E
Na^+	0	25 ± 3.2	1 ± 0.5	6 ± 2.2	10 ± 1.6	10 ± 1.6
	1	24 ± 2.6	1 ± 0.5	4 ± 2.2	9 ± 1.1	9 ± 1.1
	5	24 ± 2.7	1 ± 0.6	5 ± 1.8	11 ± 1.0	11 ± 1.0
	7	24 ± 3.1	1 ± 0.4	4 ± 1.8	12 ± 1.3	12 ± 1.3
	10	20 ± 3.6	1 ± 0.6	7 ± 3.8	8 ± 0.9	8 ± 0.9
	25	22 ± 1.9	1 ± 0.6	6 ± 2.4	13 ± 1.7	13 ± 1.7
K^+	0	16 ± 1.7	1 ± 0.4	6 ± 2.4	10 ± 1.3	10 ± 1.3
	1	12 ± 2.0	<1 ± 0.4	6 ± 2.0	9 ± 1.1	10 ± 1.1
	5	20 ± 1.9	1 ± 0.4	5 ± 1.3	12 ± 1.3	12 ± 1.3
	7	39 ± 4.5***	2 ± 0.8	6 ± 2.0	12 ± 2.1	12 ± 2.1
	10	57 ± 8.4****	9 ± 2.4***	15 ± 4.0	13 ± 1.9	13 ± 1.9
	25	81 ± 5.0****	10 ± 1.4****	12 ± 1.8	13 ± 2.2	13 ± 2.2
Li^+	0	10 ± 1.9	1 ± 0.4	14 ± 3.0	13 ± 1.7	13 ± 1.7
	1	20 ± 2.9	3 ± 0.6	17 ± 2.2	11 ± 2.5	11 ± 2.5
	5	25 ± 3.3**	4 ± 0.5***	17 ± 2.6	14 ± 2.2	14 ± 2.2
	7	29 ± 5.4*	3 ± 0.5	11 ± 2.8	15 ± 2.1	15 ± 2.1
	10	50 ± 6.0***	7 ± 1.4***	14 ± 4.8	10 ± 1.6	10 ± 1.6
	25	65 ± 6.8****	6 ± 1.8*	9 ± 1.8	17 ± 2.3	17 ± 2.3
Fe^{3+}	0	9 ± 1.4	<1 ± 0.4	9 ± 4.4	27 ± 2.9	27 ± 2.9
	1‡	98 ± 1.1****	2 ± 0.4	2 ± 0.4	3 ± 0.6****	3 ± 0.6****
	5‡	83 ± 4.7****	1 ± 0.6	1 ± 0.4	2 ± 0.5****	2 ± 0.6****
	7‡	64 ± 7.2****	1 ± 0.3	2 ± 0.8	2 ± 0.4****	4 ± 1.5***
	10‡	70 ± 1.6****	1 ± 0.5	2 ± 1.0	2 ± 0.5****	4 ± 1.0****
	25‡	53 ± 8.7***	<1 ± 0.4	1 ± 0.5	2 ± 0.5****	4 ± 1.0****
Cr^{3+}	0	8 ± 1.6	1 ± 0.4	13 ± 5.0	17 ± 2.5	17 ± 2.5
	1‡	100 ± 0****	3 ± 1.3	3 ± 1.3*	4 ± 0.9***	4 ± 0.9***
	5‡	95 ± 2.7****	2 ± 0.5	2 ± 0.6*	2 ± 0.6***	3 ± 0.7***
	7‡	96 ± 2.4****	3 ± 0.8	3 ± 0.8*	3 ± 0.8***	3 ± 0.8***
	10‡	86 ± 5.1****	1 ± 0.3	2 ± 0.4*	2 ± 0.5***	2 ± 0.6***
	25‡	80 ± 7.0****	1 ± 0.5	2 ± 0.9*	2 ± 0.4***	3 ± 0.6***

[+] All cations were supplied as chloride salts at the stated final molar concentrations. Data are means ± s.e. for 7 replicates, after 1.5h and reassessed after 16h, based on counts of 100 spores in each replicate. Significance of difference from distilled water controls by paired-samples t-tests: *, 0.05; **, 0.02; ***, 0.01; ****, 0.001.

† In most cases, irrespective of treatment, all spores had encysted by 16h.

‡ Spores not encysted in these treatments had lysed.

Table 5.30 Effects of monovalent and trivalent cations on percentage encystment and germination of motile zoospores of *Pythium dissotocum*

Treatment and conc. (mM)[+]		1.5h			16h†	
		Encyst (E)	Germinate (G)	G/E	Germinate (G)	G/E
Na^+	0	25 ± 4.2	1 ± 0.4	4 ± 1.3	15 ± 1.8	15 ± 1.8
	1	26 ± 4.4	1 ± 0.4	5 ± 1.4	14 ± 2.4	14 ± 2.4
	5	22 ± 3.0	1 ± 0.4	6 ± 2.2	14 ± 1.9	14 ± 1.9
	7	23 ± 4.1	1 ± 0.5	6 ± 2.8	16 ± 1.2	16 ± 1.2
	10	18 ± 3.8	<1 ± 0.6	3 ± 2.0	16 ± 1.6	16 ± 1.6
	25	19 ± 4.6	1 ± 0.6	4 ± 2.4	18 ± 1.2	18 ± 1.2
K^+	0	19 ± 2.5	<1 ± 0.3	4 ± 1.5	11 ± 1.6	11 ± 1.6
	1	22 ± 3.2	1 ± 0.3	6 ± 1.6	10 ± 1.7	10 ± 1.7
	5	18 ± 4.0	1 ± 0.3	6 ± 1.6	12 ± 1.8	12 ± 1.8
	7	44 ± 6.9***	3 ± 0.6	6 ± 0.8	10 ± 1.1	10 ± 1.1
	10	64 ± 8.7***	5 ± 1.2***	8 ± 1.0	14 ± 2.1	14 ± 2.1
	25	74 ± 7.3***	8 ± 1.6***	10 ± 1.6	15 ± 1.8	15 ± 1.8
Li^+	0	15 ± 1.2	1 ± 0.3	8 ± 2.2	12 ± 1.2	12 ± 1.2
	1	14 ± 3.2	1 ± 0.4	10 ± 2.1	15 ± 3.0	15 ± 3.0
	5	12 ± 2.2	<1 ± 0.3	4 ± 2.0	11 ± 1.8	11 ± 1.8
	7	18 ± 2.8	1 ± 0.3	5 ± 0.4	17 ± 1.8	17 ± 1.8
	10	33 ± 5.4**	2 ± 0.4	5 ± 1.5	11 ± 1.9	11 ± 1.9
	25	44 ± 7.8**	3 ± 0.5*	6 ± 1.2	15 ± 1.8	15 ± 1.8
Fe^{3+}	0	12 ± 3.0	1 ± 0.4	8 ± 3.7	20 ± 3.7	20 ± 3.7
	1‡	97 ± 1.6****	3 ± 1.6	3 ± 0.6	1 ± 0.3***	3 ± 1.3***
	5‡	92 ± 2.4****	2 ± 0.7	2 ± 0.8	1 ± 0.6****	2 ± 0.8***
	7‡	65 ± 6.2****	1 ± 0.3	2 ± 0.5	1 ± 0.3***	3 ± 1.2***
	10‡	70 ± 5.7****	1 ± 0.5	2 ± 0.8	1 ± 0.5***	3 ± 1.6***
	25‡	53 ± 5.2****	2 ± 0.6	3 ± 0.8	1 ± 0.4***	3 ± 1.6**
Cr^{3+}	0	7 ± 4.8	<1 ± 0.3	6 ± 4.1	25 ± 2.8	25 ± 2.8
	1‡	100 ± 0****	4 ± 1.2	4 ± 1.2	5 ± 1.2****	5 ± 1.2***
	5‡	99 ± 9.9****	4 ± 0.9	4 ± 0.9	6 ± 1.0****	6 ± 1.0****
	7‡	89 ± 3.8****	2 ± 0.3	2 ± 0.4	2 ± 0.4****	2 ± 0.4****
	10‡	72 ± 4.1****	2 ± 0.7	2 ± 0.3	2 ± 0.5****	4 ± 1.9****
	25‡	39 ± 8.4*	<1 ± 0.3	1 ± 0.7	2 ± 0.4****	6 ± 1.8***

[+] All cations were supplied as chloride salts at the stated final molar concentrations. Data are means ± s.e. for 7 replicates, after 1.5h and reassessed after 16h, based on counts of 100 spores in each replicate. Significance of difference from distilled water controls by paired-samples t-tests: *, 0.05; **, 0.02; ***, 0.01; ****, 0.001.

† In most cases, irrespective of treatment, all spores had encysted by 16h.

‡ Spores not encysted in these treatments had lysed.

It had been demonstrated that addition of EGTA in the first minutes after induction of encystment suppressed spore germination (Table 5.14). So an experiment was done similar to that above, to determine whether cations that elicit germination of non-adhered spores (Tables 5.16-5.18), could overcome suppression of germination caused by EGTA. In order to do this EGTA with or without an equimolar concentration of salts was added immediately after induction of encystment, to spores in Eppendorf tubes (Table 5.31) or to motile spores on glass slides (Table 5.32-5.34).

The vortex-encysted spores held in suspension (Table 5.31) germinated poorly in distilled water but significantly less well in the presence of EGTA, EGTA+K^+ or EGTA+Na^+. In most cases, after 16h, germination was significantly enhanced by EGTA+Ca^{2+}, EGTA+Mg^{2+} and EGTA+Sr^{2+} whereas in every case the presence of the divalent cations significantly enhanced germination of cysts above those treated with EGTA alone. Overall, Ca^{2+} had a more pronounced effect (particularly after 1.5h) than Mg^{2+} and this more than Sr^{2+}.

The equivalent experiment with motile spores (Tables 5.32-5.34) was difficult to assess because spores remained motile after 1.5h in water but were caused to encyst by EGTA in the presence or absence of salts. The only cations that significantly increased germination, expressed as a percentage of encysted spores were Ca^{2+}, Mg^{2+} and Sr^{2+}, and even these ions did not cause significant effects in all cases.

After 16h, however, all spores had encysted and the treatment effects on germination were clear (Table 5.32-5.34). Compared to water controls, germination was significantly reduced, in most cases, by EGTA, EGTA+Na^+ and EGTA+K^+ and significantly enhanced by EGTA+Ca^{2+} and EGTA+Mg^{2+}. Treatment with EGTA+Sr^{2+} significantly increased spore germination of *P. aphanidermatum* and *P. dissotocum* but had no effect on *P. catenulatum.*

5.8 Effects of lanthanum and EGTA on cyst germination

EGTA has been demonstrated to inhibit zoospore germination (Table 5.14) whereas Ca^{2+} enhances germination of both *Pythium* (Tables 5.16-5.18) and *Phytophthora* spores (Byrt *et al.,* 1982a). In view of this the effects of La^{3+}, a calcium-channel blocker (Godfraind *et al.,* 1986), on germination of suspended cysts were studied in the presence and absence of cations or nutrients (Tables

Table 5.31 Effects of EGTA (7mM final concentration) with or without ions on germination of *Pythium* zoospores that were pre-encysted by agitation[+]

Treatment[†]	*P.aphanidermatum*		*P. catenulatum*		*P. dissotocum*	
	1.5h	16h	1.5h	16h	1.5h	16h
Water	35 ± 6.7	38 ± 6.5	20 ± 2.7	30 ± 2.4	24 ± 1.6	29 ± 2.3
EGTA	3 ± 0.8***	15 ± 2.0***	5 ± 1.0***	15 ± 1.7***	4 ± 1.6****	11 ± 1.9****
Na^+ + EGTA	4 ± 1.3***	13 ± 2.2***	6 ± 1.2***	16 ± 1.9***	5 ± 1.1****	11 ± 2.0****
K^+ + EGTA	7 ± 1.4***	14 ± 2.3***	6 ± 1.1***	16 ± 3.7***	7 ± 1.4****	13 ± 2.4****
Ca^{2+} + EGTA	48 ± 6.5	67 ± 4.4***	37 ± 4.3***	55 ± 5.9***	37 ± 5.4***	54 ± 7.1**
Mg^{2+} + EGTA	55 ± 6.8	67 ± 5.3*	28 ± 4.6	45 ± 5.6*	26 ± 2.5	43 ± 4.2**
Sr^{2+} + EGTA	40 ± 4.8	54 ± 4.8	25 ± 2.8	41 ± 3.5**	22 ± 2.5	42 ± 5.0**

[†] All cations were supplied as chloride salts, at a final concentration equivalent to that of EGTA (7mM).

[+] Data are percentage germination (means ± s.e. for 9 replicates, after 1.5h and reassessed after 16h) based on counts of 100 spores in each replicate. Significance of difference from distilled water controls by paired-samples t-tests: *, 0.05; **, 0.02; ***, 0.01; ****, 0.001.

Table 5.32 Effects of EGTA (7mM final concentration) with or without ions on percentage encystment and germination of motile spores of *Pythium aphanidermatum*[+]

Treatment[‡]	1.5h			16h[†]	
	Encyst (E)	Germinate (G)	G/E	Germinate (G)	G/E
Water	3 ± 1.5	< 1 ± 0.4	6 ± 5.6	22 ± 3.8	22 ± 3.8
EGTA	98 ± 1.8****	4 ± 0.8***	4 ± 0.8	11 ± 1.1**	11 ± 1.1**
Na^+ + EGTA	100 ± 0****	3 ± 0.9*	3 ± 0.9	9 ± 2.0**	9 ± 2.0**
K^+ + EGTA	96 ± 2.8****	3 ± 0.9**	4 ± 0.9	12 ± 2.2	12 ± 2.2
Ca^{2+} + EGTA	100 ± 0****	56 ± 4.5****	56 ± 4.5****	63 ± 4.9****	63 ± 4.9****
Mg^{2+} + EGTA	100 ± 0****	63 ± 6.4****	63 ± 6.4****	68 ± 4.4****	68 ± 4.4****
Sr^{2+} + EGTA	99 ± 1.0****	48 ± 3.4****	48 ± 3.4****	61 ± 3.7***	61 ± 3.7***

‡ All cations were supplied as chloride salts, at a final concentration equivalent to that of EGTA (7mM).

† In most cases, irrespective of treatment, all spores had encysted by 16h.

+ Data are means ± s.e. for 9 replicates, after 1.5h and reassessed after 16h, based on counts of 100 spores in each replicate; significance of difference from distilled water controls by paired-samples t-tests: *, 0.05; **, 0.02; ***, 0.01; ****, 0.001.

Table 5.33 Effects of EGTA (7mM final concentration) with or without ions on percentage encystment and germination of motile spores of *Pythium catenulatum*+

	1.5h			16h†	
Treatment‡	Encyst (E)	Germinate (G)	G/E	Germinate (G)	G/E
Water	28 ± 5.6	3 ± 1.0	10 ± 3.1	22 ± 3.8	22 ± 3.8
EGTA	88 ± 4.3****	5 ± 1.2	6 ± 1.6	10 ± 1.6*	10 ± 1.6*
Na^+ + EGTA	92 ± 4.2****	5 ± 1.8	6 ± 1.8	11 ± 2.3*	11 ± 2.3*
K^+ + EGTA	90 ± 3.9****	5 ± 0.9	6 ± 1.0	11 ± 1.5**	11 ± 1.5**
Ca^{2+} + EGTA	93 ± 4.0****	24 ± 4.2****	26 ± 5.5****	45 ± 4.0***	45 ± 4.0***
Mg^{2+} + EGTA	92 ± 4.2****	15 ± 2.3***	16 ± 2.3	30 ± 3.2**	30 ± 3.2**
Sr^{2+} + EGTA	95 ± 3.1****	12 ± 3.9***	13 ± 2.0	25 ± 3.6	25 ± 3.6

‡ All cations were supplied as chloride salts, at a final concentration equivalent to that of EGTA (7mM).

† In most cases, irrespective of treatment, all spores had encysted by 16h.

+ Data are means ± s.e. for 9 replicates, after 1.5h and reassessed after 16h, based on counts of 100 spores in each replicate; significance of difference from distilled water controls by paired-samples t-tests: *, 0.05; **, 0.02; ***, 0.01; ****, 0.001.

Table 5.34 Effects of EGTA with or without ions on percentage encystment and germination of motile spores of *Pythium dissotocum*+

		1.5h		16h†	
Treatment‡	Encyst (E)	Germinate (G)	G/E	Germinate (G)	G/E
Water	15 ± 4.1	2 ± 1.2	10 ± 2.9	19 ± 1.7	19 ± 1.7
EGTA	90 ± 4.5****	4 ± 0.6	5 ± 0.9	10 ± 1.5***	10 ± 1.5***
Na^+ + EGTA	97 ± 2.9****	6 ± 1.4	6 ± 1.4	10 ± 1.6***	10 ± 1.6***
K^+ + EGTA	94 ± 3.1****	5 ± 1.2**	6 ± 1.2	10 ± 1.4**	10 ± 1.4**
Ca^{2+} + EGTA	90 ± 4.2****	9 ± 1.4***	9 ± 1.7	39 ± 2.5****	39 ± 2.5****
Mg^{2+} + EGTA	94 ± 3.6****	9 ± 1.4***	9 ± 1.7	48 ± 6.9***	48 ± 6.9***
Sr^{2+} + EGTA	94 ± 3.5****	11 ± 1.7***	12 ± 2.2	30 ± 3.7*	30 ± 3.7*

‡ All cations were supplied as chloride salts, at a final concentration equivalent to that of EGTA (7mM).

† In most cases, irrespective of treatment, all spores had encysted by 16h.

+ Data are means ± s.e. for 9 replicates, after 1.5h and reassessed after 16h, based on counts of 100 spores in each replicate; significance of difference from distilled water controls by paired-samples t-tests: *, 0.05; **, 0.02; ***, 0.01; ****, 0.001.

5.35, 5.36). La^{3+} or SDW (control) was added to Eppendorf tubes 10 min after induction of encystment, and post-treatments were applied 10 min later. In a repeat of this experiment, spores were centrifuged to remove the initial treatments before the post-treatments were applied (Table 5.36).

Both methods gave similar results. Germination of control cells (pre-treated with SDW) was significantly enhanced by post-treatment (after 10 min) with Ca^{2+}, Sr^{2+}, Mg^{2+} or nutrients (MEP; comprising malt extract and peptone) but suppressed by La^{3+} or La^{3+} with excess Ca^{2+}. Spores initially treated with La^{3+} germinated below the level of water controls and this suppression was not overcome by post-treatment with Ca^{2+} or Sr^{2+}. However, Mg^{2+} partially restored germination of La^{3+} treated-cells and MEP significantly enhanced germination of La^{3+}- treated cells.

In similar experiments to those above, cysts were treated 10 min after induction of encystment with La^{3+}, EGTA or SDW and then post-treated (20 min) with amino acids that are known to stimulate germination or with amino acids that do not stimulate germination (see Table 5.6).

The results (Tables 5.37, 5.38) obtained were similar whether or not cells were centrifuged and washed to remove the initial treatments before post-treatment. Water treated (control) spores were stimulated to germinate by asparagine, glutamate, aspartate or Ca^{2+} but the low basal level of germination was suppressed by post-treatment with La^{3+} or EGTA. In all cases pre-treatment with La^{3+} or EGTA suppressed germination below that of the corresponding cells pre-treated with SDW. Suppression of germination elicited by EGTA (pre-treatment) could only be overcome by post-treatment with Ca^{2+} which then significantly elevated germination levels above those of untreated controls. In contrast, suppression of germination in the presence of La^{3+} was not overcome by Ca^{2+} but was overcome by post-treatment with asparagine, glutamate and aspartate, which then enhanced germination above water controls but not above corresponding spores pre-treated with SDW.

5.9 Effects of Ca^{2+} modulators on cyst germination of *P. aphanidermatum*

Cysts were pre-treated, 10 min after induction of encystment, with a range of potential Ca^{2+} modulators and then post-treated after 20 min with cations or amino acids.

Spores pre-treated with water were stimulated to germinate by Ca^{2+},

Table 5.35 Effects of lanthanum and post-treatment with other substances on cyst germination by *Pythium aphanidermatum* +

Pre-Treatment	Post-treatment (10mM unless stated)	Germination (%) 1.5h	16h
Water	Water	19 ± 2.0	22 ± 2.9
	Ca^{2+}	47 ± 4.6*	51 ± 3.6**
	Sr^{2+}	36 ± 3.0*	40 ± 2.7*
	Mg^{2+}	39 ± 3.7*	42 ± 3.5**
	La^{3+} (1.5mM)	4 ± 1.1**	5 ± 1.3*
	La^{3+} (1.5mM) + Ca^{2+}	7 ± 1.1*	9 ± 0.9*
	MEP†	42 ± 2.0**	47 ± 2.2**
La^{3+} (1.5mM)	Water	8 ± 1.3	11 ± 1.2
	Ca^{2+}	12 ± 2.4	14 ± 2.2
	Sr^{2+}	12 ± 1.3	14 ± 1.8
	Mg^{2+}	23 ± 3.3*	27 ± 3.3*
	La^{3+} (1.5mM)	5 ± 1.2	8 ± 1.3
	La^{3+} (1.5mM) + Ca^{2+}	8 ± 1.0	12 ± 0.9
	MEP†	40 ± 7.3*	46 ± 2.2**

+ Zoospores encysted by agitation were treated after 10 min with La^{3+} and post-treated, at 20 min, as shown. Data are means ± s.e. for 7 replicates, after 1.5h and reassessed after 16h, based on counts of 100 spores in each replicate. Significance of difference from matching controls post-treated with distilled water by paired-samples t-tests: *, 0.01; **, 0.001.

† MEP; malt extract (3%) + peptone (1%).

Table 5.36 Effects of lanthanum and post-treatment (after centrifugation) with other substances on cyst germination by *Pythium aphanidermatum* +

Pre-Treatment	Post-treatment after washing (10mM unless stated)	Germination (%) 1.5h	16h
Water	Water	13 ± 2.2	21 ± 3.8
	Ca^{2+}	41 ± 5.2***	52 ± 7.4***
	Sr^{2+}	34 ± 5.7**	41 ± 5.9*
	Mg^{2+}	34 ± 4.2***	39 ± 4.6*
	La^{3+} (1.5mM)	9 ± 2.6	12 ± 2.8*
	La^{3+} (1.5mM) + Ca^{2+}	7 ± 1.6	10 ± 2.1****
	MEP†	39 ± 4.5***	46 ± 5.3**
La^{3+} (1.5mM)	Water	12 ± 2.1	15 ± 1.8
	Ca^{2+}	10 ± 2.1	13 ± 2.7
	Sr^{2+}	10 ± 1.9	13 ± 2.1
	Mg^{2+}	20 ± 3.5	26 ± 5.1
	La^{3+} (1.5mM)	9 ± 2.1	13 ± 1.7
	La^{3+} (1.5mM) + Ca^{2+}	9 ± 1.5	13 ± 2.1
	MEP†	33 ± 5.3***	41 ± 5.2***

+ Zoospores encysted by agitation were treated after 10 min with La^{3+}, then centrifuged, washed and resuspended in distilled water immediately prior to post-treatment, at 20 min, as shown.

Data are means ± s.e. for 7 replicates, after 1.5h and reassessed after 16h, based on counts of 100 spores in each replicate.

Significance of difference from matching controls post-treated with distilled water by paired-samples t-tests: *, 0.05; **, 0.02; ***, 0.01; ****, 0.001.

† MEP; malt extract (3%) + peptone (1%).

Table 5.37 Rescue of lanthanum- or- EGTA- suppressed germination of cysts of *Pythium aphanidermatum* by calcium and amino acids+

Pre-treatment	Post-treatment	Experiment 1		Experiment 2	
	(10mM unless stated)	1.5h	16h	1.5h	16h
Water	Water	21 ± 1.4	25 ± 1.1	20 ± 1.9	25 ± 1.7
	EGTA (7mM)	8 ± 0.8***	10 ± 0.8***	9 ± 1.6**	13 ± 2.2**
	La^{3+} (1.5mM)	9 ± 0.8*	12 ± 1.0***	9 ± 1.0**	15 ± 1.3**
	Ca^{2+}	61 ± 4.6***	64 ± 5.1***	57 ± 5.3***	63 ± 3.9***
	Asparagine	71 ± 4.8***	75 ± 3.4***	53 ± 5.3***	61 ± 5.2***
	Glutamine	23 ± 1.8	25 ± 1.3	-	-
	Glutamic acid	-	-	46 ± 4.2**	53 ± 4.7**
	Aspartic acid	-	-	48 ± 3.9***	56 ± 4.7***
	Lysine	-	-	18 ± 2.8	28 ± 1.5
EGTA (7mM)	Water	8 ± 1.0	11 ± 1.0	11 ± 0.9	15 ± 1.5
	EGTA (7mM)	9 ± 0.8	12 ± 0.7	10 ± 0.9	14 ± 1.3
	La^{3+} (1.5mM)	7 ± 0.8	9 ± 0.6	7 ± 1.0	12 ± 1.3
	Ca^{2+}	56 ± 5.4***	60 ± 4.5***	38 ± 3.5***	42 ± 3.7***
	Asparagine	10 ± 1.0	13 ± 1.6	13 ± 2.1	19 ± 2.4
	Glutamine	9 ± 0.8	13 ± 1.8	-	-
	Glutamic acid	-	-	10 ± 1.4	16 ± 1.0
	Aspartic acid	-	-	14 ± 1.9	17 ± 2.2
	Lysine	-	-	8 ± 1.1	14 ± 1.9
La^{3+} (1.5mM)	Water	9 ± 0.9	12 ± 1.3	10 ± 1.0	14 ± 1.5
	EGTA (7mM)	6 ± 0.5**	8 ± 0.8	9 ± 0.8	14 ± 1.8
	La^{3+} (1.5mM)	6 ± 1.1*	9 ± 0.9	8 ± 1.0	13 ± 1.6
	Ca^{2+}	9 ± 1.6	11 ± 0.7	14 ± 2.3	17 ± 2.1
	Asparagine	43 ± 4.3***	48 ± 3.9***	37 ± 4.6**	42 ± 3.7**
	Glutamine	10 ± 1.1	13 ± 0.8	-	-
	Glutamic acid	-	-	33 ± 2.4***	38 ± 3.3***
	Aspartic acid	-	-	40 ± 4.1***	46 ± 5.2***
	Lysine	-	-	9 ± 1.1	14 ± 1.3

+ Zoospores encysted by agitation were treated 10 min later with La^{3+} or EGTA then post-treated at 20 min as shown.

Data are percentage germination (means ± s.e. for 7 replicates, after 1.5h and reassessed after 16h) based on counts of 100 spores in each replicate, for two separate experiments.

Significance of difference from matching controls post-treated with distilled water by paired-samples t-tests: *, 0.05; **, 0.01; ***, 0.001.

Table 5.38 Rescue of lanthanum- or EGTA- suppressed germination of cysts of *Pythium aphanidermatum* by calcium and amino acids, and cells were washed to remove the initial treatments[+]

Pre-treatment	Post-treatment	Experiment 1		Experiment 2	
	(10mM unless stated)	1.5h	16h	1.5h	16h
Water	Water	21 ± 3.4	25 ± 2.7	19 ± 2.0	24 ± 2.2
	EGTA (7mM)	6 ± 2.0*	11 ± 1.3**	9 ± 1.1**	12 ± 1.3*
	La^{3+} (1.5mM)	9 ± 1.8*	14 ± 1.8**	9 ± 1.3**	13 ± 1.1*
	Ca^{2+}	53 ± 7.2**	62 ± 6.6**	60 ± 5.4***	63 ± 5.5***
	Asparagine	61 ± 11.0**	66 ± 9.9**	51 ± 5.9**	60 ± 6.1**
	Glutamine	17 ± 3.2	23 ± 4.2	-	-
	Glutamic acid	-	-	46 ± 4.4**	55 ± 3.8***
	Aspartic acid	-	-	48 ± 4.0***	52 ± 4.1***
	Lysine	-	-	19 ± 2.2	24 ± 2.0
EGTA (7mM)	Water	11 ± 1.8	16 ± 2.7	8 ± 1.0	13 ± 1.4
	EGTA (7mM)	10 ± 2.4	16 ± 2.6	8 ± 0.5	13 ± 1.5
	La^{3+} (1.5mM)	8 ± 1.1	15 ± 2.6	7 ± 0.8	11 ± 0.6
	Ca^{2+}	47 ± 6.1**	56 ± 6.5***	34 ± 2.7***	44 ± 2.7***
	Asparagine	11 ± 2.8	16 ± 2.7	10 ± 0.9	11 ± 1.7
	Glutamine	10 ± 1.5	14 ± 1.8	-	-
	Glutamic acid	-	-	12 ± 1.6	16 ± 1.5
	Aspartic acid	-	-	9 ± 0.8	14 ± 1.1
	Lysine	-	-	8 ± 1.3	11 ± 1.1
La^{3+} (1.5mM)	Water	10 ± 2.3	16 ± 2.2	10 ± 1.0	13 ± 1.3
	EGTA (7mM)	7 ± 1.8	12 ± 1.6	9 ± 1.0	12 ± 0.6
	La^{3+} (1.5mM)	8 ± 1.6	14 ± 1.7	10 ± 1.0	14 ± 0.9
	Ca^{2+}	13 ± 2.6	19 ± 3.1	10 ± 1.3	15 ± 1.0
	Asparagine	36 ± 7.6**	45 ± 7.9**	33 ± 4.1**	42 ± 4.8**
	Glutamine	9 ± 2.1	17 ± 3.0	-	-
	Glutamic acid	-	-	27 ± 1.8***	32 ± 1.8***
	Aspartic acid	-	-	25 ± 2.0***	30 ± 1.3***
	Lysine	-	-	9 ± 1.1	13 ± 1.6

[+] Zoospores encysted by agitation were treated 10 min later with La^{3+} or EGTA and then centrifuged, washed and resuspended in distilled water before post-treatment at 20 min as shown.

Data are percentage germination (means ± s.e. for 7 replicates, after 1.5h and reassessed after 16h) based on counts of 100 spores in each replicate, for two separate experiments.

Significance of difference from matching controls post-treated with distilled water by paired-samples t-tests: *, 0.02; **, 0.01; ***, 0.001.

Mg^{2+}, Sr^{2+} and asparagine, were unaffected by lysine and were suppressed by each of the Ca^{2+} antagonists (Table 5.39). In most cases pre-treatment with a Ca^{2+} modulator significantly lowered germination levels compared to those for matching cysts pre-treated with water. Suppression of germination by early (10 min) treatment with dibucaine and TFP (both calmodulin antagonists; Bereza *et al.,* 1982) verapamil (a Ca^{2+} channel blocker; Godfraind *et al.,* 1986) and amiloride (which regulates Na^{+}/Ca^{2+} flux; Godfraind *et al.,* 1986) could only be overcome by post-treatment with asparagine which then significantly enhanced germination above the level of water controls, *i.e.* pre- and post - treatment with water (Table 5.39). In contrast, suppression of germination by A23187 (a Ca^{2+} ionophore; Pressman, 1976) or TMB-8 (an intracellular Ca^{2+} antagonist; Owen & Villereal, 1982) could not be overcome by any post-treatment (Table 5.39).

In a similar experiment zoospore cysts were pre-treated with SDW, a mixture of chemicals found to suppress germination (comprising verapamil (30μm), dibucaine (50μM), amiloride (20μM), TFP (5μM) and La^{3+} (0.75mM), or DMSO (which was used to dissolve A23187 and was, therefore, included as a control for the effects of A23187) then post-treated (20 min) with cations or amino acids and the effects on germination analysed (Table 5.40).

Germination of cells pre-treated with SDW or DMSO was significantly enhanced by Ca^{2+}, Mg^{2+} and asparagine, unaffected by lysine, and suppressed by post-treatment with the combined antagonist solution (Table 5.40). Suppression of germination by pre-treatment with the antagonist solution was only overcome by post-treatment with asparagine although this level of germination was still significantly lower than the corresponding cells pre-treated with SDW and then post-treated with asparagine.

5.10 Effects of Ca^{2+} modulators on cyst germination of *P. aphanidermatum* and *P. dissotocum*

This experiment was similar to one above, except that it involved a comparison of *P. aphanidermatum* and *P. dissotocum*. Cysts were pre-treated, 10 min after induction of encystment, with dibucaine (50μM) or verapamil (30μM) and then post-treated (20 min) with Ca^{2+}, asparagine or alanine. These amino acids were selected because both normally induces germination of *P. aphanidermatum* and *P. dissotocum* (See Table 5.6), but alanine induces germination of only

Table 5.39 Effects of calcium modulators on germination of cysts of *Pythium aphanidermatum* [+]

Post-treatments [†]	Pre-treatment (μM)						
	Water	Verapamil (30)	TFP (5)	Dibucaine (50)	A23187 (5)	Amiloride (20)	TMB-8 (30)
Water	24 ± 3.4	10 ± 1.6	9 ± 4.0	5 ± 1.4	5 ± 0.9	6 ± 1.1	11 ± 2.3
Ca^{2+}	55 ± 5.5***	6 ± 1.1	10 ± 2.5	8 ± 1.4	9 ± 1.3	5 ± 1.5	9 ± 2.8
Mg^{2+}	39 ± 3.9***	7 ± 1.5	12 ± 3.0	6 ± 1.4	6 ± 1.6	6 ± 2.4	9 ± 2.8
Sr^{2+}	46 ± 2.9***	9 ± 1.8	15 ± 4.0	6 ± 1.0	3 ± 1.7	8 ± 3.6	7 ± 1.4
Asparagine	66 ± 5.7****	53 ± 5.6****	75 ± 6.5***	61 ± 9.7***	6 ± 1.4	69 ± 4.6****	6 ± 1.9
Lysine	19 ± 1.4	10 ± 1.5	10 ± 2.6	8 ± 2.4	5 ± 1.1	7 ± 1.2	8 ± 1.1
Verapamil	8 ± 1.5**	8 ± 1.0	-	-	-	-	-
TFP	12 ± 2.3*	-	12 ± 2.0	-	-	-	-
Dibucaine	11 ± 2.1**	-	-	5 ± 1.1	-	-	-
A23187	6 ± 1.2***	-	-	-	4 ± 1.3	-	-
Amiloride	10 ± 2.3	-	-	-	-	6 ± 1.4	-
TMB-8	11 ± 2.8**	-	-	-	-	-	6 ± 1.0

[+] Zoospores encysted by agitation were treated 10 min later with calcium modulators or water (control) then post-treated at 20 min as shown.
Data are percentage germination (means ± s.e. for 5 replicates, after 16h) based on counts of 100 spores in each replicate.
Significance of difference from matching controls post-treated with distilled water by paired-samples t-tests: *, 0.05; **, 0.02; ***, 0.01; ****, 0.001.
[†] 10mM final concentration except for calcium modulators, which were used at the same concentrations as for pre-treatments.

Table 5.40 Reversal of suppression of germination by Ca^{2+} modulators in *Pythium aphanidermatum* cysts[+]

	Pre-treatment		
Post-treatment (10mM unless stated)	Water	DMSO[‡]	Ca^{2+} modulators[†]
Water	24 ± 2.2	27 ± 2.9	5 ± 1.4
Ca^{2+} modulators[†]	5 ± 0.9***	7 ± 1.2***	2 ± 0.7
Ca^{2+}	50 ± 4.4**	43 ± 3.8**	5 ± 0.9
Mg^{2+}	38 ± 3.6*	40 ± 4.4*	2 ± 0.5
Asparagine	65 ± 5.5**	69 ± 5.8**	28 ± 3.6**
Lysine	19 ± 1.8	22 ± 2.0	3 ± 0.6

[+] Zoospores encysted by agitation were treated 10 min later with a combination of Ca^{2+} modulators then post-treated at 20 min as shown.

Data are percentage germination (means ± s.e. for 5 replicates, after 16h) based on counts of 100 spores in each replicate.

Significance of difference from matching controls post-treated with distilled water by paired-samples t-tests: *, 0.02; **, 0.01; ***, 0.001.

[†] Ca^{2+} modulators: verapamil (30μM), dibucaine (50μM), amiloride (20μM) TFP (5μM) and La^{3+} (0.75mM).

[‡] Dimethylsulphoxide (0.05% w/v)

Table 5.41 Reversal of suppression of cyst germination caused by verapamil and dibucaine in *Pythium aphanidermatum* and *P. dissotocum* cysts[+]

	P.aphanidermatum Pre-treatments			*P. dissotocum* Pre-treatments		
Post-treatment (10mM)	Water	Verapamil (30μM)	Dibucaine (50μM)	Water	Verapamil (30μM)	Dibucaine (50μM)
Water	20 ± 3.8	9 ± 0.9	6 ± 0.7	23 ± 2.6	7 ± 2.3	4 ± 1.0
Ca^{2+}	42 ± 5.4*	7 ± 1.1	7 ± 1.4	54 ± 3.8****	9 ± 2.5	4 ± 1.9
Asparagine	64 ± 9.2*	63 ± 7.6***	45 ± 6.1***	66 ± 6.3***	47 ± 6.1***	52 ± 8.8***
Alanine	17 ± 2.4	12 ± 2.6	8 ± 2.1	41 ± 5.0**	26 ± 3.4*	28 ± 4.4***

[+] Zoospores encysted by agitation were treated 10 min later with verapamil or dibucaine and then post-treated at 20 min as shown.

Data are percentage germination (means ± s.e. for 5 replicates, after 16h) based on counts of 100 spores in each replicate.

Significance of difference from matching controls post-treated with distilled water by paired-samples t-tests: *, 0.05; **, 0.02; ***, 0.01; ****, 0.001.

P. dissotocum (Table 5.6). The findings (Table 5.41) confirmed this: germination of spores pre-treated with SDW was enhanced by post-treatment with Ca^{2+} or asparagine, and also by alanine for *P. dissotocum* only. Pre-treatment of both fungi with dibucaine or verapamil suppressed germination compared to that in water controls. In the case of *P. aphanidermatum* this suppression was overcome only by post-treatment with asparagine, whereas for *P. dissotocum* it was overcome by post-treatment with either asparagine or alanine.

5.11 Discussion

The experiments in this section investigated encystment and germination of zoospores in response to sugars, amino acids and ions. It has been shown that nutrients and cations elicit a range of responses between the different fungi and that in the case of germination a complex relationship exists between the capacity of cations and nutrients to stimulate germination. In addition, Ca^{2+} modulators were found to inhibit cyst germination but this suppression, in most cases, could be overcome by subsequent addition of amino acids whereas cations had little effect. A correlation between adhesion and germination of cysts was also demonstrated.

Of the sugars and amino acids tested (Tables 5.1, 5.2, 5.4) only L-glutamate and L-aspartate elicited encystment of motile spores. No other L- or D-amino acid nor any of the sugars had an effect on encystment. In further tests (Table 5.3) the threshold levels of L-glutamate and L-aspartate eliciting encystment of *Pythium aphanidermatum* zoospores were found to be 5 and 6mM respectively, in both cases above the corresponding chemotactic threshold values of 1 and 4mM respectively (Table 4.2).

This work confirms the findings of Royle & Hickman (1964b) who demonstrated that spores of *Pythium aphanidermatum* were induced to encyst by glutamate and aspartate, but now extends the finding to three *Pythium* spp., which behaved identically in this respect. It contrasts with work on *Phytophthora palmivora* which was not induced to encyst by glutamate or aspartate (Byrt *et al.*, 1982b). The finding that none of the sugars tested could elicit encystment of any of the three *Pythium* spp. was surprising because saccharide residues, notably fucose, have been implicated in the encystment of *Phytophthora cinnamomi* (Hinch & Clarke, 1980) and *Pythium aphanidermatum* (Longman & Callow,

1987) on roots of maize and cress respectively. Both of these studies showed that root treatments which blocked or removed terminal fucosyl residues (*i.e.* treatment with fucose binding lectin or fucosidase enzyme respectively) reduced zoosporic encystment on roots which was primarily in the zone of elongation. More recently Kelleher *et al.* (1991) claimed to demonstrate the presence of a fucose receptor on the zoospore surface of *Phytophthora cinnamomi* and implicated this in encystment (but for a critique see Hardham, 1992) However, these reports of the involvement of fucosyl residues in encystment could not be confirmed in experiments in Sections 3.3 and 5.1 of this thesis.

The findings of Hinch & Clarke (1980), Longman & Callow (1987) and Kelleher *et al.* (1991) do, however, suggest that encystment may be receptor-mediated. In an attempt to examine this zoospores were pre-incubated in a background concentration of amino acids, in an adaptation of the method used to study chemotactic receptors (Section 4.1.2; Adler, 1969), to try and block any receptors that mediate encystment. In no case, however, (Table 5.5) could background levels of amino acids prevent glutamate-mediated encystment, in contrast to similar experiments whereby background levels of amino acids reduced or blocked taxis towards L-glutamate. Although there is no absolute evidence that the amino acids negating taxis share the same chemoreceptors as L-glutamate, the lack of any blocking of encystment might indicate that the processes mediating chemotaxis and encystment are different. This contrasts with the view of Jansson & Thiman (1992), working on *Catenaria anguillulae*, that the receptors mediating chemotaxis and encystment, in response to Con A, are the same. However Con A is widely known to induce encystment of a large number of zoosporic fungi (*e.g.* Hardham & Suzaki, 1986; Kerwin *et al.,* 1991), perhaps acting as a chaotropic (cell-damaging) agent.

In contrast to encystment, a wide range of nutrients elicited cyst germination (Tables 5.6, 5.8). L-glutamate, L-aspartate and L-asparagine elicited germination of all three *Pythium* spp. extending the findings of Jones *et al.* (1991) for *P. aphanidermatum*. In addition, L-alanine stimulated germination of both *P. catenulatum* and *P. dissotocum*, whereas L-glutamine and L-methionine stimulated germination of *P. catenulatum* and *P. dissotocum* respectively. Interestingly the majority of amino acids stimulating germination were also chemotactic (Table 4.1), but this was not invariably true because L-glutamine (chemotactic) did not induce germination of *P. aphanidermatum* and *P. dissotocum* nor L-alanine of *P. aphanidermatum*.

With reference to sugars, glucose and sucrose enhanced germination of all three fungi whereas maltose and fructose had a more limited effect and galactose weakly inhibited *P. dissotocum*. Similarly, Jones *et al.* (1991) had reported that cysts of *P. aphanidermatum* were stimulated to germinate, after 2h, by a sugar mixture, comprising glucose, galactose, fructose, mannose, maltose, raffinose and sucrose.

Most of the compounds eliciting germination had no effect on encystment. Byrt *et al.* (1982b) also reported that several sugars and amino acids capable of eliciting germination, of *P. cinnamomi* spores, had no effect on encystment. They also found that these compounds had to be added at the time of encystment (as was done above) in order to stimulate germination; a delay of 10 min or more led to no response.

Amino acids and sugars were found to promote germ-tube growth of germinating *Pythium* cysts (Table 5.9). In all such cases the spores had been induced to germinate, independently of added nutrients, by adhesion to glass slides (see later). Addition of L-asparagine, L-glutamine, glucose and sucrose significantly increased germ-tube lengths of all three *Pythium* spp. whereas L-alanine, L-glutamate, L-lysine and fructose enhanced germ-tube elongation of at least one of the fungal species. With regard to the amino acids tested, the responses were restricted to L-forms; the corresponding D-forms either had no effect or were inhibitory. Interestingly, there was no absolute correlation between compounds stimulating germination and those promoting germ-tube growth. For each of the *Pythium* spp. L-asparagine, glucose and sucrose stimulated both germination and germ-tube growth. However, L-glutamine stimulated germ-tube growth of all three fungi but had no effect on germination of *P. aphanidermatum* or *P. dissotocum* (Table 5.6); similarly, L-alanine, L-lysine and fructose promoted germ-tube elongation of *P. aphanidermatum* cysts but had no effect on germination (Table 5.6, 5.8). Conversely, L-alanine elicited germination of *P. catenulatum* and *P. dissotocum* cysts (Table 5.6) but had no effect on germ-tube growth. Similarly, Barash *et al.* (1965) have reported differences between the capacity of nutrients to stimulate germination and to stimulate germ-tube growth. For example, L-glutamine had no significant effect on germination of *Phytophthora* cysts but significantly elevated the rate of germ-tube elongation. The results of Barash *et al.* (1965) and those obtained with the three *Pythium* spp. (Table 5.9) would be explained if germinated cysts can take up and metabolise nutrients, thereby promoting germ-tube growth, and if these transport functions are absent from the ungerminated cyst. Then germination

per se would depend on the presence of surface located receptors that are not, themselves, involved in nutrient uptake. Penington *et* al. (1989) have reported that uptake of glucose and glutamate, into zoospores of *P. palmivora*, is only observed after germ-tube emergence. This would, therefore, coincide with the period when zoospore germlings have infected a host plant and are able to use host-derived nutrients to support further growth and infection. Such transport systems involving sugars (Jennings, 1974) and amino acids (Mateos & Sanchez, 1990) have been reported for fungal hyphae.

Induction of zoospore differentiation may be triggered by receptors (Hardham & Suzaki, 1986; Estrada-Garcia *et al.*, 1990b; Kelleher *et al.*, 1991) or more generally, by depolarisation of the spore membrane as has been shown for cations (Jen & Haug, 1981).

Of the cations tested here for their effect on encystment of *Pythium* spp. (Tables 5.10-5.12, 5.25-5.30) only Na^+ had no effect. In general, for the three fungi tested the divalent cations Ca^{2+},Sr^{2+},$Mn^{2+}$and Mg^{2+} elicited encystment whereas Ba^{2+}, Cr^{3+} and Fe^{3+} caused both encystment and cell lysis. Lower levels of encystment were also observed with Li^+ and K^+. These findings agree in part with Byrt *et al.* (1982a), working with *P. cinnamomi*, who found that Ca^{2+},Mg^{2+},Sr^{2+},Ba^{2+},Na^+,K^+ and Fe^{3+} to varying degrees, elicited encystment. The stronger responses were obtained with Ca^{2+}, Sr^{2+}, Mn^{2+} and Fe^{3+}, the latter two cations also causing cell damage. Similar work involving *P. palmivora* has shown that the divalent cations Ca^{2+}, Mg^{2+}, and Sr^{2+} (Grant *et al.*, 1986) stimulate encystment, as do Ba^{2+} and Mn^{2+} (Grant *et al.*, 1985). In contrast to the results obtained with *Pythium* spp. (Tables 5.10, 5.28-5.30) Na^+ was also found to elicit encystment of *P. palmivora* (Grant *et al.*, 1986). In this case it was proposed that, early in encystment, Ca^{2+} is displaced from sites on or in the spores, enabling Na^+ uptake through a Ca^{2+} gated monovalent ion translocator, and that Na^+ uptake elicits encystment by interfering with the water expulsion vesicle and water regulation. This sequence of events is unlikely to happen in the *Pythium* spp. tested, as Na^+ had no effect on encystment. Indeed an absolute requirement for Na^+ has still to be confirmed for differentiation of *P. palmivora* spores (Iser *et al.*, 1989).

Cations were also found to elicit germination of *Pythium* spp. (Tables 5.16-5.30). Ca^{2+}, Mg^{2+}, and Sr^{2+} enhanced germination, compared to distilled water controls, when added to both pre-encysted (Table 5.16-5.18) and motile spores (Table 5.25-5.27) whereas Na^+ and K^+ elicited weak germination responses of spores (Table 5.28-5.30). Mn^{2+} and Li^+ were wholly ineffective,

and Fe^{3+}, Cr^{3+} and Ba^{2+} inhibited germination of all spores (Tables 5.16-5.18, 5.22, 5.24, 5.25-5.30).

Similar results have been reported whereby Ca^{2+} and Sr^{2+} elicit germination of both *P. cinnamomi* (Irving & Grant, 1984) and *P. palmivora* (Grant *et al.*, 1986) whereas K^+ had no effect on germination of either fungus and Li^+ had no effect on germination of *P. palmivora*. In contrast to *P. aphanidermatum*, Mg^{2+} had no effect on germination of *P. cinnamomi* spores (Byrt *et al.*, 1982a) whereas Na^+ elicited germination of *P. palmivora* spores (Grant *et al.*, 1986). In contrast to both *Pythium* spp. and *P. cinnamomi*, germination of *Blastocladiella emersonii* spores were enhanced by K^+, Na^+, Cs^+ and Rb^+, whereas Ca^{2+} and Mg^{2+} were less effective (Soll & Sonneborn, 1972).

The results on adhesion, obtained with spores of *P. aphanidermatum*, (Table 5.13) confirm the findings of Gubler *et al.* (1989) for spores of *P. cinnamomi*. It has long been known (Sing & Bartnicki-Garcia, 1975a, b) that cysts are naturally adhesive when newly formed, and if they come into contact with a surface will adhere permanently to it. But cysts that do not come into contact with a surface soon lose their adhesiveness. This is despite the fact that they retain on their surfaces the glycoprotein released early during encystment and which is thought to be responsible for adhesion. The solution to this paradox was provided by Gubler *et al.* (1989) who showed that the natural adhesiveness of young cysts is abolished by adding EGTA, whereas adhesiveness can be restored to older cysts by addition of Ca^{2+}. The experiments on *Pythium* confirm these findings and suggest that adhesion is Ca^{2+} dependent.

It is likely that the Ca^{2+} that facilitates "natural" adhesion is released from the cells early during encystment, as suggested by the report of Irving *et al.* (1984) that spores of *P. palmivora* release up to 40% of their intracellular Ca^{2+} within 2 min of the induction of encystment. The loss of adhesiveness when cysts are held in suspension (Table 5.13) could then be due to dispersion of the released Ca^{2+}, which would explain the relatively short time during which adhesiveness is retained (Sing & Bartnicki-Garcia, 1972; Gubler *et al.*, 1989). Adding further to this it has now been demonstrated that there is a close correlation between adhesion and germination of *Pythium* cysts (Table 5.13), both of these factors being influenced in the same way by age of the cysts, Ca^{2+} and EGTA. Adhered cysts germinated rapidly and in large numbers whereas

non-adhered cysts germinated poorly. However, adhesion was shown not to be an absolute requirement for germination because Ca^{2+} (and other cations and amino acids) stimulated germination of cysts retained in suspension (*e.g.* Table 5.37) to levels comparable to those of firmly attached cysts (Table 5.13). Also, cysts that had been allowed to adhere and were then physically removed germinated as well as did those that remained attached (Table 5.15). These and other experiments demonstrated that Ca^{2+} alone is necessary for germination, with or without adhesion and even in the absence of exogenous organic nutrients. A similar mechanism may operate in differentiation of the rust fungus *Uromyces appendiculatus*, whereby contact with a specific leaf topography induces appressorium formation which may be under Ca^{2+} control (Zhou *et al.*, 1991).

It was shown, further, that *Pythium* cells adhering early during encystment are independent of exogenously supplied Ca^{2+} whereas cells not adhering early require a Ca^{2+} supplement for adhesion or germination. For *P. aphanidermatum*, the critical time for this change seemed to be 5-7 min (Table 5.14), which coincides with the period when the cyst wall of *P. aphanidermatum* is fully developed (Deacon & Mitchell, 1985); after this time the cells germinated poorly without an exogenous trigger and EGTA had no effect. However, up to 4 min after induction of encystment, when zoospores are still reported to be adhesive (Gubler *et al.*, 1989), the spores germinated well when added to slides but were suppressed by EGTA.

The suppression of germination by EGTA was further analysed by adding EGTA alone or in combination with equimolar concentrations of cations to motile (Table 5.32-5.34) or pre-encysted spores (Table 5.31). In each case suppression elicited by EGTA was overcome by the presence of Ca^{2+}, Mg^{2+}, or Sr^{2+} whereas Na^{+} and K^{+} had no effect. The Ca^{2+} channel blocker La^{3+} (Godfraind *et al.,* 1986) also suppressed germination but its effect was partly overcome by Mg^{2+} whereas Ca^{2+} and Sr^{2+} had no effect (Table 5.35, 5.36). This specific role of Mg^{2+} as opposed to Ca^{2+} was the only instance in which such an effect was noted, because Mg^{2+} normally acts as a substitute for Ca^{2+} in zoospore differentiation but not better than it. The likely explanation is that Mg^{2+} was taken up by cells through a different transport system to that for Ca^{2+} (or Sr^{2+}) which was blocked by lanthanum.

In explanation of these findings, and consistent with work on *P. palmivora* (Iser *et al.*, 1989), it is suggested that Ca^{2+} is rapidly reabsorbed by cells after it has been released (Iser *et al.*, 1989) and then stimulates

germination. Thus in the case of suspended cysts EGTA suppresses germination by sequestering Ca^{2+} released from the spores or from the background medium, whereas La^{3+} acts as a Ca^{2+} channel blocker preventing Ca^{2+} uptake. This would suggest that germination of spores in water controls is also dependent on Ca^{2+} uptake. Interestingly the necessity of Ca^{2+} in germination of suspended cysts, in the presence of EGTA or water, could be satisfied by Sr^{2+} and Mg^{2+}, indicating a non-specific response to divalent cations.

The hypothesis, in short, is that Ca^{2+} plays a central role in triggering germination, but its effects can be substituted for by Mg^{2+} or Sr^{2+} and that the Ca^{2+} released early during encystment both serves to adhere the cyst (by interaction with the released glycoprotein) and serves to trigger germination when it is reabsorbed. All this, as demonstrated here, occurs independently of the availability of exogenous organic nutrients. However organic nutrients (some amino acids and sugars) also can stimulate germination. Notable in this was the finding (Table 5.6) that glutamate, aspartate and asparagine caused germination of zoospore cysts of *P. aphanidermatum*, as in the work of Jones *et al.* (1991), but could not overcome suppression of germination elicited by EGTA (Tables 5.37, 5.38). Only Ca^{2+} was then effective (Tables 5.37, 5.38). However, amino acids (and malt extract+ peptone) could at least partly overcome La^{3+}-suppression of germination (Tables 5.35-5.38) whereas Ca^{2+} could not do so. Together, all these results suggest that there was an absolute requirement for Ca^{2+} uptake for germination (or at least, an absolute requirement for Ca^{2+} to be present in the external environment) and that selected amino acids could trigger germination by facilitating Ca^{2+} uptake even when this was otherwise blocked.

Further tests served to support this conclusion. Ca^{2+} modulating drugs were found to suppress germination of suspended (non-adhered cysts) below even the low control levels in water (Table 5.39). However, suppression of germination by dibucaine and TFP (both calmodulin inhibitors, Bereza *et al.*, 1982), verapamil (a Ca^{2+} channel blocker; Godfraind *et al.*, 1986), amiloride (which regulates Na^{+}/Ca^{2+} flux; Godfraind *et al.*, 1986) and a combination of all of these modulators plus La^{3+} (Tables 5.39, 5.40) could at least partly be overcome by addition of asparagine (a known stimulator of germination of control cysts) but not by lysine (non-stimulatory in controls). In contrast, post-treatment with cations including Ca^{2+}, which elicited significant germination of water controls, could not overcome suppression of germination elicited by the Ca^{2+} modulators. The exceptions to the ability of specific amino acids to

overcome the effects of Ca^{2+} modulators were in the case of spores treated with A23187 (a Ca^{2+} ionophore, Pressman, 1976) and TMB-8 (an intracellular Ca^{2+} antagonist, Owen & Villereal, 1982). The effects of A23187 (Table 5.39) perhaps could not be overcome because this ionophore would serve to equilibrate Ca^{2+} levels inside and outside of the cells, preventing the internal accumulation of Ca^{2+} at levels sufficient to elicit germination. The effects of TMB-8 (Table 5.39) could be explained on the assumption that intracellular antagonism of Ca^{2+}-mediated processes blocked germination even when Ca^{2+} was taken up by the cells.

It was noted above that Ca^{2+} must be available in order for asparagine or other specific amino acids to trigger germination. So the simplest explanation, consistent with all the data, is that these amino acids opened Ca^{2+} channels that were independent of those blocked by La^3 or verapamil. The presence of asparagine-gated cation channels has not previously been reported but equivalent glutamate-gated Ca^{2+} channels are widely reported in other organisms (Gilbertson *et al.,* 1991; Hollmann *et al.*, 1991). An alternative suggestion involving symport of Ca^{2+} with asparagine can be excluded because Penington *et al.* (1989) showed that amino acids are only transported into cysts at the germling stage.

If the above hypothesis is correct then asparagine must also have opened Ca^{2+} channels that were closely linked to calmodulin (because asparagine overcame the effect of the calmodulin inhibitors TFP and dibucaine) or otherwise enabled Ca^{2+} to trigger germination without the intervention of the Ca^{2+} binding protein calmodulin, which is known to be present in spores of *P. cinnamomi* (Gubler *et al.,* 1990) and is almost certainly present in *Pythium* spp.

Amiloride, which regulates Na^+/Ca^{2+} flux, is known to block Na^+ channels (Godfraind *et al.,* 1986). It is unlikely, however, that suppression of germination elicited by amiloride was due to Na^+ imbalance or blockage of Na^+ channels, despite the suggestion (Grant *et al.*, 1986) that Na^+ is involved in germination of *P. palmivora,* because Na^+ had no effect on germination of *P. aphanidermatum* (Table 5.19). However, amiloride may also block Ca^{2+} channels (Tytgat, *et al.*, 1990) suggesting once more that asparagine, which rescued the suppression of germination elicited by amiloride, overcomes suppression of germination by opening Ca^{2+} channels. Amiloride is also reported (Manev *et al.,* 1990) to block glutamate-gated cationic channels. Although this may cast doubt, in the case of amiloride, that asparagine

overcomes suppression of germination by gating Ca^{2+} channels, this observation was made with rats and the corresponding effects of amiloride on fungal spores are not documented.

Almost all the work on Ca^{2+} involvement in germination was done with *P. aphanidermatum.* However, it was supported by an experiment in which *P. aphanidermatum* and *P. dissotocum* were compared directly (Table 5.41). Then two amino acids, which were known to have different abilities to trigger germination of control cysts were also found differentially to overcome the suppression of germination caused by dibucaine or verapamil. This specificity of effect served to reinforce the view that amino acids eliciting germination can operate through Ca^{2+}-mediated processes, according to the presence of specific germination-triggering receptors on individual fungal isolates.

Taken collectively the results in this section of the thesis suggest a central role of Ca^{2+} in stimulating germination, whereby Ca^{2+} released early in encystment, which may also facilitate zoospore adhesion, is rapidly reabsorbed by the spore to trigger germination. Early adhesion may prevent dispersal of released Ca^{2+}, which in turn stimulates germination by acting on a "control centre" near the pre-determined point of germ-tube outgrowth. *In vivo*, spores dock and encyst with precise orientation on roots, such that the point of germ-tube outgrowth is located next to the host (Mitchell & Deacon, 1986; Paktitis *et al.,* 1986). Adhesion at this point would ensure that the released Ca^{2+} does not rapidly diffuse away from this region, and the rapid resorption of Ca^{2+}, demonstrated for cysts of *P. palmivora* (Irving *et al.,* 1984), would ensure that the cyst receives a Ca^{2+} signal leading to germination and host penetration. *In vitro* experiments demonstrate that treatments sequestering or inhibiting Ca^{2+} uptake suppress germination (Tables 5.37-5.39) whilst non adhered spores, due to Ca^{2+} limitation, show low basal levels of germination. *In vivo*, as with *in vitro* experiments, non-adhered spores of *P. aphanidermatum* deprived of Ca^{2+} might be stimulated to germinate by specific amino acids such as asparagine, glutamate or aspartate, which are present in root diffusates (Moody *et al.,* 1988), acting via opening of Ca^{2+} channels. If so, then even the relatively few spores that do not dock correctly could still detect the presence of a root due to it's amino acids diffusates. They might then germinate due to the opening of high-affinity Ca^{2+} channels and the emerging germ-tube could show tropism towards nutrients present in root diffusates (Jones *et al.*, 1991), leading to host infection.

A similar mechanism, whereby contact with a surface facilitates an influx of cations leading to appressorium germination, may occur in the fungus *Uromyces appendiculatus* (Zhou *et al.,* 1991). In this case bean leaf stomata provide a topographical signal, opening a mechanosensitive ion channel, which appears to allow an influx of ions, including Ca^{2+}, that trigger differentiation. Similarly, differentiation can be inhibited by the mechanosensitive ion channel blocker Gd^{3+} which can be partly overcome in the presence of Ca^{2+}.

Most recently Zhang *et al.* (1992) have suggested that blocking or removal of exogenous Ca^{2+} by verapamil and EGTA respectively, suppresses germination by preventing the production of phosphatidic acid (PA), synthesised via a stimulus-activated phospholipase D, which may act as a second messenger in eliciting germination of *P. palmivora.* Further work will be necessary to see if this is also true of *Pythium* spp.

6. GENERAL DISCUSSION

The work in this thesis has involved the study of factors influencing the behaviour and infection process of three different root-pathogenic *Pythium* spp.- *P. aphanidermatum*, *P. catenulatum* and *P. dissotocum*. It has been shown that cations and calcium-modulating drugs can influence the motile behaviour of zoospores and that *Pythium* spp. respond chemotactically to amino acids and sugars. In addition, zoospores are induced to encyst in the presence of amino acids, uronic acids, cations and plant-derived compounds such as polysaccharides and root mucilage. Similarly, zoospores were stimulated to germinate in the presence of various sugars, amino acids, uronates and divalent cations. In particular, a central role of calcium in both adhesion and germination of zoospore cysts is shown, and germination *per se* can be inhibited by calcium-modulating drugs but their effects can be overcome by amino acids. The role of exogenous factors, which presumably interact with zoosporic receptors, in determining both the zoospore infection cycle and localised accumulation and encystment, *in vivo*, are discussed.

The motile characteristics of zoospores of *Pythium aphanidermatum* were found to be influenced by divalent cations whereas monovalent and trivalent cations had no effect (Tables 4.13-4.21). The presence of Ca^{2+}, Ba^{2+}, Mg^{2+}, Mn^{2+} or Sr^{2+}, supplements in the zoospore suspending medium, caused zoospores of *P. aphanidermatum* to change from their normal helical swimming mode with periodic abrupt turns (Figure 4.3) to a perpetual circular swimming mode. Conversely, the Ca^{2+}-chelator EGTA (Schmid & Reilley, 1957) suppressed random changes of direction and caused zoospores to swim in continuous straight paths of reduced helical amplitude (Figure 4.4). Zoospores in the presence of Ca^{2+} or EGTA were locked into the perturbed modes of swimming behaviour, described above, and were unable to respond chemotactically to the attractant L-glutamate (Table 4.22). In addition, spores in the presence of Ca^{2+} or EGTA were occasionally observed to collide, but were merely deflected and did not deviate from their perturbed modes of behaviour. Interestingly, cations in combination with EGTA produced differing responses which were concentration-dependent. In general, however, monovalent cations in combination with EGTA caused spores to swim in a non-deviating straight path and adopt patterns of motility elicited by EGTA (Tables 4.13-4.15). Conversely, Ca^{2+} together with an equivalent concentration of EGTA induced spores, initially, to swim in perpetual circles but they later

reverted to straight swimming typical of EGTA. In the case of Ba^{2+}, Mg^{2+} and Mn^{2+}, however, spores were able to revert to normal swimming patterns. Similarly, Fe^{3+} totally suppressed patterns of motility elicited by EGTA and spores displayed normal motile behaviour.

Ca^{2+}-modulating drugs also affected the motility patterns of zoospores of *P. aphanidermatum* and four distinct modes of motile behaviour were observed (Table 4.23; Figures 4.5-4.10). The Ca^{2+} entry-blockers lanthanum and verapamil (Godfraind *et al.*, 1986) caused perpetual circular swimming. The Ca^{2+} ionophore A23187 (Pressman, 1976) and amiloride, which is a Na^{+}/Ca^{2+} flux inhibitor (Godfraind *et al.*, 1986), caused spores to swim in a "jerky" manner with frequent changes of direction (Table 4.23; Figures 4.9, 4.10). The calmodulin inhibitors trifluoperazine and dibucaine (Bereza *et al.*, 1982) caused a reduction of the zoospore swimming speed (Table 4.23) coupled with a "posterior" drift of the spore (figures 4.7, 4.8) which resembled that of a handbrake turn of a vehicle.

The most reasonable explanation for these perturbed modes of behaviour is that the cations and the Ca^{2+} modulators must have altered the normal behaviour of the zoospore flagella. It has been proposed that the anterior flagellum of *P. palmivora* provides swimming thrust whilst the posterior flagellum acts as a rudder eliciting directional change (Holwill, 1982; Carlile, 1983). If this is true for *Pythium* spp. then it is likely that treatments with Ca^{2+} and Ca^{2+} modulating-drugs had differential effects on the two flagella. Sequestration of Ca^{2+} by EGTA may have influenced the posterior flagellum with the result that it remained in a trailing position, preventing the spore from changing direction. Conversely, divalent cations (including Ca^{2+}) and the Ca^{2+} channel-blockers might have induced circular swimming by causing permanent deflection of the posterior flagellum. Boitano & Omoto (1992) reported that trout sperm, in the presence of Ca^{2+}, are induced to swim in a circular fashion due to their posterior flagellum being permanently bent. The observation that circular swimming is induced by both Ca^{2+} and Ca^{2+}-entry blockers suggests that a relatively high external to internal ratio of Ca^{2+} is associated with this mode of motility. However, other divalent cations, such as Ba^{2+} and Sr^{2+}, could mimic the patterns of motility elicited by Ca^{2+} and also induced circular swimming. Indeed Ba^{2+} may have acted as a Ca^{2+} channel-blocker (Bloodgood, 1991), inducing circular motion akin to that caused by lanthanum and verapamil. Ba^{2+} and Sr^{2+} have also been found to mimic the motile effects elicited by Ca^{2+} on the ctenophore *Beroe*, by activating motility and microtubule sliding of the flagella (Tamm, 1989). Mg^{2+} and Mn^{2+} are similarly

known to substitute for Ca^{2+} in the activation of motility of trout sperm (Boitano & Omoto, 1992).

The perpetual jerking of zoospores treated with amiloride or A23187 (Figures 4.9, 4.10) was indicative of continual threshing of the posterior flagellum. This may be a result of repeated depolarisation of the posterior flagellar membrane due to an imbalance of ion flux, in accordance with the assumption that random turns, during normal motility, are associated with temporary membrane depolarisation (Carlile, 1983). Spiral drifting and a reduction of speed of zoospores treated with the calmodulin antagonists, trifluoperazine and dibucaine (Figures 4.7, 4.8), would be consistent with a reduction of thrust generated by the anterior flagellum while the posterior flagellum remains largely unaffected. This observation may be explained by the finding that zoospores of *P. cinnamomi* are reported to have calmodulin localised at the base of their anterior flagellum (Gubler *et al.,* 1990) and there is no reason to believe that the same is not true of *Pythium* spp.

The findings, therefore, demonstrate a central role of Ca^{2+} in determining the motile behaviour of zoospores, consistent with Ca^{2+}-mediation of motility and chemotaxis in other organisms. For example the presence of Ca^{2+} and EGTA can govern chemotaxis by influencing the switch between tumbling and smooth swimming of *Bacillus subtilis* (Ordal 1977). Similarly, localised accumulation of zoospores in the vicinity of host roots and substrates, *in vivo*, is likely to involve direct interaction of zoospore surface chemoreceptors and attractant molecules leading to signal transduction and other intracellular events in which Ca^{2+} is involved. Transmembrane signalling is important in ciliary and flagellar action, whereby Ca^{2+} levels regulate protein phosphorylation, which determines axonemal behaviour (Bloodgood, 1991).

Chemotaxis of fungal zoospores has been analysed for different species of *Phytophthora* (*e.g.* Khew & Zentmyer, 1973; Halsall, 1976) but direct comparisons involving different *Pythium* spp. have not previously been reported. Here it was shown that all three *Pythium* spp. showed identical chemotactic responses to amino acids (Tables 6.1-6.3): they showed positive taxis to L-alanine, L-asparagine, L-aspartic acid, L-glutamine and L-glutamic acid whereas no response was observed to D-amino acids or other L-amino acids. Identical responses towards the same amino acids were surprising because Halsall (1976) found different chemotactic responses with different species of *Phytophthora*, and different isolates of *P. aphanidermatum* also are reported to differ in taxis to amino acids- the examples in separate studies (Royle & Hickman, 1964b;

Chang-Ho & Hickman, 1970; Jones *et al.,* 1991). One of the common features in previous work, however, is that both Royle & Hickman (1964b) and Jones *et al.* (1991), reported chemotaxis of *P. aphanidermatum* to L-glutamate and L-aspartate. The isolate of *P. aphanidermatum* used by Jones *et al.* (1991) was also the one used in this thesis. The ecological significance of differences in taxis to amino acids among *Pythium* spp. is unknown. According to van der Plaats-Niterink (1981) the three *Pythium* spp. used here have different host ranges, but with some overlap such as sugar beet and tomato. It may be that they show general, non-specific, taxis to the common amino acids released by these and other hosts and that their host-ranges are determined after the chemotactic phase.

Despite the comments above and the similar chemotactic responses of *P. aphanidermatum*, *P. catenulatum* and *P. dissotocum* to amino acids these three fungi responded differently in chemotaxis towards sugars. All showed taxis to mannose, but glucose and fructose attracted only two of them and maltose and sucrose attracted only *P. aphanidermatum* (Tables 6.1-6.3). The possibility that these differential responses to sugars might be related to host range and to the sugars released in the root exudates of different plants merits further study. Certainly, there are characteristic differences in the root mucilages of different plants (Moody *et al.*, 1988; Ray *et al.,* 1988; Chaboud & Rougier, 1990) but the significance of this is discussed later. A final point of note in relation to the compounds that elicit taxis is that none of the three *Pythium* spp. showed positive taxis to ethanol. This contrasts with the behaviour of several *Phytophthora* spp. For example *P. cinnamomi* was first shown to be attracted to ethanol (Allen & Newhook, 1973) then *P. palmivora* (Cameron & Carlile, 1978). Carlile (1983) records unpublished work by Benjamin & Newhook which showed that many other (but not all) *Phytophthora* spp. showed taxis to it, and the number of "responsive" *Phytophthora* spp. was extended by Morris & Ward (1992) who also found that *Pythium irregulare* was positively tactic to ethanol. The significance of taxis to ethanol and other volatile metabolites (Cameron & Carlile, 1978) has been suggested to be that these are fermentation products of roots in wet soils (where root metabolism will soon create anoxic conditions at the root surface). Such wet soil conditions are characteristically associated with infection by *Pythium* and *Phytophthora* spp., so it is suggested (Allen & Newhook, 1973; Carlile, 1983) that ethanol or similar volatile fermentation products could be common chemoattractants in field conditions. Against this, however, is the fact that not all fungi responded to them (*e.g.* the three *Pythium* spp. used here), and the rate of release of amino acids and other common

Table 6.1 Stimulatory effects of L-amino acids and sugars at different stages of the developmental sequence of zoospores of *P. aphanidermatum*

Compound	chemotaxis	encystment	germination	germ-tube growth
L-glutamate	+	+	+	+
L-aspartate	+	+	+	n.d.
L-glutamine	+	0	0	+
L-asparagine	+	0	+	+
L-alanine	+	0	0	+
L-Lysine	0	0	0	+
Other L-amino acids†	0	0	0	0
D+mannose	+	0	0	0
Sucrose	+	0	+	+
Fructose	+	0	0	+
Maltose	+	0	+	0
D+glucose	0	0	+	+
Other sugars‡	0	0	0	0

† Other L-amino acids tested but found to have no stimulatory effect were: Proline, Leucine, Tryptophan, Histidine, Methionine, Phenylalanine, Arginine, Serine, Isoleucine, Hydroxyproline, Threonine, Valine, Glycine.

‡ Other sugars tested but found to have no stimulatory effect were: L+arabinose, D-arabinose, D+xylose, L-fucose, D+galactose.

n.d.; not determined.

Table 6.2 Stimulatory effects of L-amino acids and sugars at different stages of the developmental sequence of zoospores of *P. catenulatum*

Compound	chemotaxis	encystment	germination	germ-tube growth
L-glutamate	+	+	+	0
L-aspartate	+	+	+	n.d.
L-glutamine	+	0	+	+
L-asparagine	+	0	+	+
L-alanine	+	0	+	0
Other L-amino acids†	0	0	0	0
D+mannose	+	0	0	0
Sucrose	0	0	+	+
Fructose	0	0	+	n.d.
D+glucose	+	0	+	+
Other sugars‡	0	0	0	0

† Other L-amino acids tested but found to have no stimulatory effect were: Proline, Leucine, Lysine, Tryptophan, Histidine, Methionine, Phenylalanine, Arginine, Serine, Isoleucine, Hydroxyproline, Threonine, Valine, Glycine.

‡ Other sugars tested but found to have no stimulatory effect were: Maltose, L+arabinose, D-arabinose, D+xylose, L-fucose, D+galactose.

n.d.; not determined.

Table 6.3 Stimulatory effects of L-amino acids and sugars at different stages of the developmental sequence of zoospores of *P. dissotocum*

Compound	chemotaxis	encystment	germination	germ-tube growth
L-glutamate	+	+	+	+
L-aspartate	+	+	+	n.d.
L-glutamine	+	0	0	+
L-asparagine	+	0	+	+
L-alanine	+	0	+	0
L-Methionine	0	0	+	0
Other L-amino acids†	0	0	0	0
D+mannose	+	0	0	0
L-fucose	+	0	0	0
Sucrose	0	0	+	+
Fructose	+	0	+	+
Maltose	0	0	+	0
D+glucose	+	0	+	+
Other sugars‡	0	0	0	0

† Other L-amino acids tested but found to have no stimulatory effect were: Proline, Leucine, Lysine, Tryptophan, Histidine, Phenylalanine, Arginine, Serine, Isoleucine, Hydroxyproline, Threonine, Valine, Glycine.

‡ Other sugars tested but found to have no stimulatory effect were: L+arabinose, D-arabinose, D+xylose, D+galactose.

n.d.; not determined.

components of root exudates would also be enhanced in waterlogged soil conditions. Indeed there may be an even more fundamental argument against a major role of ethanol as a chemotactic agent in the root zone. The site of maximum production of fermentation products should be the most metabolically active zone (the meristem) and not the root elongation zone or zone of emerging root hairs which are typically the most attractive zones for zoospores.

In order for zoospores to detect attractant compounds, they must have some form of receptor mechanism. In an investigation of receptor activity, the specificity of a proposed receptor detecting L-glutamate was analysed (Tables 4.6-4.11) using the method of Adler (1969) which has also been used successfully for zoospores of the rumen chytrid *Neocallimastix frontalis* (Orpin & Bountiff, 1978). In this thesis it was found that background concentrations of the chemoattractants L-glutamine, L-alanine and L-glutamate blocked or reduced chemotaxis of all three *Pythium* spp. to L-glutamate. L-asparagine, as a background, also blocked chemotaxis of *P. aphanidermatum* to L-glutamate, and L-aspartic acid had no effect in any case. In reciprocal tests, the presence of L-glutamate in the zoospore suspension negated taxis of *P. aphanidermatum* (the only fungus tested) to L-asparagine, L-glutamine and L-alanine but had no effect on attraction towards L-aspartate (Table 4.12). These results suggest the active involvement of at least two different receptors, whereby one receptor detects L-glutamate, L-alanine, L-glutamine and, in the case of *P. aphanidermatum*, L-asparagine; the other receptor detects L-aspartate in addition to, perhaps, L-asparagine in the case of *P. catenulatum* and *P. dissotocum*. This analysis follows that commonly used in such competition studies (Adler, 1969). However, non-chemoattractive L-amino acids, as well as D-forms in the case of *P. aphanidermatum*, also negated taxis to L-glutamate. As these "blocking" agent are unlikely to have acted via receptors, it seems that at least some background amino acids can interfere with chemotaxis in other ways. Such observations have also been made for zoospores of *Phytophthora* spp. (Khew & Zentmyer, 1973) and for *Escherichia coli* (Mesibov & Adler, 1972). The possibility was investigated that the blocking agents might negate chemotaxis by altering zoospore motility, in the same way as Ca^{2+} and EGTA supplements prevented taxis to L-glutamate by inducing, respectively, perpetual circular and perpetual straight swimming modes. However, (Tables 4.24, 4.25) it was found that none of the background amino acids that were tested had any effect on the motile behaviour of zoospores of *P. catenulatum* or *P. dissotocum*, even though these tested compounds had interfered with chemotaxis to L-

glutamate when used in identical conditions. Their actions in blocking chemotaxis remain unexplained.

In general, zoospores of *Phytophthora* and *Pythium* do not show host-specific or substrate-specific taxis (for exceptions see Chi & Sabo, 1978; Morris & Ward, 1992). But there are several reports that zoospores are differentially induced to encyst by host roots (Mitchell & Deacon, 1986a; Goldberg *et al.*, 1989) and insoluble substrata (Mitchell & Deacon, 1986b). In tests here, of all the amino acids and soluble sugars tested only L-glutamate and L-aspartate were found to induce encystment- a response common to all three species (Tables 6.1-6.3). The fact that this was stereospecific, *i.e.* not elicited by D-glutamate or D-aspartate strongly suggests that it was receptor-mediated and not caused by pH or other chemical or physical properties. The threshold concentrations of L-glutamate and L-aspartate that induced encystment were 5 and 6mM respectively (Table 5.3) -higher than the corresponding threshold levels required to elicit chemotaxis (1 and 4mM; Table 4.2). This has parallels with work on root-infecting bacteria. Root derived flavonoids elicit both taxis and expression of the *Sym* plasmid (Caetano-Anolles, 1988) whereas acetosyringone has a similar multiple role in *Agrobacterium tumefaciens* (Ashby *et al.*, 1988). In other words, a single compound can serve to attract motile cells to roots and the same compound near the source can activate other functions. The same might be true of L-aspartate and L-glutamate in root exudates, if these compounds are, indeed, involved in encystment *in vivo*.

An alternative or additional factor that might induce encystment at the root surface is the presence of an "inductive" surface or specific root surface-located residues. This was first reported by Hinch & Clarke (1980), who noted that the zone of maximum encystment of zoospores of *P. cinnamomi* (actually assessed as adhesion rather than encystment) on maize roots was also the zone of maximum binding of a fucose-specific lectin. It was thus suggested that localisation of adhesion (? encystment) on roots was mediated by fucosyl residues which were similarly localised on the roots. Longman & Callow (1987) made a similar report for adhesion of *P. aphanidermatum* to the zone of elongation of cress and in both studies a variety of evidence was assembled to support the involvement of fucosyl residues (see Introduction). However, the results reported here, which substantiate the work of Jones *et al.* (1991), raise doubts about the role of simple saccharide residues in mediating localised accumulation and encystment- at least for zoospores of the isolate of *P. aphanidermatum* used here on roots of wheat. When wheat roots coated in a

double layer of calcium alginate gel were added to zoospore suspension, the zoospores accumulated at the root cap and in the zone of young root hairs by 90 sec. However, by 10 min the zoospores had vacated the root cap region, without encysting, and swam to the root hair zone where zoospores were rapidly induced to encyst in large numbers (Figures 3.1-3.4). Significantly, the root hair zone was the only site at which sustained attraction was observed- it continued to attract further spores- thus suggesting, perhaps, synergism between the factors eliciting taxis and encystment. These results, which were also obtained with non-gelled wheat roots (Figures 3.7, 3.8), were surprising because it was assumed that root slime, and hence its terminal saccharide residues, would be masked by the double layer of alginate gel. Treatment of gelled and non-gelled wheat roots with lectins and enzymes, in an attempt respectively, to block and to remove exposed saccharide residues had no effect on the localised accumulation or encystment in the root hair zone. Similarly, gelled and non-gelled wheat roots treated with FITC-labelled lectins (including fucose binding protein) showed no evidence of localisation of specific saccharide residues that could account for localisation of encystment. In short, all the evidence in this thesis casts serious doubt about the role of specific saccharide residues, including fucose, in the localisation of encystment by *P. aphanidermatum* on wheat roots- or even for a role of root surface slime at all, because alginate gel could serve as a suitable surface on which zoospores could encyst on roots.

Surface-mediated events must also participate in encystment, however, because treatment of non-gelled roots with the dyes methylene blue and India ink (Table 3.1) significantly reduced the number of zoospores encysting on roots but had no effect on accumulation of motile spores in the immediate root vicinity (Table 3.2). In contrast, similar attempts to block encystment of zoospores on gelled roots were unsuccessful (Table 3.4). The mode of action of India ink, which is often used for negative staining of bacterial exopolysaccharides, is unclear but the particles could be precipitated from the ink by treatment with acid, suggesting that they are charged. Methylene blue is known to be an ionic dye, so it too may interfere with charge-mediated events involved in encystment (Mitchell & Deacon, 1986c).

Typically, zoospores were observed to encyst at the bases of root hairs of non-gelled wheat roots (Figure 3.9), in accordance with the work of Jones *et al.* (1991). In examination of the effects of root surface mucilage *in vitro*, zoospores of *P. catenulatum* and *P. dissotocum* were induced to encyst by both tomato and wheat root mucilage whereas only wheat mucilage mediated encystment of *P. aphanidermatum*. The differential encystment response elicited

by tomato mucilage was surprising because all three fungi are reported to infect tomato plants (van der Plaats-Niterink, 1981; Goldberg *et al.*, 1989). The compounds causing this encystment are possibly polyuronates, which are major components of root mucilage (Moody *et al.*, 1988; Ray *et al.*, 1988; Chaboud & Rougier, 1990) and are reported to cause encystment of fungal zoospores (Grant, *et al.*, 1985). Alginate also is a polyuronate, composed of guluronic acid and mannuronic acid (Haug, 1974), and was found to be a conducive surface for zoosporic encystment in root studies (Section 3.3). Of the uronic acids tested for induction of encystment *in vitro*, poly-D-galacturonic acid, sodium alginate, D-galacturonic acid and D-glucuronic acid all were effective for all three *Pythium* spp. (Table 3.8). But at the highest concentrations tested ($25mgml^{-1}$) D-galacturonic acid and poly-D-galacturonic acid also caused cell lysis. Similarly, Grant *et al.* (1985) demonstrated that all four of these uronic acids elicited encystment of *Phytophthora palmivora*. Jones *et al.* (1991) in addition showed that sodium alginate induced encystment of *P. aphanidermatum*.

In contrast to the results discussed thus far, differential responses were obtained for the three different *Pythium* spp. in tests on induction of encystment by purified polysaccharides of plant origin. *P. catenulatum* was induced to encyst by five of the eight compounds examined, whereas *P. dissotocum* responded to three and *P. aphanidermatum* to only two of them (Table 3.1). Therefore, each fungus showed a unique pattern of encystment. For example, only *P. catenulatum* was induced to encyst by the fucose containing polysaccharides, fucoidan and fucosylated xyloglucan, but it was not so induced by the non-fucosylated xyloglucan which caused encystment of *P. dissotocum*. Conversely, all three fungi were induced to encyst by gum arabic, a polymer comprising D-glucuronic acid, ß-D-galactose and α-L-arabinose and none were encysted by an arabinogalactan (*i.e.* lacking the uronic acid). These tests were limited by the availability of relatively pure, characterised polysaccharide, and thus it is possible that minor unidentified compounds elicited some of these effects. Further work could examine this possibility by testing partial hydrolysates so as to characterise the active groups or sequences. However, these tests illustrate that the ability to respond to fucosylated polysaccharides is species-specific, and perhaps isolate-specific, whereas all species responded similarly to uronates and uronic acid-containing compounds such as gum arabic. The differential responses almost certainly reflect differences in the range of zoospore surface proteins (Hardham & Suzaki, 1986) linked to signal transducers for encystment.

In addition to polysaccharides, zoospores of the three fungi showed

differential responses to chitinous and cellulosic materials (Table 3.10). Zoospores of *P. dissotocum* were induced to encyst by both cellulose and chitin whereas *P. catenulatum* and *P. aphanidermatum* were induced to encyst only by cellulose and chitin respectively. These results reflect similar differences among other zoosporic fungi (Mitchell & Deacon, 1986b). Notably, in all cases zoospores were not attracted to cellulose or chitin but were induced to encyst after random contact. Interestingly, both *P. catenulatum* and *P. dissotocum* were induced to encyst by both cellulose and a mixed-linkage glucan (ß1,3 and ß1,4 linkages) whereas *P. aphanidermatum* encysted in response to neither.

With regard to induction of encystment *in vivo*, it is likely that zoospores of *P. aphanidermatum* encyst locally on roots because spores are attracted to localised root regions by chemotaxis, presumably in response to root diffusates. The spores are then induced to encyst by root surface components, blockable by treatment with methylene blue or Indian ink, suggesting ionic involvement and thus a role of uronates. Other saccharide residues of root slime, in defined sequences or configurations, might act also in this role and perhaps synergistically with chemoattractants, such as L-glutamate and L-aspartate, which also can elicit encystment *in vitro*. Such synergystic effects were clearly seen in studies involving wheat roots coated in alginate gel because encystment was localised to zones of maximum release of chemoattractants even though the roots were uniformly coated with the same gel polymer, and even though alginate can alone, induce encystment *in vitro*. Similarly, Royle & Thomas (1973) recognised synergism but in a different context: the encystment of downy mildew zoospores on stomata involved the combined roles of chemical and topographical signals.

These findings suggest that tests on the induction of encystment *in vitro* may not be strictly applicable to conditions *in vivo*. An indication of this is also given by the fact that cysts induced to form when spores make contact with alginate beads *in vitro* germinate with random orientation relative to the bead, whereas germination *in vivo* is always orientated towards the host (Jones *et al.*, 1991). The point of germ-tube outgrowth from a cyst is fixed (pre-determined), so some factor *in vivo* must serve to orientate the zoospore as it encysts. This role could be served by synergism between root diffusates and uronate residues- the attractants bringing the zoospore to the root so that encystment receptors dock the spore onto the root surface gel as it encysts. The germ-tube would arise immediately adjacent to the root, enabling rapid penetration and infection of the root. It seems relevant in this context that, of a bank of monoclonal antibodies (MAbs) raised against cell surface components of zoospores of *P.*

cinnamomi, only the class of MAb that bound specifically to the surface of both flagella induced rapid encystment (Hardham & Suzaki, 1986). Indeed, these MAbs caused encystment also of another fungus, *P. drechsleri*, even though they had not been raised against it. Estrada-Garcia *et al.* (1990b) also found that of a bank of MAbs raised against *P. aphanidermatum*, only those that bound to both flagella caused rapid encystment when added to zoospores *in vitro*.

With regard to germination, zoospores of the three *Pythium* spp. showed similar responses to amino acids and simple sugars (Table 6.1-6.3). For example, all were induced to germinate in the presence of L-asparagine, L-glutamate, L-aspartate, glucose and sucrose although L-alanine and fructose stimulated germination of *P. catenulatum* and *P. dissotocum* only whereas maltose enhanced germination of *P. aphanidermatum* and *P. dissotocum* only (Tables 6.1-6.3). These findings agree with those of Jones *et al.* (1991), using the same isolate of *P. aphanidermatum*, who found enhanced germination in the presence of L-glutamate, L-asparagine, L-aspartate and sugar mixtures. As with chemotaxis and encystment, germination responses were stereospecific being limited to L-forms of amino acids. Notably a wide range of amino acids and sugars were capable of enhancing germination whereas only L-glutamate and L-aspartate elicited encystment. Byrt *et al.* (1982b) also reported that several amino acids and sugars capable of causing germination had no effect on encystment of spores of *P. cinnamomi*. In contrast, of the complex polysaccharides examined for their effects on germination, gum arabic elicited both encystment and germination of *P. aphanidermatum* and *P. catenulatum* but elicited encystment only of *P. dissotocum* spores (Table 3.11). Similarly, arabinoxylan and methylglucuronoxylan elicited both encystment and germination of- *P. aphanidermatum* and *P. catenulatum*- respectively whereas non-fucosylated xyloglucan had a similar effect on spores of *P. dissotocum*. However, of all the complex polysaccharides examined, collectively, there was no absolute tendency for compounds to elicit both encystment and germination (Table 3.11). Similarly, of the four uronic acids examined, all of which induced encystment, only sodium alginate strongly enhanced germination of *Pythium* cysts (Table 3.9) whereas poly-D-galacturonic acid, D-glucuronic acid and D-galacturonic acid either had no effect or were inhibitory to germination. In contrast, Grant *et al.* (1985) found that sodium alginate inhibited germination whereas galacturonic acid stimulated germination of *P. palmivora*. This uronate was implicated as being the active component of pectin which elicits both encystment and germination (Zhang *et al.* 1990).

The effects of cations on encystment and germination were also

studied here. Of the cations tested for their effects on encystment, Ca^{2+}, Sr^{2+}, Mg^{2+} and Mn^{2+} elicited encystment of all three fungi whereas Ba^{2+}, Fe^{3+} and Cr^{3+}, in addition to encystment, also induced cell lysis (Tables 5.10-5.12). Conversely, weaker responses were observed with L^{+} and K^{+} whereas Na^{+} had no effect. Similarly, Byrt *et al.* (1982a) and Grant *et al.* (1985), working with *P. cinnamomi* and *P. palmivora* respectively, found that strong encystment responses were obtained with cations- notably Ca^{2+}, Mg^{2+} and Sr^{2+}. Ca^{2+}, Mg^{2+} and Sr^{2+} enhanced germination of all three *Pythium* spp. (Tables 5.16-5.24) whereas other cations had little effect or, in the case of Ba^{2+}, Cr^{3+} and Fe^{3+}, were inhibitory to germination. Parallel results are also reported for *Phytophthora* spp: Ca^{2+} and Sr^{2+} enhanced germination of both *P. cinnamomi* (Irving & Grant, 1984) and *P. palmivora* but, in contrast to the *Pythium* spp, *P. cinnamomi* was unaffected by Mg^{2+} and cysts of *P. palmivora* were induced to germinate by Na^{+}.

Interestingly, results obtained here with *P. aphanidermatum* demonstrated a close relationship between adhesion of zoospore cysts and the ability to germinate, and also suggested that Ca^{2+} plays an active role in both of these processes. Ca^{2+} was found to promote adhesion of zoospore cysts, thus confirming a similar report for *P. cinnamomi* (Gubler *et al.,* 1989). The results demonstrated that new cysts of *P. aphanidermatum* naturally adhered to a glass surface, but this was prevented by EGTA. Conversely, older cysts (by 10 min) lost their ability to adhere, but this can be restored by addition of Ca^{2+} (Table 5.13). Gubler *et al.* (1989) suggested that similar loss of adhesiveness with ageing of *P. cinnamomi* was due to dispersion of Ca^{2+} released by zoospores early in the encystment process (Irving *et al.,* 1984). Evidently, Ca^{2+} interacts with the glycoprotein cyst coat to confer on it adhesive properties and in the normal course of encystment on a (host) surface, the Ca^{2+} released in the first few minutes of encystment will interact with the newly released cyst coat glycoprotein to attach the cyst to the surface.

In vitro, zoospore cysts normally, require an external stimulus for germination (*e.g.* Grant *et al.*, 1986). However, cysts of *P. aphanidermatum* that had adhered to a glass surface immediately after induction of encystment needed no further trigger for germination (Table 5.13), whereas equivalent young cysts, but non-attached, required an exogenous stimulant such as divalent cations or amino acids (*e.g.* Tables 5.36, 5.37). The capacity of young cysts to germinate was retained during a delay of 4-5 min in contacting a surface during which time the cysts still had the capacity to adhere, but was lost by 10 min at which time the cyst also had lost the ability to adhere. These findings suggested

close association between adhesion and germination. Nevertheless, this was not absolute because adhesion for the initial 10 min was sufficient to enable germination, and cysts could then be dislodged without effect on subsequent germination. These findings can be related to the zoospore infection process, *in vivo*, by a simple model. Zoospores dock precisely on roots such that the fixed point of germination is adjacent to the host (Hardham & Gubler, 1990; Jones *et al.*, 1991) and the glycoprotein secreted over the germination site adheres the cyst in this orientation (Gubler & Hardham, 1990). As on slides, adhesion *in vivo* probably involves Ca^{2+}, released during encystment, and the adhesive should prevent dispersion of the released Ca^{2+} from the germination site, perhaps by ionic bridging but at least by lengthening the diffusion path. Ca^{2+} could then be reabsorbed and trigger germination. Thus, docking would lead automatically to germination, independent of exogenous triggers, such as when cysts adhere and germinate on glass. Further, the orientation of germination would lead automatically to host-penetration. Therefore, it is proposed that the zoospore has an in-built reporter system to signal that it has docked properly, and infection follows by a Ca^{2+}-mediated cascade.

The proposed requirement for Ca^{2+} in mediating germination is strengthened by the observation that the Ca^{2+}-chelator EGTA (Schmid & Reilley, 1957) inhibited germination, presumably by sequestering extracellular Ca^{2+} (Tables 5.37, 5.38). In addition, the Ca^{2+} channel-blockers lanthanum and verapamil (Godfraind *et al.,* 1986) also inhibited germination suggesting that uptake of Ca^{2+} into the spore is a requisite for germination (Tables 5.37- 5.39). TMB-8, an intracellular Ca^{2+} antagonist (Owen & Villereal, 1982) also inhibited germination, demonstrating that Ca^{2+}- mediated events within the cyst are needed for germination. Lastly, supporting this view, addition of trifluoperazine and dibucaine, both of which inhibit the Ca^{2+}-binding protein calmodulin (Bereza, *et al.*, 1982), also inhibited germination. Calmodulin is reported to be present at the base of the anterior flagellum of *P. cinnamomi*, and the base of this flagellum is located near the point of germ-tube outgrowth. It is thus tempting to suggest that the "control centre" within the cyst which detects Ca^{2+} and elicits germination, may be linked to the localisation of calmodulin or a similar regulatory protein. Supporting this view both the Ca^{2+} ionophore A23187 (Pressman, 1976) and amiloride, which controls Na^{+}/Ca^{2+} flux (Godfraind *et al.*, 1986), inhibited germination as would be expected by a disruption of membrane events that maintain ionic levels, in particular Ca^{2+}, within the cyst.

Despite all this evidence that links Ca^{2+} to germination, specific

amino acids such as L-asparagine, can trigger germination *in vitro*. The question arises as to whether they do so independently or through Ca^{2+}- mediated events. This point has still not been fully resolved, but a key line of evidence was the finding that asparagine and other "stimulatory" amino acids were unable to trigger germination in the presence of EGTA (Tables 5.37, 5.38), suggesting that at least a basal level of Ca^{2+} uptake is necessary for germination. In further tests it was found that asparagine (and other "stimulatory" amino acids) could rescue spores that had been inhibited to germinate by Ca^{2+} channel blockers (lanthanum and verapamil) and amiloride (which in addition to modulating Na^{+}/Ca^{2+} flux, might also in some cases block Ca^{2+} channels- Tytgat *et al.*, 1990). Asparagine also overcame the inhibition of germination caused by the calmodulin inhibitors but it did not overcome suppression caused by the intracellular Ca^{2+} antagonist TMB-8. In short, all the evidence points to the ability of asparagine and similar germination-triggering amino acids to overcome the suppression of germination when Ca^{2+} uptake from the external medium is inhibited by various drugs, but the amino acids cannot overcome the effect of an internal Ca^{2+} antagonist, nor can they act when Ca^{2+} is unavailable in the external medium. The simplest explanation of this is that the amino acids somehow enable Ca^{2+} to be taken up, even when the normal channels are blocked and that Ca^{2+} is still the key germination trigger. There are two possible ways in which this could occur- either the amino acids facilitate Ca^{2+} uptake by symport, when they themselves are taken up by membrane transport processes, or the amino acids remain outside the cyst but bind to specific receptors and in this way "gate" the entry of Ca^{2+}. It is unlikely that the mode of rescue of germination by asparagine is through amino acid uptake into the spore because Penington *et al.* (1989) found no significant uptake of labelled glutamate by zoospores or zoospore cysts of *P. palmivora* until the germ-tube had emerged. This, therefore, precludes the uptake of Ca^{2+} by symport, with amino acids, and indicates that instead the asparagine acted through receptors on the cyst membrane to open Ca^{2+} channels, allowing Ca^{2+} entry into the cell which in turn triggered germination. Ca^{2+}-channel-linked glutamate receptors are widely reported in other organisms (*e.g.* Gilbertson *et al.*, 1991; Hollman *et al.*, 1991) and L-glutamate was one of the amino acids that could overcome suppression of cyst germination caused by the Ca^{2+} channel blocker lanthanum. However, to date no Ca^{2+} channels linked to asparagine have been reported in other organisms, and this needs further investigating. A receptor mediated role of amino acids in enabling Ca^{2+} uptake is supported by the finding that L-alanine stimulated germination of *P. dissotocum* but not *P. aphanidermatum*,

and could also overcome suppression of germination caused by verapamil or dibucaine in *P. dissotocum* but not *P. aphanidermatum*.

If the hypothesis here is correct, then the question arises as to the role that specific amino acids might have in triggering germination *in vivo*. Clearly, they are not essential, because Ca^{2+} alone (absorbed shortly after adhesion to the root) would be sufficient to enable germination. However, if amino acids have the ability to open Ca^{2+} channels, then they could synergise Ca^{2+} uptake during the docking sequence on roots. This could explain the common observation (*e.g.* Jones *et al.,* 1991) that cysts germinate rapidly with almost 100% efficiency on roots, in contrast to the maximal of approximately 70% in this study (Table 5.13). The ability of Ca^{2+} to stimulate germination of fungal cells is not restricted to zoospores. Gadd & Brunton (1992) recently reported that exogenous Ca^{2+} (5mM) induced germ-tube formation from yeast-like cells of the Dutch elm disease pathogen *Ophiostoma ulmi*. As with the results obtained for *Pythium aphanidermatum*, germ-tube formation of *O. ulmi* was suppressed by treatment with EGTA, the calmodulin inhibitor trifluoperazine and the Ca^{2+} ionophore A23187, which prompted the authors to suggest that Ca^{2+} uptake by *O. ulmi* is required for germ-tube formation. This perhaps reflects a general requirement of fungal cells for Ca^{2+} in induction of germ-tube formation.

In addition to being able to stimulate germination of zoospore cysts, amino acids and sugars were also found to promote germ-tube extension in conditions (adhesion to glass slides) where the cysts germinated independently of added nutrients (Table 5.9). L-asparagine, L-glutamine, glucose and sucrose significantly enhanced germ-tube growth of all three fungi, whereas each of L-alanine, L-glutamate and L-lysine increased germ-tube elongation of at least one fungus. As in previous examples, stimulatory responses elicited by amino acids were restricted to L-forms, whereas D-forms either had no effect or were inhibitory to germ-tube growth. Interestingly, there was no absolute correlation between the compounds that stimulate germination of cysts and those that promoted germ-tube growth, confirming a previous report for *Phytophthora* spp. (Barash *et al.,* 1965). For example, L-glutamine stimulated germ-tube outgrowth of all three *Pythium* spp. but had no effect on germination of *P. aphanidermatum* and *P. dissotocum* (Tables 5.6, 5.9). Conversely, L-alanine elicited germination of *P. catenulatum* and *P. dissotocum* cysts but had no effect on germ-tube growth. The ability of sugars and amino acids to enhance germ-tube growth is presumably due to transport of these nutrients into the cyst, which is likely to occur only at the germling stage (Penington *et al.,* 1989). Such

uptake does not involve receptors *per se* but transport proteins (Jennings, 1974; Mateos & Sanchez, 1990).

The major theme of this study was to determine the exogenous factors that control or influence each of the different stages of the zoospore infection process from motility and chemotaxis to encystment and cyst germination. This has involved screening of a wide range of compounds of both known composition, *e.g.* amino acids, sugars, cations, and largely unknown composition, *e.g.* root mucilage, and analysis of their effects on zoospore developmental responses *in vitro*. Some remarkable common patterns have emerged, in that of all the compounds tested, only L-glutamate and L-aspartate elicited the "whole" pre-penetration sequence of each of the three fungi- chemotaxis and cyst germination, extending the report by Jones *et al.* (1991) for the same isolate of *P. aphanidermatum* as used here.

The stereospecificity of the responses strongly suggests that they operate through receptors, and enable one to exclude other possible influences based on pH, ionic charge and chemical properties. This is compatible with receptor-mediated chemotaxis in many other organisms (Armitage & Lackie, 1990) and with the induction of encystment by specific lectins and monoclonal antibodies of *Pythium* and *Phytophthora* spp. (Hardham & Suzaki, 1986; Estrada-Garcia *et al.*, 1990b). As already noted, the possibility that uptake of sugars and amino acids is required to elicit a response is unlikely because Penington *et al.,* (1989) found no uptake of glutamate or glucose by zoospores or cysts of *P. palmivora* until the germ-tube had emerged.

The results here suggest that the three *Pythium* isolates had chemotactic receptors detecting L-aspartate, L-glutamate, L-asparagine, L-glutamine and L-alanine, but differed in chemotactic receptors for sugars (Tables 6.1-6.3). Similarly, all three fungi had germination-mediating receptors for L-aspartate, L-glutamate, L-asparagine, glucose and sucrose, but differed in receptors for L-alanine, L-glutamine, L-methionine, maltose and fructose (Tables 6.1-6.3). But the encystment receptors- at least for monomers- were confined to L-glutamate and L-aspartate, so it may be assumed that L-glutamate and L-aspartate receptors are present and active throughout the whole developmental process of the three *Pythium* spp. Significantly, perhaps, glutamate and aspartate are known to be released from roots of most if not all plants (Rovira, 1965) and are among the most abundant amino acids commonly found in root diffusates (Chang-Ho & Hickman, 1970). Therefore, these amino acids may play an important role in eliciting zoospore responses *in vivo* and this may be coupled to the fact that some *Pythium* spp., in general, have extremely

wide host ranges (Mitchell & Deacon, 1986a).

Apart from these two compounds, each of the *Pythium* spp. responded to a different range of compounds at different developmental stages. Rai & Strobel (1966) also reported similar observations for *Aphanomyces cochliodes*, and Jones *et al.* (1991) reported that *P. aphanidermatum* differed in its responses to compounds not only in taxis, encystment and germination but also in germ-tube tropism.

A summary of these chemoresponses is shown in Table 6.1 for the isolate of *P. aphanidermatum* used here and by Jones *et al.* (1991) and for which the most evidence is available. In addition, the proposed location of receptors eliciting each of the developmental responses of *P. aphanidermatum*, as will now be discussed, are shown in Table 6.4. If, as has been argued throughout this thesis, these chemoresponses are receptor-mediated, then a different range of receptors must be present (or active) at the different developmental stages. In simple terms, the transition from a zoospore to a cyst and then to a germling is a transition of a flagellate cell to a cell that has shed its flagella, to the same cell with a germ-tube. The receptors inducing germination must clearly be present on the somatic (cyst) membrane. They may be present throughout the motile stage or are alternatively inserted when the zoospore peripheral vesicles fuse with the cell membrane during encystment (Estrada-Garcia, 1989; Gubler & Hardham, 1991). The receptors that mediate germ-tube tropism are almost certainly located at the germ-tube tip. Their insertion perhaps coincides with the ability of the germ-tube to take up nutrients (Penington *et al.*, 1989), even though they may not function directly in nutrient uptake, because Musgrave *et al.* (1977) working with *Achlya bisexualis*, found no obvious relationship between the chemotropic thresholds of different amino acids and the Km and Vmax values for uptake of the same compounds by older germlings.

The locations of chemotactic receptors are more difficult to deduce. They could be on the flagella, as in the case of chemoreceptors for sexual agglutinin of *Chlamydomonas* (Bloodgood, 1991) and chemosensors on the cilia of olfactory epithelium (Lancet, 1988) or they could be on the soma as in the case of receptors for acetate, folate and cAMP of *Paramecium* (Van Houten, 1990). It is tempting to propose that chemotactic receptors are located on the flagella because zoospores responded to a wider range of amino acids in taxis than in germination. However, it is possible that chemotactic receptors for L-alanine and L-glutamine, which do not function in eliciting germination, may be located on the zoospore soma but are lost during surface changes in the process of encystment. In any case it is likely that the receptors eliciting chemotaxis are

Table 6.4 Examples of responses of *P. aphanidermatum* to compounds during zoospore taxis, cyst germination, germ-tube tropism and germ-tube growth, with proposed locations of chemoreceptors and transport systems. Data from this study and Jones *et al.*, (1991)

	Taxis	Germination	Tropism	Germ-tube growth	Interpretation
L-glutamate	+	+	+	+	Receptors on soma, germ-tube membrane and perhaps on the the flagella, transport system in germ-tube membrane
L-asparagine	+	+	0	+	Receptors on soma, and perhaps on flagella, transport system in germ-tube membrane
L-alanine	+	0	+	+	Receptors on soma or flagella, or both, receptor and transport system in germ-tube membrane
L-glutamine	+	0	0	+	Receptors on flagella or soma or both, transport system in germ-tube membrane
L-leucine	0	0	+	0	Receptor only on germ-tube membrane

different from those eliciting encystment, because only two of the nine chemotactic compounds, of *P. aphanidermatum* were able to induce encystment. Moreover, chemotaxis to, for example L-glutamate could be blocked by isotopic backgrounds of other amino acids whereas L-glutamate-induced encystment was not blocked by the same compounds (Tables 4.6, 4.5). The location of at least some receptors that elicit encystment may be on the flagella, because Hardham (1989) demonstrated that a monoclonal antibody which bound flagella and not to any other surface component of *P. cinnamomi* could specifically cause encystment *in vitro*. In addition this monoclonal antibody was found to elicit encystment by other *Pythium* and *Phytophthora* spp. suggesting that the antigen that it recognises (? an encystment-mediating receptor) is almost universally present in these fungi.

In future it may be possible to analyse receptor mediated responses and to determine the locations of these receptors by use of mutants which do not respond to well-defined stimuli at different stages of the infection process. It is difficult to isolate such mutants, which would almost certainly be recessive to wild type, because of the diploid nature of Oomycetes. The work here may serve to facilitate future studies on species chemosensory-deficient mutants, because these could be selected for at various stages of the developmental sequence and, once obtained compared for their range of chemoresponses at the various developmental stages.

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