ROLE OF CALCIUM IN ZOOSPORE BIOLOGY OF *PHYTOPHTHORA* AND *PYTHIUM* SPECIES

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A Thesis presented for the
Degree of Doctor of Philosophy
University of Edinburgh
1997

DECLARATION

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

Adrian J. Warburton

Acknowledgement

I thank the Ministry of Agriculture, Fisheries and Food for funding this project.

I wish to express my sincere gratidude to Dr Jim Deacon for his supervision during the project, and especially for his guidence during the writing of this thesis.

I also would like to thank the following people for their help and advice, because without them much of this work would not have been possible. Dr Nick Read (ICMB) for allowing me use of the confocal microscope, Dr Dave Dryden (ICMB) for allowing me to use his spectrofluorimeter and for helpful discussions on the spectral properties of everything, and Dr Andy Mount (Chemistry) for a whirlwind tour of the Debye-Huckel limiting law, and physical chemistry in general.

I also wish to express my sincere thanks to Patrick Taylor for being a great friend and for always being available for a well-earned trip to the boozer, and for making the past year bearable. The same goes for Pete McMullen, Richard Inwood, Benson Durham, Stephen Poole and Russ Atkinson. The Goodfellas.

Thanks also go to the many people who I have worked with in the lab over the past few years, in particular, Dave Grayson for valuable scientific contributions, for being a great laugh, and for just being "around".

I would also like to thank my ever-growing family including Paul, Nadine, Emma and Grandma Edie. In particular, I thank my Mum and Dad for constant support and encouragement during my years at university (it's all over now). For these reasons, I dedicate this thesis to them.

ABSTRACT

The calcium ion (Ca^{2+}) has been implicated in many aspects of the behaviour of zoospores of phytopathogenic members of the Oomycota, so manipulation of calcium levels or interference with calcium-mediated processes offers the potential for controlling several important diseases caused by these fungi. The work in this thesis was designed to study the role of Ca^{2+} in zoospore biology, and extends previous findings by comparing effects of Ca^{2+} and Ca^{2+}-modulating treatments on zoospores and zoospore cysts of *Pythium* spp., *Phytophthora* spp. and *Aphanomyces euteiches.*

Zoospore motility was studied by videomicroscopy to compare the behaviour of *Phytophthora infestans*, *Ph. parasitica* and *Ph. palmivora*, *Pythium aphanidermatum*, *Py. dissotocum*, *Py. catenulatum* and *A. euteiches.* Zoospores of all fungi swam in an extended helix, but the *Phytophthora* spp. and *A. euteiches* made frequent changes of direction when swimming in water or buffer, whereas the *Pythium* spp. did not do so. Chelation of external Ca^{2+} with EGTA or BAPTA buffer caused zoospores of all fungi to swim in a straight path, reduced random turning, and prevented the spores from changing direction when they collided with obstacles. *Phytophthora* zoospores accumulated at the surface of suspensions, but this was abolished in the presence of EGTA, whereas *Pythium* zoospores always accumulated at the base of suspensions. Isotropic backgrounds of some amino acids (e.g. L-glutamic acid) induced an irregular motility pattern; this was overridden by EGTA but restored by subsequent addition of Ca^{2+}. These findings are discussed in relation to host-location behaviour by zoospores.

A role of Ca^{2+} in germination of cysts, induced by agitation of zoospores of *Py. aphanidermatum* and *Ph. parasitica*, was demonstrated by reduced or suppressed germination in the presence of Ca^{2+}-uptake inhibitors (La^{3+}, verapamil), Ca^{2+} chelators (BAPTA), calmodulin-antagonists (trifluoperazine, calmidazolium) or compounds that affect intracellular Ca^{2+} stores (caffeine, TMB-8). The critical concentrations and times of application of these treatments were determined, and some divalent cations and organic nutrients (sugars, amino acids) were shown partly to reverse the suppression of germination.

Attempts to load an esterified form of the Ca^{2+}-sensitive fluorochrome fura-2 into motile zoospores were unsuccessful. However, non-esterified fura-2, added to the bathing solution of zoospores of *Ph. parasitica*, enabled fluxes of Ca^{2+} to be monitored by fluorimetry when zoospore populations were induced to encyst by agitation. The Ca^{2+} level of the bathing medium decreased significantly in the first 3-4 minutes after encystment, then increased progressively over 20-30 min, to significantly higher levels than those initially, indicating early uptake and then release of Ca^{2+} from encysting cells. These changes were markedly altered by Ca^{2+}-modulating treatments: the presence of TMB-8 caused an initial slight (significant) reduction of the external Ca^{2+} concentration when zoospores encysted, followed by a slow further reduction; the presence of verapamil and La^{3+} caused an initial increase in the external Ca^{2+} concentration, and a slow continued increase thereafter. Thus, a link was demonstrated between Ca^{2+} fluxes and zoospore development into cysts.

Esterified Calcium Green-1, a Ca^{2+}-sensitive fluorescent dye, was successfully loaded into zoospores of *Py. aphanidermatum*, and was detected by laser scanning confocal microscopy of both motile zoospores and during the transition of zoospores into cysts. However, the dye was rapidly compartmentalised in the cells and could not be used to monitor changes in cytosolic Ca^{2+} concentrations.

TABLE OF CONTENTS

CHAPTER 1

Introduction

1.1 General Introduction

There are five groups of zoosporic fungi or fungus-like organisms - Chytridiomycota, Hypochytridiomycota, Plasmodiophoromycota, Labyrinthulomycota and Oomycota (Fuller, 1987; Alexopoulos *et al*, 1996). A criterion for grouping all these fungi is their ability to produce zoospores. Fungal zoospores are asexual, wall-less, flagellate, motile cells capable of dispersal in the presence of free water. A major basis for separation of the subdivision Mastigomycota into these five groups is the variation in flagellation of the cells, that is, the number, type and insertion of flagella on the swimming zoospore (Table 1.1).

These organisms are abundant in soil and aquatic environments (Sparrow, 1960), some can also be found on aerial plant surfaces (Buczacki, 1983) and even in anaerobic conditions of the rumen (Orpin & Bountiff, 1978). Zoosporic parasites occur on a diverse range of hosts and are receiving increasing attention, for example, as economically important pathogens of crop plants, including *Pythium* spp. and *Phytophthora* spp. in soil and *Ph. infestans* and powdery mildew pathogens on leaves.

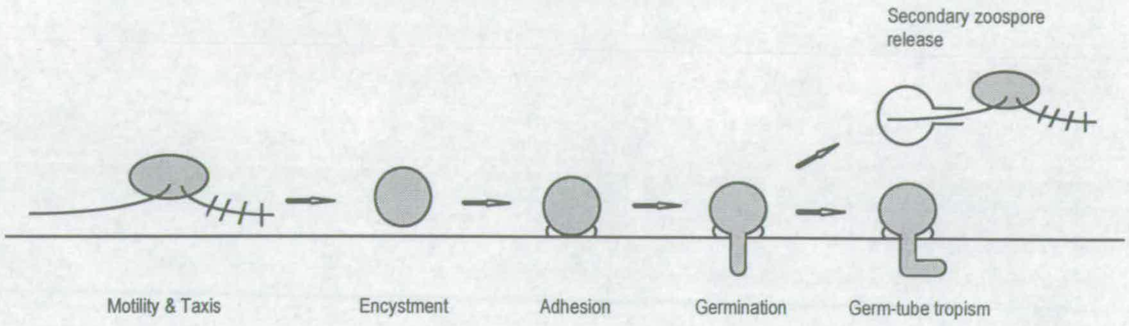
Dispersion of zoospores is limited unless aided by moving water (Newhook *et al.*, 1981; Wilkinson *et al.*, 1981) so the ability of root pathogens to locate root tips, wounds or even individual root cells (eg. Goldberg *et al.*, 1989; Shushkoff, 1989) may lie in the role of motile zoospores as precise homing agents, or site-selection agents, through detection of environmental signals (Deacon & Donaldson, 1993). The role of zoospores as site-selection agents may enable the downy mildew fungus *Pseudoperenospora humuli* (Oomycota) to precisely locate stomata of hop leaves (Royle & Thomas, 1971), *Catenaria anguillulae* (Chytridiomycota) to accumulate around the mouth and other body orifices of its nematode host (Jansson & Thiman, 1992; Deacon & Saxena, 1997a), and other species to selectively colonise non-living substrates in natural environments (eg. Couch, 1939; Park, 1974; Mitchell & Deacon, 1986).

Table 1.1 Flagellation characteristics of some zoosporic fungi and fungus-like organisms.

Group	Flagellation characteristics	Examples
Chytridiomycota	uniflagellate: posterior, whiplash	*Blastocladiella* spp *Allomyces* spp *Coelomomyces* spp *Catenaria* spp
Hypochytridiomycota	uniflagellate: anterior, tinsel	*Rhizidiomyces* spp
Plasmodiophoromycota	biflagellate: anterior, whiplash posterior, whiplash	*Plasmodiophora* spp
Labyrinthulomycota	biflagellate: anterior, tinsel posterior, whiplash	*Labyrinthula* spp
Oomycota	biflagellate: anterior, tinsel posterior, whiplash	*Pythium* spp *Phytophthora* spp *Achlya* spp *Saprolegnia* spp *Aphanomyces* spp

An understanding of the mechanisms involved in the homing responses of zoospores is central if zoosporic fungi are to be exploited or controlled. The behaviour of *Phytophthora* (Oomycota) zoospores and the sequence of developmental stages leading to host location and infection as shown in Figure 1.1 have been discussed by Carlile (1983) and recently updated to include *Pythium* spp. (Oomycota) by Deacon and Donaldson (1993). Essentially, the homing sequence begins with release of zoospores from a sporangium and results in penetration of host tissues. Motile, flagellate zoospores are released from the sporangium and, in the absence of external stimuli, can remain motile for many hours, utilising endogenous lipid reserves (Bimpong, 1975). Motile zoospores may be responsive to a diverse range of stimuli which can elicit taxes so that the zoospores accumulate at sites suitable for colonisation or avoid unfavourable environments. Usually, when zoospores locate a suitable site they encyst; the motile wall-less spore becomes a walled cyst. The cyst may then develop into a germling with a hyphal apex. Germ-tubes can show tropism to or from external stimuli, thereby maximising recognition and subsequent colonisation of the target. These stages in which zoospores locate and colonise their target may be disrupted, thus reducing or eliminating successful infection.

Figure 1.1 The homing sequence of a typical phytopathogenic zoospore (Oomycota). Adapted from Deacon & Donaldson (1993).

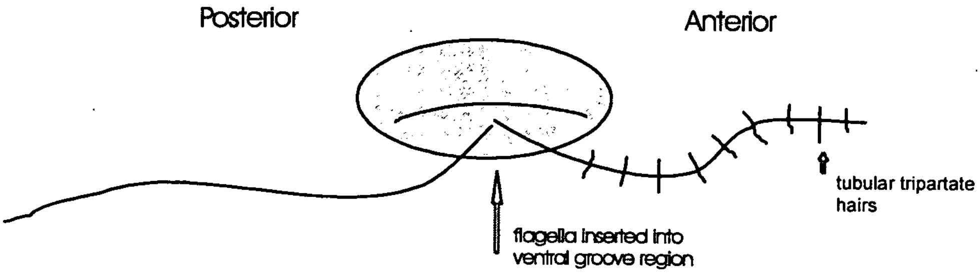


The natural sequence of events and the factors involved in each of these stages are reviewed below. In addition, due to recent persuasive arguments for the participation of calcium ions (Ca^{2+}) in the developmental stages of zoosporic fungi of the Oomycota (eg. Griffith *et al.*, 1988; Deacon & Donaldson, 1993; Jackson & Heath, 1989; 1993), an account of a possible role for calcium ions (Ca^{2+}) in a cell signalling capacity will be included in this chapter.

1.2 Motility

Zoospores of the Oomycota are biflagellate; the point of insertion of the two flagella lies deep within a groove which runs along the ventral surface of the zoospore (Figure 1.2). Cytoplasmic microtubules are found in the complex of "rootlets" anchoring the flagella into the zoospore soma. The centrally positioned pyriform nucleus has the narrower end oriented toward the groove region of the zoospore and the two basal bodies of the flagella. The major rootlet consisting of eight fused microtubules extends from the basal bodies along the longitudinal axis of the zoospore to the end of the cell in *Ph. parasitica* (Reichle, 1969). Microtubules arise at regular intervals on both sides of the rootlet to form a rib-like frame just inside the plasma membrane. Anti-calmodulin antibodies have been used to show location of calmodulin at the extreme base of the anterior flagellum, whereas only a weak immunostaining reaction for calmodulin is found elsewhere along the lengths of both flagella (Gubler *et al.*, 1990). Additionally, centrin, another Ca^{2+}-binding protein, has been localised at the anterior flagellar rootlet (Hardham, 1992). The anterior, or tinsel flagellum bears fine "flimmer" hairs - termed "mastigonemes" in several studies. However, this terminology is considered obsolete (Dick, 1997) and a flagellum bearing these tubular tripartate hairs is now called a straminipilous flagellum. The posterior whiplash flagellum is smooth and longer than the anterior flagellum. The anterior flagellum contributes 10 times the swimming thrust of the posterior flagellum due to the presence of tubular tripartate hairs (Holwill, 1982) and the action of the posterior flagellum is thought to be responsible for stabilising the swimming zoospore or acting as a rudder leading to a change in direction of zoospore swimming - it is seen to bend by 90° to the direction of movement during a turn (Holwill, 1982; Carlile, 1983). During uninterrupted forward swimming, however, both flagella undulate in a plane as sinusoidal waves are propagated at the base of the flagellum and progress towards the apex (Hickman, 1970; Holwill, 1982). Both *Pythium* (Cahill *et al.*, 1996; Donaldson & Deacon, 1993a) and *Phytophthora* (Allen & Newhook, 1973; Cahill *et al.*, 1996; Ho & Hickman, 1967) zoospores follow a helical path, rotating about their long axis as they swim.

Figure 1.2 Simplified diagram of a typical zoospore of the Oomycota showing insertion of the two flagella.



Barash *et al.* (1965) reported that motile *Ph. drechsleri* zoospores metabolise ^{14}C-labelled compounds such as D-glucose, acetate and L-glutamate. However, Carlile (1983) argued that metabolism of these compounds probably occurred during cyst germination rather than by motile zoospores. Furthermore, Bimpong (1975) found that *Ph. palmivora* zoospores contained endogenous lipid reserves which were reduced after zoospores had been swimming for several hours. She suggested, therefore, that endogenous lipids were the main source of energy for zoospore motility. Carlile (1983) suggested that nutrients may act as encystment triggers rather than as energy sources for the maintenance of zoospore motility.

Studies on zoospore swimming speeds have involved measurement of the actual swimming speed of zoospores along their helical path or the speed in which they travel from place to place. The actual swimming speed of zoospores is dependent upon temperature. Several studies involving *Phytophthora* spp. (eg. Ho & Hickman, 1967; Allen & Newhook, 1973) and *Pythium* spp. (Donaldson & Deacon, 1993a) have been reported, a linear displacement of around 100-150μm s^{-1} (at room temperature) being typical. Presumably, a higher metabolic rate would account for faster swimming at high temperatures. Interestingly, zoospores of the Oomycota are reported to make frequent, random changes of direction every 2-4s, thus interrupting the straight, helical swimming path. Furthermore, Newhook *et al.* (1981), studying *Ph. cinnamomi* zoospores swimming through coarse wet sand, concluded that the rate at which zoospores moved through the sand (unaided dispersion) was only a little higher than the rate of diffusion of a small molecule such as HCl.

Zoospores swimming in soil water are likely to encounter many obstructions, especially when carried through soil by free-flowing water. To test whether collision with obstacles might induce rapid encystment, Young *et al.* (1979), working with *Ph. cinnamomi*, investigated the effects of "ideal soils" composed of glass microbeads on zoospore motility. These workers found that zoospores could be passed through columns of glass microbeads without affecting motility as long as the size of the passages between the beads was greater than the zoospore dimensions. In extension of these findings, Newhook *et al.* (1981) found that motile zoospore suspensions could pass through cores of undisturbed soils and be retrieved, still motile, to about 75% of the original number.

Some *Phytophthora* spp., in particular *Ph. infestans*, produce zoospores that remain motile for only relatively short (~5min) periods under laboratory conditions. In the case if *Ph. infestans*, the importance of motility in the natural environment should not be underestimated since zoospores can not only infect the potato tuber through soil but also the leaf surface where particulate matter may be far less abundant.

It seems probable, therefore, that zoospores can be transported by water through soils, and remain motile long enough in natural soils or on leaves for motility to play a useful role in the homing sequence of zoosporic fungi.

1.2.1 Role of Ca^{2+} in motility

Several studies have shown that ion supplements can alter the motile behaviour of swimming zoospores (eg. Allen & Harvey, 1974; Thomas & Butler, 1989; Ersek *et al.*, 1991). Thomas and Butler (1989) found that Ca^{2+} caused zoospores of *Achlya* to swim in straight paths. Lithium ions caused immobilisation of *Ph. infestans* (Ersek *et al.*, 1991), this effect being reversed with subsequent Ca^{2+} addition. An apparent role of Ca^{2+} in zoospore motility is consistent with many studies on other types of cell which have implicated Ca^{2+} in flagellar activity (eg. Bloodgood, 1991; Boitano & Omoto, 1992) and in the motile responses of bacteria (Eckert, 1972; Ordal, 1977), algae (Harz & Hegemann, 1991), slime moulds (Newell *et al.*, 1990) and marine invertebrates (Tamm, 1989). Furthermore, a recent study by Donaldson and Deacon (1993a) found that Ca^{2+} mediates the locomotory attributes and thus, presumably, the homing behaviour of *Pythium* zoospores. Addition of Ca^{2+} or other divalent cations to *Py. aphanidermatum* zoospores was reported to change the normal helical motility pattern to one of perpetual circular swimming. Amiloride, a Na^+/Ca^{2+} membrane flux inhibitor in animal systems, caused jerky swimming and calmodulin antagonists (eg. dibucaine) caused a significant reduction in swimming speed. These workers proposed that calmodulin antagonists resulted in loss of swimming speed by exerting their effects on the anterior flagellum - as mentioned above, the calcium-binding protein calmodulin is intensely localised at the base of the anterior flagellum (Gubler *et al.*, 1990). Erratic, jerky swimming is suggested to be caused by repeated depolarisations of the membrane of the posterior flagellum (Allen & Newhook, 1973; Carlile, 1983) thus affecting its rudder function.

Sensory perception of environmental stimuli is linked to changes in intracellular free Ca^{2+} levels in numerous cell types studied to date. Although this has yet to be established fully for zoospores, the surfaces of eukaryotic flagella are thought to have many functions including cell-substrate or cell-pathogen adhesion, force transduction for locomotion, regulation of axonemal behaviour via Ca^{2+} concentration and also sensory reception such as mechanoreception and chemoreception (Bloodgood, 1991). Donaldson and Deacon (1993a) found that zoospores in altered swimming modes (eg. caused by the Ca^{2+}-chelator EGTA) failed to respond chemotactically to gradients of amino acids and did not show avoidance responses during collisions, suggesting perturbation of presumed chemoreception and mechanoreception.

1.3 Kinesis and Taxis

The external environment may influence the motile behaviour of cells and organisms by kinesis or taxis. Such responses can contribute either to the accumulation of cells, including zoospores, at a particular site or their movement away from it. The various forms of kineses and taxes of microorganisms and the relative contribution of these processes in site selection by zoospores are outlined below (see reviews by Carlile, 1980a; 1980b; 1983; Dunn, 1990; Deacon & Donaldson, 1993).

1.3.1 Kinesis

Kineses are unidirectional locomotory reactions in response to environmental stimuli (Dunn 1990). Orthokinesis is a response whereby the speed of an organism is affected and it may lead to accumulation of an organism at specific sites. An extreme form of this response may describe the accumulation of zoospores at specific sites on a root surface by encystment. Klinokinesis is a reaction by which a change in the frequency or amount of turning of a motile organism per unit time occurs. This may result in trapping of an organism, such as zoospores, at a source of attractant (Royle & Hickman, 1964a).

1.3.2 Taxis

The term taxis (strictly topotaxis) is used to describe directed orientation reactions in response to environmental stimuli. If the direction is towards the stimulus, taxis is said to be positive, if away from the stimulus, the reaction is negative. Often there is insufficient information to show whether accumulation of fungal zoospores occurs by kinesis or taxis or both, so the term taxis is used in a general way to cover both types of response. Zoospores show taxes to a wide range of stimuli, outlined below.

1.3.2.1 Phototaxis

Phototaxis describes the movement of an organism in response to light. Response to light has been observed in some zoosporic fungi (eg. Petri, 1925; Carlile, 1970) and positive phototaxis can be demonstrated with zoospores of the marine chytrid *Rhizophydium littoreum* (Amon, 1984; Kazama, 1972). The precise mechanism of the response is poorly understood; however, the significance of positive phototaxis may be to keep zoospores close to water/soil surfaces.

1.3.2.2 Geotaxis

Geotaxis is the movement of an organism in response to gravity. Zoospores of *Ph. cactorum*, *Ph. nicotianae* and *Ph. megasperma* (Cameron & Carlile, 1977; Ho & Hickman, 1967) tend to swim upward and accumulate just below the water-air interface of a zoospore suspension. The environmental significance of this for Oomycota zoospores could be that they maintain a position within soils where host plant rootlets are abundant. Negative geotaxis is common in eukaryotic microorganisms, as shown by many algae and protozoa (Carlile, 1980a). Little evidence exists for a role of negative geotaxis in zoospores of the Oomycota (Cameron & Carlile, 1977) and Carlile (1983) argued against this being a true sensory response; rather it was suggested to result from the morphology of the zoospore and the nature of flagellation, causing zoospores to tilt upwards as they swim.

1.3.2.3 Rheotaxis

Katsura and Miyata (1971) reported that *Ph. capsici* zoospores showed positive rheotaxis, swimming towards the mouth of a capillary tube from which a water current was emanating. Positive rheotaxis against gentle water currents percolating downwards may serve to keep zoospores near the soil surface and thus close to roots.

1.3.2.4 Adelphotaxis

Adelphotaxis refers to attraction between zoospores which may result in auto-aggregation, possibly in response to chemical signals released from aggregating spores. Several studies have reported this phenomenon in *Ph. palmivora* (Ko & Chase, 1973), *Ph. capsici* (Ko & Chase, 1974), *Ph. drechsleri* (Porter & Shaw, 1978) and the water mould *Achlya heterosexualis* (Thomas & Peterson, 1990). These authors concluded that auto-aggregation occurred in response to a heat-stable "aggregation factor". Recently, Reid *et al.* (1995) showed that, at high cell densities, zoospores of *Ph. palmivora* auto-aggregated to form multicell clumps of cysts and that individual zoospores exhibited a tactic response towards aggregates. Populations of *Py. catenulatum* and *Py. dissotocum* zoospores also formed autoaggregates, but *Py. aphanidermatum* did not do so. Furthermore, the recruitment of zoospores to an aggregate appeared to be a genus-specific phenomenon since *Pythium* zoospores were not attracted to pre-formed aggregates of *Ph. palmivora* zoospores.

Reid *et al.* (1995) suggested that autoaggregation was Ca^{2+}-dependent since *Ph. palmivora* zoospores were attracted to and aggregated around crystals of $CaCO_3$, and an increase in Ca^{2+} concentration adjacent to large aggregates could be detected by Ca^{2+}-sensitive microelectrodes. Treatments that reduced the level of Ca^{2+} in the medium surrounding the zoospores, or that interfered with Ca^{2+} uptake mechanisms, completely inhibited aggregation. The attraction of zoospores to clusters of cysts might well be in response to simple substances such as Ca^{2+}, known to be released during zoospore encystment (Irving *et al.*, 1984). However, Ca^{2+} ions are probably not the sole substance involved in an autoaggregation response because aggregates of one genus did not attract individual zoospores of another (Reid *et al.*, 1995). It is possible that Ca^{2+} plays an important role in conjunction with other factors, but the precise role of Ca^{2+} in autoaggregation remains to be clarified.

Reid *et al.* (1995) suggested that autoaggregation might reinforce a direct attraction of zoospores to root exudates or electrical gradients, and that formation of aggregates of zoospores at localised sites on host tissues might improve the chance of host colonisation by directly increasing the inoculum level. Nevertheless, the ecological significance of autoaggregation is unclear, because the phenomenon only occurs in suspensions of very high zoospore densities of $5x10^5$ ml^{-1}.

1.3.2.5 Electrotaxis

Electrotaxis describes the movement of an organism in response to an electrical field and is distinct from responses to an ionic current (galvanotaxis). The effects of electrical fields on zoospore motility have been studied by Troutman and

Wills (1964), Ho and Hickman (1967), Katsura and Miyata (1971), Khew and Zentmyer (1974), Morris *et al.* (1992) and Morris & Gow (1993).

Miller *et al.* (1988) detected ion currents generated at root tips, root hairs and wounded tissues in *Nicotiana tabacum*; these sites are highly susceptible to infection from *Phytophthora* zoospores. *Ph. parasitica* zoospores have been reported to move cathodotactically (Troutman & Wills, 1964), and in agreement with this, Miller *et al.* (1988) found that the sites where *Ph. parasitica* had been reported to accumulate were electrically cathodic. The cathodic nature of the root at these attractive sites was suggested to be due, in part, to an inward flow of protons. However, these same zones are also major sites of release of soluble nutrients known to attract zoospores. Indeed, it has been suggested that zoospores may use electrical gradients in addition to chemical ones to locate sites for host infection *in vivo* (Troutman & Wills, 1964).

Morris *et al.* (1992) argued that these earlier studies on electrotaxis did not take into account possible artefacts such as electrolytic products and pH gradients generated around the electrodes. By applying electrical gradients to zoospores that were isolated from electrodes and their products, Morris *et al.* (1992) demonstrated that *Ph. palmivora* zoospores exhibited electrotaxis to field strengths equivalent to those found at root tips and wound sites (Miller *et al.*, 1988). The implication is that there may well be a role for electrotaxis in host-location by zoospores (Gow *et al.*, 1992), but most likely in combination with responses to chemical signals.

Fluxes of protons may lead to the establishment of pH gradients in the rhizosphere. To test whether pH gradients in the rhizosphere were related to host location, Morris *et al.* (1995) used capillary tubes filled with solutions of different pH to examine the tactic response of *Ph. palmivora* zoospores. Although zoospores were attracted to capillary tubes containing acidic buffers, it was concluded that root-generated pH gradients had little, if any, role in zoospore attraction to host roots because disruption of the pH gradient around roots using buffers had no effect on the pattern of zoospore accumulation.

It is possible that currents of ions other than protons may be utilised by zoosporic pathogens for successful infection of roots (Gow, 1993). For example, currents of Ca^{2+} have been detected around the roots of some plants (Miller *et al.*, 1988). As outlined in this chapter, a role for Ca^{2+} has been implicated in motility, adhesion, encystment and germination of *Pythium* and *Phytophthora* zoospores, and so spacial gradients of Ca^{2+} may be important for host location and infection.

In a more recent study on *in vitro* electrotaxis of *Pythium* and *Phytophthora* zoospores by Morris and Gow (1993), the anterior flagellum of cathodotactic *Py. aphanidermatum* zoospores was found to be electropositive relative to the posterior flagellum, whereas the anterior flagellum of the anodotactic zoospores of *Py. dissotocum*, *Py. catenulatum* and *Ph. palmivora* was found to be electronegative relative to the posterior flagellum; the electrical dipole was thought to result from the relative charge of proteins/glycoproteins associated with the anterior and posterior flagella. Morris and Gow (1993) suggested that the existence of an electrical dipole may be involved in orientation of zoospores in electric fields (electro-topotaxis). Furthermore, Morris & Gow (1993) found that *Phytophthora palmivora* zoospores increased their rate of turning in electrical fields of 5 mV cm^{-1}, comparable in size to those found around roots (Miller *et al.*, 1988). Morris & Gow (1993) suggested that increased zoospore turning in the vicinity of roots could facilitate accumulation of zoospores at the root surface.

Since the orientation of the posterior flagellum (believed to have a rudder function in steering motile biflagellate zoospores - Carlile, 1983) was greatly affected by electrical fields, Morris and Gow (1993) believe that this may play a role in dictating the direction of the electrotactic response. Zoospores approaching a root are likely to experience stronger electrical fields which could in turn increase the turning frequency of zoospores. An increase in zoospore turning frequency (trapping) in proximity to roots has been reported previously (Royle & Hickman, 1964a; Jones *et al.*, 1991) and could enhance accumulation of zoospores at the root surface.

Calcium ions are important in the normal swimming pattern of *Achlya* (Thomas & Butler, 1989) and *Pythium* (Donaldson & Deacon, 1993a) zoospores; however, the mechanisms by which electric fields lead to changes in the frequency of abrupt turns remains unclear. One possible mechanism is through modulation of voltage-operated Ca^{2+} channels (VOCs). Electrotaxis of the unicellular alga *Chlamydomonas* (Dolle & Nultsch, 1987) and of *Paramecium* (Eckert, 1972; Machemer, 1974; Preston, 1990) depends upon an exogenous supply of Ca^{2+}. In *Paramecium*, mechanosensitive channels open in response to collision of the cell with an obstacle. An influx of Ca^{2+} occurs causing depolarisation of the cell membrane, and this leads to opening of further VOCs. The resulting elevation in cytosolic Ca^{2+} concentration is believed to alter ciliary beating in an "avoidance reaction". Ca^{2+} is then rapidly pumped out of the cell by Ca^{2+}-ATPases to restore normal cytosolic Ca^{2+} levels and thus the original pattern of cilia beating and swimming behaviour. Reagents affecting Ca^{2+} transport or calmodulin function had no apparent effect on electrotaxis of *Pythium* or *Phytophthora* zoospores (Morris & Gow, 1993). However, many of the Ca^{2+} modulating treatments used caused premature encystment and sometimes lysis. The duration of swimming was thus reduced, and presumably, the opportunity of the zoospores to respond to electrical fields was also reduced. Therefore, a role for Ca^{2+} in zoospore electrotaxis can not yet be ruled out.

1.3.2.6 Chemotaxis

Chemotaxis is the movement of an organism in response to chemical compounds such as ions, amino acids, sugars or volatile compounds. Chemotaxis is the most widely studied aspect of zoospore tactic behaviour; the subject has thus been extensively reviewed (Hickman & Ho, 1966; Hickman, 1970; Zentmyer, 1970; Carlile, 1980a; Carlile, 1983; Deacon & Donaldson, 1993).

1.3.3 Attraction to roots

Experiments carried out *in vitro* have shown that when root systems or severed roots are placed in zoospore suspensions, the zone of root elongation and wound sites are generally most attractive to zoospores (eg. Zentmyer, 1961; Royle & Hickman, 1964a; Mitchel & Deacon, 1986; Hinch & Clarke, 1980; Longman & Callow, 1987; Jones *et al*, 1991). *In vivo* studies by Mehrotra (1972) supported this view, using roots growing in soil against a cellulose acetate membrane. Zoospores of both *Ph. drechsleri* and *Ph. megasperma* var *sojae* accumulated along the outside of the membrane where it was in contact with roots of safflower and soybean seedlings. Furthermore, when a zoospore suspension was added to roots growing in soil in pots, and the roots were subsequently removed and stained with Calcofluor White, the fluorescent dye clearly demonstrated accumulation of zoospores on the roots.

In spite of variations attributable to, for example, differences in the type of root used and conditions in which seedlings are grown in culture, responses of many zoosporic pathogens towards roots are similar to those described by Royle and Hickman (1964a) for *Py. aphanidermatum* on pea roots, as follows. Zoospores swimming in the absence of stimuli displayed a gliding, helical motility pattern. When entire, unwounded pea roots were placed in zoospore suspensions, the direction of the zoospore swimming path changed so that they swam towards the root - typically towards the region behind the root tip. Attracted zoospores were also seen to undergo "trapping" in the immediate vicinity of the root whereby the unidirectional, but otherwise typical motility pattern became irregular and jerky, thus confining the zoospores within an area close to the root surface. When entire but wounded roots were placed in a zoospore suspension, initial accumulation occurred preferentially at the wound site, with attraction to the undamaged tissues of the elongation zone only resuming some time later.

Despite some reports, such as by Goldberg *et al.* (1989) who reported that *Py. dissotocum* zoospores were attracted only to the root cap region of cotton and not to wound sites, attractive zones on roots are probably the major sites of nutrient release, with wound sites releasing the greatest quantities of attractive substance(s).

Exudates from the elongation zone of healthy, intact roots and from wound sites most likely contain a wide range of low molecular weight compounds. Attraction to roots by zoospores may occur by positive chemotaxis to these substances diffusing away from roots. Most plants will release sugars, amino acids and organic acids, but compounds which have specific stimulatory or inhibitory activity on microorganisms are likely to be exuded at very low levels (Rovira, 1969). Plant species and age will affect root exudation, but factors such as the moisture and temperature of the soil in which roots are grown may also be important (Rovira, 1969).

The selective stimulation of some bacteria by plant roots is highlighted in the symbiotic association between the root nodule bacterium *Rhizobium* and its legume host. In a study by Gulash *et al.* (1984), clouds of *R. meliloti* occurred at the surface of young alfalfa roots. The bacteria appeared to be swimming toward a localised source of attractant at a very restricted region just behind the root tip; this region is often an attractive region to root-infecting zoosporic plant pathogens. Furthermore, Gaworzewska & Carlile (1982) found that *R. leguminosarium* showed chemotaxis to a wide range of amino acids, sugars and organic acids; many of these compounds are also attractive to fungal zoospores.

1.3.4 Attraction to root exudates and to pure compounds

Chemotaxis studies can be conducted *in vitro* using capillary model systems; much information has accumulated in this way on the substances which are attractive and non-attractive to zoosporic pathogens.

Several workers have studied the responses of zoospores to root exudates (reviewed by Carlile, 1983). Fractionation of root exudates (eg. Chang-Ho & Hickman, 1970) has shown the cationic fraction, containing a wide range of amino acids, to be most attractive. Consistent with this, many *Pythium* and *Phytophthora* spp. show positive chemotaxis to one or more of L-glutamate, L-aspartate, L-asparagine and L-glutamine (eg. Khew & Zentmyer, 1973; Jones *et al.*, 1991), combinations of amino acids often being more attractive than single amino acids in capillary tube assays. Indeed, many fungi appear to respond to the common components of root exudates (eg., Chang Ho & Hickman, 1970; Jones *et al.*, 1991; Khew & Zentmyer, 1973; Rai & Strobel, 1966). In contrast, there is little information on the chemoattractants of zoospores of leaf-infecting fungi such as *Ph. infestans* or powdery mildew pathogens.

Allen and Newhook (1973, 1974) found that *Ph. cinnamomi* zoospores showed positive chemotaxis to ethanol *in vitro*. In extension of this, Cameron and Carlile (1978) found that *Ph. palmivora* also responded to ethanol. *Ph. palmivora* was attracted to a range of aldehydes, organic acids and alcohols, the most potent attractants having 4-6 carbon atoms. It was argued, therefore, that volatile compounds, arising from fermentation products of roots in waterlogged soils, may be significant as powerful zoospore attractants *in vivo* (Cameron & Carlile, 1978).

Cameron and Carlile (1978, 1981) found isovaleraldehyde to be the most powerful chemoattractant of *Ph. palmivora*, having a chemotactic threshold at 1μM. They characterised the isovaleraldehyde receptor by testing the ability of other compounds to displace [^{3}H] isovaleraldehyde that had been adsorbed to the surface of *Ph. palmivora* zoospores. Only unlabelled isovaleraldehyde and structurally related compounds such as valeraldehyde and isobutyraldehyde could displace [^{3}H] isovaleraldehyde, these compounds also being chemoattractants *in vitro*. Other attractants such as asparagine could not displace [^{3}H] isovaleraldehyde, suggesting that they operate by different surface receptors.

Morris and Ward (1992) found that the host-adapted soybean parasite, *Ph. sojae*, showed chemotaxis to ethanol, glutamine, asparagine and aspartic acid but also responded chemotactically to the host-specific isoflavone compounds, daidzein and genistein, at concentrations as low as 10nM; other *Phytophthora* spp. did not do so. Furthermore, Horio *et al.* (1992) found strong attraction of *Aphanomyces cochlioides* zoospores to cochliophilin A, a flavone isolated from spinach roots.

Although these workers provided no data on attraction of this isolate of *A. cochlioides* toward other compounds, Rai & Strobel (1966), working with a different isolate, showed attraction of *A. cochlioides* to compounds such as gluconic acid and glucose. In relation to this, studies on bacteria such as *Agrobacterium tumefaciens* (Ashby *et al.*, 1988) and *Rhizobium* spp. (Caetano-Anolles *et al.*, 1988), have suggested that in addition to general attraction to root diffusates, there exists a high-affinity chemotactic response to specific compounds. Whether this phenomenon applies in general to host-location by zoosporic fungi is not know.

If, for example, two attractive compounds share the same chemoreceptor, then if one compound is present in a high enough concentration to saturate the receptor the response to the other compound will be blocked (Adler, 1969; 1975). Khew & Zentmyer (1973) found that a background of either aspartic or glutamic acids reduced the chemosensitivity of five *Phytophthora* spp. to the other amino acid. A similar effect has also been noted for *Pythium* spp. (Donaldson & Deacon, 1993b), whereby a background of, for example, L-glutamic acid and/or L-glutamine could block or reduce attraction to capillary tubes containing L-glutamic acid.

1.3.5 The mechanisms of chemotaxis

The essence of receptor function is transduction of a stimulus into an informational signal and transmission of that signal from the site of recognition to the appropriate effector within the cell. The receptor proteins that mediate chemotaxis in bacteria, and the mechanisms that control flagellar activity so that bacteria move toward favourable environmental conditions, have been the focus of extensive studies (eg. see reviews - Adler, 1975; Armitage, 1992; Stock & Surette, 1996).

Bacteria, such as *Escherichia coli* and *Salmonella typhimurium*, respond to a chemical gradient by a change in the turning frequency in such a way as to suppress a “tumbling” response as the gradient increases. In order to sense such a gradient a temporal sensing mechanism is needed to compare changes in concentration. Berg and Brown (1972) and MacNab and Coshland (1972) observed a change in the tumbling frequency of bacteria. By changing the concentration of attractants it was demonstrated that the chemotactic mechanism of *E. coli* and *S. typhimurium* involved temporal sensing. In both organisms each cell has a family of transmembrane proteins with receptor functions. These proteins are regulated by methyl esterification and are therefore termed methyl-accepting chemotactic proteins (MCPs). Each MCP mediates responses to a specific set of attractant and repellent stimuli. MCPs are composed of an extracytoplasmic sensory domain that binds stimulatory ligands, connected by a membrane spanning sequence to an intracellular signalling domain (Wang & Koshland, 1980). In the cytoplasm of *E. coli* and *S. typhimurium* there are six Che gene products: CheA, CheB, CheR, CheW, CheY and CheZ, all of which act in signal transduction. The MCPs signal through formation of a ternary complex with CheW and CheA proteins. In the absence of attractant the flagella cause tumbly swimming behaviour due to the passage of phosphoryl groups along the Che proteins to the flagellar motor. Attractant binding to the sensory domain of MCPs causes their methylation thus inhibiting the kinase activity of the MCP-CheW-CheA complex, leading to smooth swimming. Methylation and demethylation of MCPs thus controls kinase activity.

Counterclockwise rotation of flagella in these bacteria is associated with smooth motility. Clockwise rotation is associated with tumbling swimming. The signal transduction network modulates the frequency of transition between these states. In a constant environment a cell typically moves in a random walk of runs of approximately 1s punctuated by tumbles of 0.1s (Berg & Brown, 1972). When a cell detects that it is swimming toward increasing attractant or decreasing repellent concentrations, it tumbles less frequently, thereby biasing its random walk in the preferred direction.

Fewer studies have been done with eukaryotic cells, but evidence is accumulating which suggests that receptor-mediated systems for chemotaxis also exist in these organisms.

Van Outen (1979) found that accumulation of the ciliated protozoan *Paramecium* in response to attractants is similar to the biased random walk of bacteria, but it differed to some extent in that *Paramecium* modulates its swimming speed as well as frequency of turning whereas bacteria modulate turns only. These cells respond to changes in the concentration of attractants and adapt in uniform distributions of stimuli. Both the speed of forward swimming and the frequency of turns are controlled by membrane potential whereby the level of membrane potential determines the frequency and angle of ciliary beating, hence swimming speed. Action potentials cause a transient reversal of beating thereby initiating a change in the direction of motility. During the Ca^{2+} action potential, Ca^{2+} enters the ciliary compartment through VOCs; this leads to a rise in Ca^{2+} level, and the ciliary beat then reverses causing a turn. Attractive compounds hyperpolarise the cell as the cell moves up a gradient of attractant, and an increase in speed occurs concomitant with a decrease in turning frequency.

Zoospores have a net negative charge on the cell surface (Khew & Zentmyer, 1974). In the absence of a stimulus, Allen and Newhook (1973) observed that zoospore flagella are extended fore and aft. Electrostatic repulsion between adjacent negative charges along the surface of a flagellar membrane could oppose the natural tendency for all proteins to adopt a random coil configuration of high entropy (Allen & Harvey, 1974), thereby preventing the flagella from collapsing which would result in loss of motility. Ethanol suppresses random turning activity in *Ph. cinnamomi* (Allen & Newhook, 1974). Doetsch (1972) and Eckert (1972) theorised that substances which induce positive chemotaxis cause initial hyperpolarisation of the plasma membrane. When a zoospore is attracted towards ethanol (Allen & Newhook, 1973) hyperpolarisation of the membrane must occur as it encounters ethanol of increasing concentration; at the same time, spontaneous depolarisations that accompany flagella reorientation would be suppressed. In extension of this, Cameron and Carlile (1980) found that *Ph. palmivora* zoospores were repulsed by monovalent and certain divalent cations. They proposed that the approach of cations to the cell surface reduced the surface potential and hence changed the transmembrane potential, resulting in turning due to altered flagellum activity. In support of this, *Paramecium* exhibits negative chemotaxis away from repellents as a result of cell depolarisation (Van Outen, 1979).

Carlile (1980a) proposed that, during chemotaxis, the zoospore may orientate in the direction of receptors receiving greatest stimulation (assuming that there are evenly distibuted receptors over the membrane surface) until chemoreceptors are stimulated symmetrically with respect to the zoospore long axis; it may then proceed directly up the gradient of attractant. Alternatively, the distribution of receptors may control the gradients of chemoresponse from anterior to posterior as for *Paramecium* (Van Outen, 1979), just as depolarising and hyperpolarising mechanoreceptor channels appear to be arranged in gradients from anterior to posterior (Machemer, 1989). Hardham (1992) proposed that chemoreceptors may be located on the flagella of zoospores where they could interact with Ca^{2+}-binding proteins, thereby eliciting directional changes. In support of this, transmembrane signalling occurs in both cilia and flagella (Brockaw, 1987; Bloodgood, 1991; Boitano & Omoto, 1992) and the effects of Ca^{2+} in altering flagellar movements and motility patterns have been mentioned above (section 1.2.1). However, information on zoospore flagellar behaviour is still relatively sparse.

Monoclonal antibodies raised against zoospore flagellar antigens (Hardham, 1994) have indicated differences between membrane components of the zoospore anterior flagellum compared to the posterior flagellum. Whether these surface molecules have receptor function has not been investigated although anti-receptor anti-sera could be useful in mapping relative chemoreceptor densities and distributions on zoospore membranes.

1.4 Encystment

Anatomical changes that occur during cyst formation have been presented for species of *Pythium* (Grove, 1970; Lunny & Bland, 1976; Grove & Bracker, 1978), *Phytophthora* (Reichle, 1969; Hemmes & Hohl, 1971; Tokunaga & Bartnicki-Garcia, 1971; Bimpong & Hickman, 1975; Hardham *et al.*, 1991) and *Aphanomyces* (Hoch & Mitchell, 1972). During encystment the naked zoospore protoplast becomes spherical in shape. A glycoprotein cyst coat is released and beneath this a microfibrillar cyst wall forms (Truesdell & Cantino, 1971). The flagella are shed, and cytoplasmic organelles and other inclusions are reorganised and occupy positions different from those found in the motile stage. Encystment can occur in response to a wide range of physical or chemical factors. Even whole populations of motile zoospores can be induced to encyst synchronously and this has not only aided research into the ultrastructural changes which occur during differentiation into cysts but also has given insights into the factors involved in initiating encystment and the events that occur during and after encystment.

1.4.1 Factors involved in initiating encystment

Fucosyl residues present in root surface slime have been implicated in cyst binding of *Py. aphanidermatum* (Longman & Callow, 1987; Estrada-Garcia, 1990a) and *Ph. cinnamomi* (Hinch & Clarke, 1980) on roots of cress and maize respectively. Root surface slime is also rich in uronic acids. Uronate-rich compounds such as poly-D-galacturonic acid, pectin and alginate have all been shown to cause encystment of zoospores of *Pythium* and *Phytophthora* spp. (eg., Irving & Grant, 1984; Grant *et al.*, 1985; Zhang *et al.*, 1990; Jones *et al.*, 1991; Donaldson & Deacon, 1993c). Kerwin *et al.* (1992) found that *Py. marinum* zoospores encysted on thalli of species of red algae (host), whereas attempts to induce encystment on green or brown algae (non-hosts) proved unsuccessful. Host surface carbohydrates were implicated in causing encystment. The addition of monosaccharide and polysaccharide residues suggested that encystment was induced specifically by sulphated or non-sulphated galactose residues and anhydrogalactose found in commercial agars and carrageenans.

Zoospores also encyst in response to factors such as those summarised in Table 1.2. The cytoskeleton, made up of an arrangement of microtubules and microfilaments (Held, 1972; Holloway & Heath, 1977), is important in maintaining the shape of wall-less zoospores (Grove & Bracker, 1978; Reichle, 1969); treatments which are known to degrade microtubules have been shown to induce encystment of zoospores (Held, 1972). Zoospores can be induced to encyst by lectin and monoclonal antibody binding to the cell surface (Table 1.2). Concanavalin A causes encystment by binding to the entire zoospore surface, including both flagella. This effect is annulled by pre-incubation of the lectin with its hapten sugar. A monoclonal antibody termed Zf-1 and raised against zoospores of *Ph. cinnamomi* specifically binds to the flagella of this and other *Pythium* and *Phytophthora* spp., and also causes encystment (Hardham & Suzaki, 1986) suggesting that specific receptors involved in triggering encystment are located on the flagellar surfaces.

Table 1.2 Some treatments known to induce encystment of fungal zoospores*, with selected references.

Physical	Temperature	Ho & Hickman (1967)
	pH	Ho & Hickman (1967)
	Hydrostatic pressure	Held (1972)
	Dilution	Ho & Hickman (1967)
	Mechanical agitation	Ho & Hickman (1967)
Chemical	Ions	Byrt *et al.* (1982)
	Amino acids	Donaldson & Deacon (1993b)
	Lectins	Hardham & Suzaki (1986) Longman & Callow (1987)
	Monoclonal antibodies	Hardham & Suzaki (1986)
	Phosphatidic acid	Zhang *et al.* (1992)
	Colchicine	Held (1972)
Host surface factors	Uronic acids	Irving & Grant (1984) Jones *et al.* (1991)
	Fucose residues	Hinch & Clarke (1980) Longman & Callow (1987) Estrada-Garcia *et al.* (1990a)
	Chitin	Mitchell & Deacon (1986)
	Cellulose	Mitchell & Deacon (1986)

* Some of the treatments affect zoospores of many different species, whereas others such as chitin and cellulose are known to act selectively.

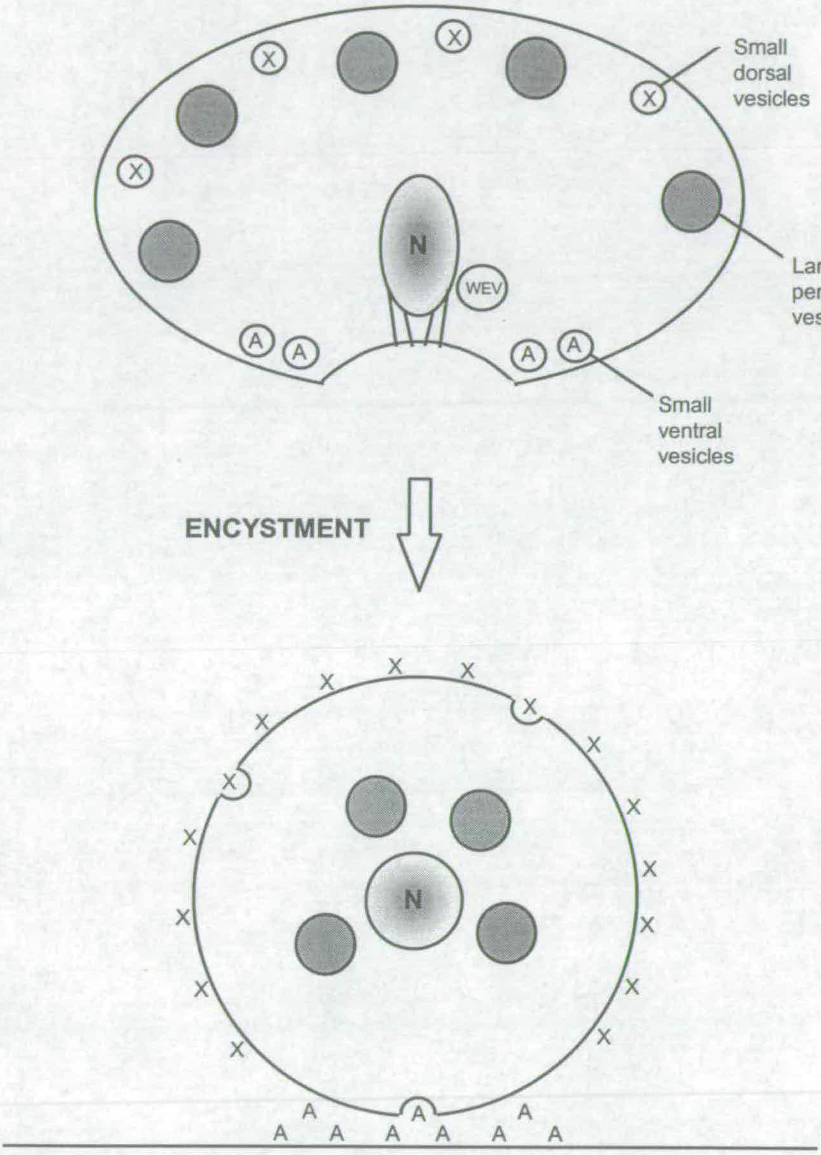
Phosphatidic acid also induces zoospore encystment; phosphatidic acid may arise via a stimulus-activated phospholipase-D and may act as a second messenger as part of an intracellular signalling cascade (Zhang *et al.*, 1992).

1.4.2 Ultrastructural changes during encystment

When *Pythium* and *Phytophthora* zoospores encyst they round up, shed their flagella and secrete a thin cyst coat. Flagellar retraction or detachment has been observed in several studies (eg., Ho & Hickman, 1967; Grove & Bracker, 1978), both phenomena being recorded in the same population for *Ph. parasitica* (Reichle, 1969). However, flagellar shedding seems to be most common in the zoospores of the Oomycota. Within the zoospores, the pre-formed peripheral vesicles fuse with the plasma membrane and deposit a glycoprotein coat and possibly other cell wall precursor materials on the spore surface (Sing & Bartnicki-Garcia, 1975a, b). Ca^{2+}-binding proteins have been visualised along the peripheral cisternae and peripheral vesicles by fixing encysting zoospores in Ca^{2+}-containing glutaraldehyde (Hemmes & Pinto da Silva, 1980). They are suggested to be involved in triggering this membrane fusion and exocytosis. Then, over the next few minutes a wall develops under the new cyst coat (Sing & Bartnicki-Garcia, 1975a).

Recently, monoclonal antibodies and lectins have been used to characterise the vesicle contents and study their movements during encystment. Monoclonal antibodies Cpa-2, Cpw-1, Lpv-1 and Vsv-1 (Hardham *et al.*, 1986; Gubler & Hardham, 1988) were raised against components of spores of *Ph. cinnamomi* and are specific for each of the types of peripheral vesicles in zoospores of this species. Cpw-1 labels the vesiculated peripheral cisternae at early stages of encystment and binds to the cyst wall once it appears at the cell surface. Cpa-2 and Vsv-1 label small peripheral vesicles which predominate beneath the spore surface on the dorsal and ventral side of the cell respectively. Both of these small vesicle types undergo rapid exocytosis following the induction of encystment (Figure 1.3). Material from the ventral vesicles covers about one third of the cyst surface; their contents are thought to function as an adhesive material which mediates attachment to an adjacent surface. Material from the dorsal surface vesicles coats the rest of the cell; their glycoproteins are thought to form the cyst coat. Lpv-1 labels the large peripheral vesicles which do not undergo exocytosis but instead, about 5 to 10 minutes after the induction of encystment, move away from the plasma membrane and become dispersed throughout the cytoplasm (Gubler & Hardham, 1988). This behaviour has also been observed in *Ph. nicotianae* and *Ph. parasitica*. These large peripheral vesicles are thought to contain a cache of protein for use during future development. Indeed, the events of encystment do not seem to require either RNA or protein synthesis (Soll & Sonneborn, 1971). So, if any proteins are required for any of the structural changes and rearrangements during encystment, then they must exist within the zoospore. However, it is thought that synthesis of new proteins is required for cyst germination (Leaver & Lovett, 1974). Also during encystment the nucleus becomes rounded and assumes a central position within the cell, the anchoring system of rootlets disappears and the large water expulsion vacuole with its associated membranes and vesicles disappears.

Figure 1.3 Simplified diagram of a zoospore in transverse section, showing the ultrastructural changes which occur during encystment. The small ventral vesicles discharge their adhesive contents at the ventral groove region. The small dorsal vesicles discharge their glycoprotein contents around the rest of the spore to form a cyst coat; beneath this, a cyst wall forms. The large peripheral vesicles and the nucleus then take up a more central location within the cyst, and the water expulsion vacuole (WEV) disappears.



1.5 Calcium and fungal morphology

1.5.1 Introduction

Calcium acts as a second messenger within eukaryotic cells and transduces cell surface primary stimuli - whether they are chemical, electrical or physical - into intracellular events. Changes in the concentration and/or distribution of intracellular free Ca^{2+}, acting through a complex system of Ca^{2+}-modulating proteins and their targets, regulate an astonishing variety of cellular processes (Jaffee, 1993). A role for Ca^{2+} as an important second messenger has long been established in many animal systems upon which the majority of research has been focused to date. There is, however, growing evidence that this ion plays an equally important role in signal transduction in plant and fungal cells (eg., Pitt & Ugalde, 1984; Hepler & Wayne, 1985; Deacon & Donaldson, 1993; Bush, 1995; Gadd, 1995; Haley *et al*, 1995; Malho & Trewavas, 1996; von Broembsen & Deacon, 1996; Deacon & Saxena, 1997b). Since Ca^{2+} may be cytotoxic, low levels of Ca^{2+} are maintained within the cytoplasm such that small controlled fluctuations are sufficient to trigger intracellular regulatory events (Carafoli, 1987; Rasmussen & Rasmussen, 1990).

In eukaryotic cells many signalling pathways exist, eg. those involving inositol lipids, cyclic AMP and diacylglycerol (DAG), and interactions occur between these different signalling systems. However, the Ca^{2+}/Ca^{2+}-calmodulin mediated effects on fungal differentiation have received much attention to date and will be considered below. However, it is now widely recognised that the Oomycota are phylogenetically distinct from chitin-walled fungi, and more closely related to diatoms and brown algae. So, calcium-mediated systems reported for fungi may not necessarily be the same as in the Oomycota.

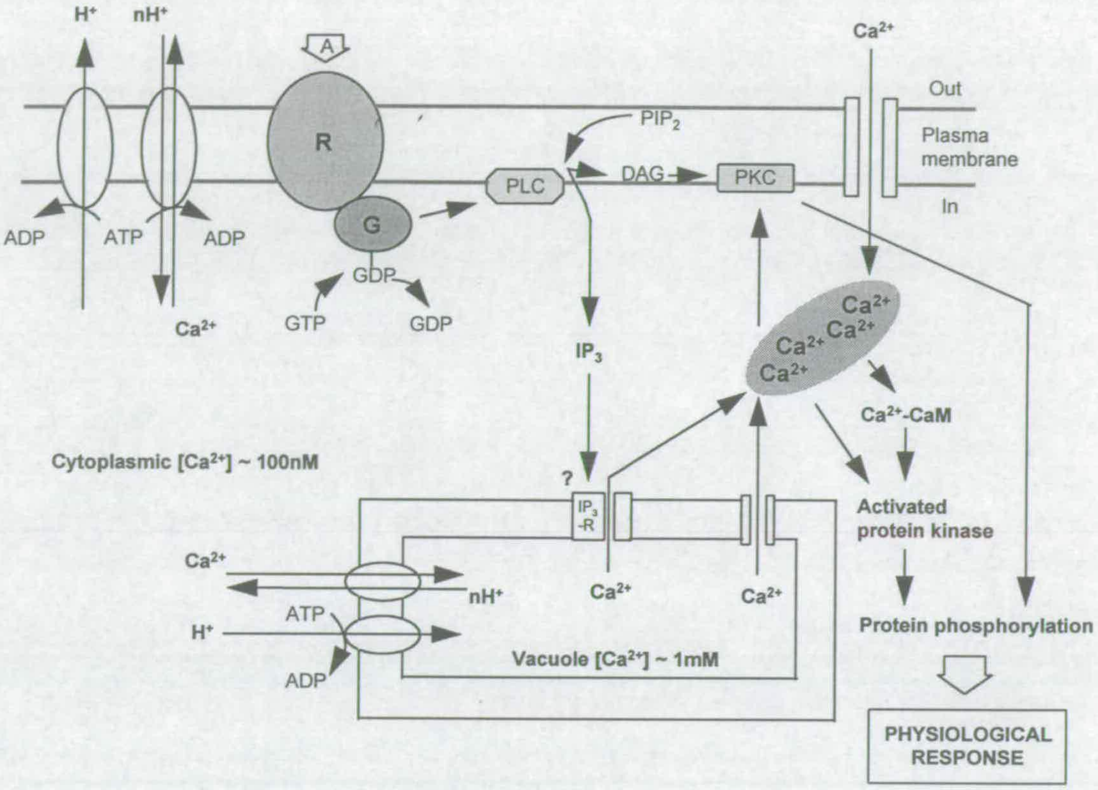


Figure 1.4 Simplified diagram in fungi of the location of Ca^{2+}-homeostatic systems and the possible events in signal transduction though G-protein linked receptors and phosphatidylinositol turnover leading to the release of Ca^{2+} from intracellular stores. The agonist (A) induces a conformational change in the receptor (R) which in turn is transmitted to the G-protein (G). The G-protein exchanges bound guanosine diphosphate (GDP) for GTP and then interacts with and activates phospholipase C (PLC) which in turn cleaves inositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). DAG may activate protein kinase C (PKC) to phosphorylate specific cellular proteins. IP_3 may act to mobilise Ca^{2+} from intracellular stores such as the vacuole. Ca^{2+} may activate protein kinases either directly or through activation of calmodulin (CaM). Ca^{2+}-influx channels of the plasma and vacuolar membranes are also shown as are the postulated Ca^{2+}-efflux transporters. Based on references: Trewavas & Gilroy (1991) and Gadd (1995).

1.5.2 Intracellular calcium regulation

Cells utilise a complex system of sequestrative and homeostatic mechanisms which maintain cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) at a low level; recorded as 100-300nM in resting fungi (eg., Miller *et al.*, 1990). Cellular Ca^{2+} homeostasis is maintained by Ca^{2+} transport proteins which fall into two classes; those that mediate efflux from the cytoplasm (Ca^{2+}-ATPases and Ca^{2+}/nH^+ antiporters) and those that mediate influx (the Ca^{2+} channels).

1.5.2.1 Ca^{2+} efflux transporters

Ca^{2+}-ATPases. In fungi it is proposed that Ca^{2+} transport is via a H^+/Ca^{2+}-ATPase located on the plasma membrane; the stoichiometric ratio being at least $2H^+/Ca^{2+}$ in *Neurospora crassa* (Miller *et al.*, 1980). The importance of ATPases in negative feedback systems that resist alterations in $[Ca^{2+}]_c$ has been demonstrated in *Saccharomyces cerevisiae* by mutations in the *PMC1* gene, which encodes a plasma membrane-type ATPase found on the yeast tonoplast membrane (Cunningham & Fink, 1994). This tonoplast ATPase in yeast is thought to be important for regulating $[Ca^{2+}]_c$ under conditions of increased Ca^{2+} influx.

Antiporters. Evidence for a role of antiporters in buffering $[Ca^{2+}]_c$ during net Ca^{2+} influx has been obtained for *N. crassa* (Miller *et al.*, 1990) and *S. cerevisiae* (Ohya *et al.*, 1991). It is proposed that antiporters function by damping large changes in $[Ca^{2+}]_c$ and maintaining the Ca^{2+} store in the vacuole, this being under the influence of a vacuolar proton gradient (Figure 1.4).

1.5.2.2 Calcium influx transporters

The principal routes of Ca^{2+} entry into fungal cells are likely to be through Ca^{2+} channels (Figure 1.4). Ca^{2+} channels may be classified by their intracellular location: those found on the plasma membrane are termed influx channels; those found on an intracellular membrane are called release channels. Five classes of

Ca^{2+} channels are recognised in animal systems (Tsien & Tsien, 1990), based on the factors involved in opening or closing them. Voltage-operated, second messenger-operated and mechanically-operated channels have been identified in plants. So far, evidence for the existence of ion channels in fungi comes mainly through the use of pharmacological agents that, in the case of animal cells, block or promote the opening of ion channels. These agents, such as verapamil, block Ca^{2+} entry by binding to the $\alpha 1$ subunit of voltage-operated channels. The use of such compounds, in studies on their effects on cell differentiation, has implicated Ca^{2+} channels in fungal development, in particular during the homing behaviour, encystment and zoospore germination in both *Phytophthora* (Iser *et al.*, 1989) and *Pythium* (Donaldson & Deacon, 1992; 1993a; 1993b) zoospores but also in spore germination and appressorium development of, for example, *Colletotrichum trifolii* (Warwar & Dickman, 1996). Furthermore, *Uromyces appendiculatus* possesses a mechanosensitive ion channel which, under conditions of membrane stress, is thought to allow influx of ions such as Ca^{2+}, thereby transducing a signal leading to further differentiation (Zhou *et al.*, 1991).

1.5.3 Compartmentation of cellular Ca^{2+}

1.5.3.1 Vacuoles

Vacuoles are thought to be the major storage organelles for Ca^{2+} in fungi (Miller *et al.*, 1990) and a source of messenger Ca^{2+} during signal transduction in *N. crassa* (Cornelius & Nakashima, 1987). A proton-motive force generated by the vacuolar membrane H^+-ATPase drives a Ca^{2+}/nH^+ antiport system (Figure 1.4) responsible for transporting Ca^{2+} across the vacuolar membrane (Anraku *et al.*, 1991); most of the vacuolarised Ca^{2+} being stored as polyphosphate (Ohsumi *et al.*, 1988). The mechanism by which Ca^{2+} is released from the fungal vacuole is not well known although it is thought to involve Ca^{2+} release channels (Corzo & Sanders, 1992). In fungi it is not clear whether localised elevations in $[Ca^{2+}]_c$ can trigger Ca^{2+} release at further points across the vacuole membrane (Berridge & Irvine, 1989) in a process termed Ca^{2+}-induced Ca^{2+}-release. However, inositol

1, 4, 5-trisphosphate (IP3)-mediated Ca^{2+} release channels exist on the vacuolar membrane in plants (Alexandre *et al.*, 1990) and have recently been described in *N. crassa* (Cornelius & Nakashima, 1987; Cornelius *et al.*, 1989).

1.5.3.2 Endoplasmic reticulum

The endoplasmic reticulum (ER), like the vacuole, is thought to function as a Ca^{2+} store. Total Ca^{2+} levels can approach micromolar concentrations in the mammalian sarcoplasmic reticulum, which contains an abundant Ca^{2+}-binding protein, calsequestrin (Campbell, 1983). Plant cells contain homologues of these low affinity Ca^{2+}-binding proteins that localise to the ER (Menegazzi *et al.*, 1993). Cell fractionation studies in *Penicillium cyclopium* have met difficulties in attempts to localise Ca^{2+} in the ER (Ugalde & Pitt, 1984) because relocalisation of Ca^{2+} to other organelles is thought to occur after disruption of the ER membranes. However, studies with yeasts have provided evidence for the existence of an intracellular Ca^{2+}-ATPase pump which appears distinct from the plasma membrane H^+-ATPase (eg., Goffeau *et al.*, 1990; Ghislain *et al.*, 1990; Halachmi *et al.*, 1992); the role of such a Ca^{2+}-ATPase is thought to be for Ca^{2+} transport into non-vacuolar organelles, the likely destination being the ER.

1.5.4 Ca^{2+}-binding proteins

Following an increase in $[Ca^{2+}]_c$ the transduction pathway continues with the formation of complexes with Ca^{2+} and Ca^{2+}-binding proteins (CaBPs) in the cytosol (Figure 1.4); Ca^{2+} can also bind to components of the cytoskeleton thereby regulating their formation and/or organisation. Calmodulin (CaM) is probably one of the most important of the CaBPs; it is apparently ubiquitous among the eukaryotes, being discovered in a range of fungi including the basidiomycota (Grand *et al.*, 1980), *N. crassa* (Ortega Perez *et al.*, 1981; Cox *et al.*, 1982) *Blastocladiella* (Gomes *et al.*, 1979) and yeasts (eg., Hubbard *et al.*, 1982). Binding of Ca^{2+} induces conformational change in calmodulin thereby enabling it to interact with a number of target proteins (Figure 1.4) including Ca^{2+}-ATPases, protein kinases, phosphatases and adenylate cyclases. Ca^{2+}/CaM complexes alone, or bound to these CaM target proteins, may produce a physiological response directly by regulation of phosphorylation reactions; in fungi and yeasts, a diverse range of physiological responses are CaM-dependent, including cell proliferation, cell cycle control and nuclear division (Anraku *et al.*, 1991). Interestingly, CaM can have an uneven distribution in some cell types and it has been postulated that localisation of CaM may cause polarised cell growth (St. Leger *et al.*, 1990; Brockerhoff & Davis, 1992).

1.5.5 Cyclic AMP

Adenosine 3',5'-cyclic monophosphate (cAMP) has been ascribed many roles as a second messenger in fungal growth and differentiation. For example, several studies have implicated cAMP in triggering spore germination. When a range of physical or chemical germination inducers were used to activate spores of *Phycomyces blakesleeanus*, a resulting transient rise in intracellular cAMP occurred (Van Laere, 1986). Additionally, sclerotium formation induced by hormones in *Rhizoctonia solani* was accompanied by elevated intracellular cAMP levels (Sharada *et al.*, 1992). Furthermore, exogenously supplied cAMP initiates mitotic division and infection structure formation in the bean rust fungus *Uromyces phaseoli* (Hoch & Staples, 1984).

Cyclic AMP binds to and regulates cAMP-dependent protein kinases (PK-As); activated PK-A can then phosphorylate regulatory proteins thus altering their biochemical properties (Cohen, 1992). Furthermore, PK-A may lead to enhanced production of other second messenger signalling molecules such as IP_3 and diacylglycerol (DAG) as found for *S. cerevisiae* (Gadd, 1995) - see below.

1.5.6 G protein-linked receptors

In fungi, detection of certain signal molecules by specific plasma membrane receptors (Figure 1.4) may result in an intracellular signal via activation of a membrane-bound GTP-binding protein (G protein). Activated G proteins may then result in changes in the activity of certain specific effectors, such as ion channels, adenylate cyclase, or enzymes involved in phosphoinositide metabolism. When agonists interact with external receptors, a phosphodiesterase (phospholipase C, PLC) is activated via a G protein-mediated process and this cleaves inositol 4, 5 bisphosphate (PIP_2) into IP_3 and DAG (Berridge, 1984; 1987; 1993); both of these molecules having second messenger functions.

1.5.7 Phosphoinositol signalling in fungi

Hydrolysis of PIP_2 results in a bifurcating signal pathway (Berridge, 1984), one pathway depending on DAG activating PKC to phosphorylate specific cellular proteins, the other pathway depending on IP_3-mediated elevation of $[Ca^{2+}]_c$, which may act via CaM to regulate target enzymes and phosphorylate a separate group of proteins (Figure 1.4). These two pathways may contribute to the final response by acting either co-operatively or synergistically. Both IP_3 and DAG have many of the properties of classical second messengers such as cAMP; they are produced rapidly, act at low concentrations and there are specific mechanisms involved in removing these messengers once the external signal is withdrawn. There is evidence of the existence of both messengers and for their involvement in signalling in yeasts and other fungi (Robson *et al.*, 1991; Hanson, 1991; Brunton & Gadd, 1991).

IP_3 has been shown to stimulate Ca^{2+} release in *N. crassa* (Cornelius *et al.*, 1989); the ability of IP_3 to mobilise Ca^{2+} from intracellular IP_3-sensitive stores is also widely known in animal and plant cells (Alexandre & Lassalles, 1992; Jaffe, 1980; Gilroy *et al.*, 1990; Hepler & Wayne, 1985).

1.5.8 Diacylglycerol and protein kinase C

DAG in animal cell membranes is short-lived since it can be rapidly phosphorylated to phosphatidic acid or hydrolysed to release arachidonic acid (Morgan, 1989). However, its transient existence allows it to play a second messenger role by transferring information from extracellular signals directly across the plasma membrane to result in protein phosphorylation. It can do this by activating a specific Ca^{2+}-dependent protein kinase (protein kinase C) as shown in Figure 1.4.

Protein kinase C (PKC) catalyses protein phosphorylation reactions (Figure 1.4) of many cellular proteins which in turn seem to control reactions such as secretion and exocytosis from various cell types. Berridge (1984) even suggests that PKC may modulate ion conductance through phosphorylating several membrane proteins relating to channels, pumps and ion exchange carriers.

1.5.9 Intracellular control of zoospore encystment

In *Phytophthora*, the transition from motile zoospore to cyst can occur within five minutes; the ultrastructural and morphological changes which happen during this time have been described in section 1.4.2. It is probable that the motile zoospore depends upon various signal transduction mechanisms to translate the external encystment stimulus into those cellular events required for differentiation into a cyst capable of germinating.

Evidence for putative second messengers has been provided for *Phytophthora*; these messengers include cyclic nucleotides, inositol phosphates, DAGs, phosphatidic acids and Ca^{2+} (Zhang *et al.*, 1992). Encystment was found to be induced by, for example, Ca^{2+} and Na^{+} (Grant *et al.*, 1986), was dependent upon low concentrations of Ca^{2+} (Griffith *et al.*, 1988), was accompanied by increases in the transmembrane flux of radiolabelled Ca^{2+} (Irving *et al.*, 1984) and Na^{+} (Iser *et al.*, 1989) and was sensitive to external pH (Griffith *et al.*, 1988). Furthermore, Ca^{2+}-rich sites have been associated with the cytoplasmic regions neighbouring the areas of fusion of peripheral vesicles with the plasma membrane in *Phytophthora* (Hemmes & Pinto Da Silva, 1980). These deposits could be removed by EGTA treatment and were not present in cysts. It is suggested that these sites are involved in the exocytosis events during encystment, when the small dorsal and ventral vesicles fuse with the plasma membrane to release their glycoprotein contents.

Encystment induced by pectin addition was followed rapidly (20s) by a marked increase in phosphatidic acid (PA) in *Phytophthora* zoospores concomitant with small elevations in cAMP and inositol phosphates (Zhang *et al.*, 1992). Exogenously supplied PA also induced encystment but only if Ca^{2+} was present in the bathing medium. Furthermore, pectin-induced encystment was slowed by the Ca^{2+} channel-blocker verapamil or when the external $[Ca^{2+}]$ was lowered with an EGTA supplement, with a corresponding reduction in the accumulation of PA in the cells. It is not yet clear how all these factors interact; however, the elements of a cell signalling mechanism are starting to emerge, and it could accommodate a role of second messengers, some of which may interact co-operatively or synergistically with calcium signal transduction.

1.6 Cyst adhesion and germination

1.6.1 Adhesion

Newly formed cysts are sticky due to the release of an adhesive material, allowing attachment to solid surfaces including roots (Sing & Bartnicki-Garcia, 1975a,b). Once adhered, the spores are not easily dislodged by washing or mild chemical treatment. This adhesiveness might be attributable to the cyst coat (glyco)protein in general or specifically to the (glyco)protein released from the ventral vesicles. Immunolabelling has revealed that the material released from ventral vesicles lies between the cyst and the root surface in about 90% of the cases (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, 1986), contain the functional adhesive material in zoospores of *Phytophthora*. A similar role has been proposed for the contents of ventrally-located K-bodies in *Saprolegnia ferax* (Lehnen & Powell, 1989). In contrast, immunochemical studies with *Py.*

aphanidermatum have suggested that the adhesive material may be located in the large peripheral vesicles which are present throughout the zoospore periphery and secreted during encystment (Sing & Bartnicki-Garcia, 1975a, b; Estrada-Garcia *et al.*, 1990b). However, Cope *et al.* (1996) recently used immunocytochemical techniques to show that during zoospore encystment, the large peripheral vesicles of *Pythium* spp. have a similar fate as for *Phytophthora* and *Saprolegnia* zoospores, and are not secreted during encystment.

It has been suggested that adhesion *in vivo* involves the interaction of proteinaceous components on the cyst surface with certain sugar residues, in particular fucose, present in root slime. Indeed, binding of *Ph. cinnamomi* and *Py. aphanidermatum* zoospores to the root elongation zone of maize and cress roots respectively, is abolished by pre-treating the roots with compounds which block or alter exposed fucosyl residues (Hinch & Clarke, 1980; Longman & Callow, 1987).

Once adhesion has occurred it is more or less irreversible, but the ability of newly encysted zoospores to adhere is short lived, in some cases lasting for only 1-4 minutes after induction of encystment (Gubler *et al.*, 1989), even though the surface glycoprotein is still present. Adhesiveness could be restored to older cysts of *Ph. cinnamomi* by addition of Ca^{2+}, whereas adhesiveness of young cysts was abolished by removal of Ca^{2+} with EGTA before they contacted a surface (Gubler *et al.*, 1989). This has also been confirmed for *Pythium* (Donaldson & Deacon, 1992). Evidence exists that *Ph. palmivora* zoospores release 30% of their Ca^{2+} within 2 minutes of the induction of encystment (Irving *et al.*, 1984). Gubler *et al.* (1989) propose that this released Ca^{2+} fixes the glycoprotein adhesive to a surface but may disperse or be reabsorbed if the cell does not contact a surface in the early stages of encystment, and then an exogenous Ca^{2+} supplement is necessary to restore adhesiveness.

1.6.2 Germination

The adhesion and germination of cysts are closely linked and findings indicate a central role for Ca^{2+} (Donaldson & Deacon, 1992). Newly formed cysts adhered and germinated well against glass slides, whereas older cysts held in suspension for 10 minutes showed poor adhesion and germination and required an exogenous Ca^{2+} or amino acid supplement to induce germination.

The findings can be related to the zoospore infection process, *in vivo*, by a simple working hypothesis (Donaldson & Deacon, 1992; Deacon & Donaldson, 1993). 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 (glyco)protein secreted over the germination site adheres the cyst in this orientation (Gubler & Hardham, 1990). The zoospore releases Ca^{2+} early in encystment (Irving *et al.*, 1984), and this is thought to interact with this glycoprotein to adhere the cyst to the host. The adhesive pad 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+} can then be reabsorbed to trigger germination, and the orientation of the cyst would lead directly to host penetration. It is proposed that the zoospore has an inbuilt reporter system to signal that it has docked properly, and this involves Ca^{2+} signalling.

This hypothesis is consistent with many *in vitro* studies which show that Ca^{2+} can trigger germination of *Phytophthora* (eg., Byrt *et al.*, 1982; Grant *et al.*, 1986; von Broembsen & Deacon, 1996; Deacon & Saxena, 1997b) and *Pythium* zoospore cysts (Donaldson & Deacon, 1992). The proposed requirement for Ca^{2+} in mediating germination is supported by the observations that the Ca^{2+}-chelator EGTA inhibits germination, as do the Ca^{2+} channel blockers La^{3+} and verapamil, suggesting that a flux of external Ca^{2+} into the spore is involved in this process. The intracellular Ca^{2+} antagonist, TMB-8, also inhibited germination, indicating that Ca^{2+}-mediated events within the spore are also involved. Lastly, and in further support of this view, calmodulin antagonists (trifluoperazine and dibucaine) also inhibit germination. The nucleus of the motile zoospore extends as a beak, and microtubules connect this to the base of the flagellar apparatus (Grove & Bracker, 1978). The extreme base of the anterior flagellum has high concentrations of locally exposed calmodulin (Ca^{2+}-binding protein) as evidenced by binding of a calmodulin-specific antibody, and centrin (another Ca^{2+}-protein) is present on the basal body connecting fibres and microtubules of the anterior microtubular rootlet (Gubler *et al.*, 1990; Hardham, 1992). This region is at or near the predicted site of future germ-tube outgrowth and may act as the "control centre" which recognises the Ca^{2+} signal for germination.

1.6.3 Role of organic compounds in germination

Despite all the evidence that links Ca^{2+} to germination, specific organic compounds can trigger germination *in vitro*. For example, *Phytophthora* spp. germinate in response to pectin, glucose, sucrose and several amino acids (Byrt *et al.*, 1982; Irving & Grant, 1984). *Py. aphanidermatum* germinates in response to, for example, certain L-amino acids and some sugars (Jones *et al.*, 1991; Donaldson & Deacon, 1993b). But the amino acids tested by Donaldson & Deacon (1993b) had no effect in the presence of EGTA, suggesting that an intake of exogenous Ca^{2+} was involved. Germination could also be prevented by Ca^{2+} entry blockers (eg., La^{3+}) but this suppression could be overcome at least partly by specific amino acids. The simplest explanation for this is that amino acids enable Ca^{2+} to be taken up even when the normal channels are blocked - possibly by interaction with ligand-gated Ca^{2+} channels.

In vivo, organic compounds in proximity to host roots could synergise the proposed signalling events involving Ca^{2+}. This could explain the common observation (eg., Jones *et al.*, 1991) that cysts germinate rapidly with almost 100% efficiency on roots, compared to the maximum of approximately 70% in *in vitro* experiments (eg., Donaldson & Deacon, 1992).

After germination, zoospores require new protein synthesis for germination (Hemmes & Hohl, 1971; Penington *et al.*, 1989) and rapid production of new RNA has also been observed. 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 (Gubler & Hardham, 1990), before the spore has been able to derive nutrients from the host. 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).

In vitro, after stimulation of germination, germ-tubes of pre-encysted spores emerge randomly in relation to an attractant source but can then reorientate themselves to factors such as nutrients (Mitchell & Deacon, 1986) and electrical fields (McGillivary & Gow, 1986). Germination of cysts that fail to dock on the host could be triggered by root exudates; subsequent germ-tubes could then reorientate towards the host by hyphal tropism.

1.7 Summary

Based on evidence from several workers, various fungi and different experimental conditions, a brief summary of our understanding of the factors involved in the different stages of the homing sequence of plant-pathogenic zoospores of the Oomycota is as follows.

1. Factors affecting motility and zoospore attraction.

- Role of calcium: external Ca^{2+} is required for maintaining the motile stage, and compounds which chelate Ca^{2+} or antagonise Ca^{2+}-mediated events alter the motile behaviour of zoospores. Other ions, such as K^+ and Mg^{2+}, can also alter the swimming pattern of zoospores.

- Zoospores accumulate around roots and in response to root exudates or pure compounds such as amino acids, sugars and alcohols. Zoospores respond to electrical fields and can also accumulate to form autoaggregates.

2. Induction of encystment.

- Zoospores can encyst in response to host surface components, such as uronic acids, fucose residues and cellulose, or to chemical treatments including amino acids, lectins and monoclonal antibodies. Furthermore, ions such as Ca^{2+} can induce encystment.

- During encystment, small dorsal and ventral vesicles fuse with the plasma membrane, whereas large peripheral vesicles migrate toward the centre of the cell; the nucleus also relocates as the cell becomes spherical in shape, and the flagella are shed or retracted. A glycoprotein coat appears on the spore surface and a wall develops beneath this.

- An efflux of Ca^{2+} followed by an uptake of Ca^{2+} accompanies the transition from motile to encysted spore.

3. Cyst orientation and adhesion.

- Zoospores orientate as they encyst against a root surface. Because zoospores have a fixed point of germination, this orientation means that the point of future germ-tube outgrowth lies adjacent to the host surface. Orientation of the zoospore, so that the ventral surface is adjacent to the root, means that the adhesive material released from the small ventral vesicles will facilitate attachment of the spore to its host.

- Ca^{2+} is required for adhesion - the postulated outward flux of Ca^{2+} during encystment may facilitate this process.

4. Germination.

- External Ca^{2+} can trigger germination and is thought to be intimately involved in an autonomous germination signal. Compounds present in root exudates, such as sugars and amino acids, synergise this autonomous signal.

5. Germ-tube tropism and secondary zoospore release.

- Germ-tubes can show tropisms to factors such as amino acids and applied electrical fields. Furthermore, Ca^{2+} is known to be involved in hyphal growth.
- Release of secondary zoospores may occur in the absence of nutrients but is suppressed in the presence of external Ca^{2+}.

1.8 Aims of the project

The literature survey above reveals growing evidence for a role of calcium ions in each of the events involved in host-location by plant-pathogenic Oomycota zoospores. The aim of this study was to extend these findings to a comparison between zoospores of three *Pythium* spp., three *Phytophthora* spp. and one *Aphanomyces* spp. while further investigating individual aspects of behaviour of *Py. aphanidermatum* and *Ph. parasitica* in greater depth. This work has involved three major lines of investigation.

1 Comparison of the motile behaviour of the different phytopathogenic fungi and investigation of the effects on motility of treatments which interfere with Ca^{2+}-mediated events.

2 Comparison of the effects of Ca^{2+}-modulating drugs on germinability of encysted zoospores of *Py. aphanidermatum* and *Ph. parasitica*.

3 The use of Ca^{2+}-reporting probes to determine the role of Ca^{2+} ions in a cell signalling capacity during encystment of *Pythium* and *Phytophthora* zoospores.

CHAPTER 2

General materials and methods

2.1 Culture of *Pythium aphanidermatum*, *Pythium dissotocum* and *Pythium catenulatum*, and production of zoospores

Pythium aphanidermatum (Edson) Fitz. (CBS 634.70) was originally isolated from tomato, *Lycopersicon esculentum*; *Py. dissotocum* Drechs. and *Py. catenulatum* Matthews were isolated from lettuce and spinach respectively, and were supplied by M.E. Stanghellini. The behaviour of some or all of these strains was reported in Goldberg *et al.* (1989) and Jones *et al.* (1991). The fungi were grown on potato-dextrose agar (PDA) and were maintained on PDA discs in sterile water at 4°C.

Production of zoospores was essentially as described by Goldberg *et al.* (1989). Cultures for zoospore production were grown on V8 agar (10ml per 9cm Petri dish), comprising 100ml V8 juice (Campbell's Soups Co.), 20g Oxoid N°3 agar and 1l HPLC water (18.88MΩ.cm). Circles, 15mm diameter, were cut from 3, 5 and 7 day-old cultures of *Py. aphanidermatum*, *Py. dissotocum* and *Py. catenulatum* respectively on plates of V8 agar incubated at 25°C. Six circles were placed in an empty Petri dish, 20ml of either sterile distilled water (SDW) or 10mM phosphate buffer (pH 7.0) was added and the plates were placed on a laboratory bench at 16-20°C.

After 10 hours the zoospore numbers were typically 10^5 ml^{-1}, assessed with a haemocytometer. Unless stated, the suspension was diluted with SDW or 10mM phosphate buffer (pH 7.0) to give a final concentration of $2x10^4$ zoospores ml^{-1}.

2.2 Culture of *Phytophthora parasitica* and production of zoospores

The isolate of *Phytophthora parasitica* Dastur (=*Ph. nicotianae* van Breda de Haan) originated from diseased vinca [*Cantharanthus roseus* (L.) G. Don] in a commercial glasshouse in Oklahoma, USA. The fungus was grown in Petri dishes containing 10ml of clarified V8 agar. This was prepared by mixing 330ml of canned Campbell's V8 juice, centrifuging at 4000 rpm for 20 minutes (MSE Mistral 1000 benchtop centrifuge) to collect the supernatant, diluting this (1:5 v/v) with HPLC water (18.88MΩ.cm) and addition of 1.5% (w/v) Difco Bacto-agar.

Production of zoospores was essentially as described by von Broembsen & Deacon (1996). Agar blocks, 2mmx2mm, were cut from the margins of 3 to 4 day-old colonies, and 15 blocks were distributed in a Petri dish containing 10ml of clarified V8 broth (as above but without agar). The dish was incubated for 24 hours at 27°C to allow young mycelial colonies to develop. Then the broth was removed by pipette and replaced by 20ml of sterile HPLC water. The dish was rotated gently to rinse the mycelial mats and left for 15 minutes before the HPLC water was removed and replaced with 20ml of fresh sterile HPLC water. The dish was rotated once more and left for a further 15 minutes before the HPLC water was removed and replaced by 10ml of mineral salts solution (see later). The mats were then incubated for 3 days at 20°C in a Gallenkamp Fi-totron 600H incubator beneath 3 fluorescent tubes (Thorn, 40W, white). The salts solution was changed once (at 24 hours) during this time. After 3 days the salts solution was removed, the mats were rinsed three times with 20ml sterile HPLC water, flooded with 15ml of 10mM Tris-Cl (made with HPLC water and Ultra-high purity Tris, ICN Biomedicals), pH 7.20 and placed in a refrigerator at *c.* 4°C for 30 minutes to trigger cleavage of the sporangium contents into zoospores. The dish was then placed on a laboratory bench at 16-20°C. Zoospores began to be released 20-30 minutes later. After 30 minutes the zoospore numbers were typically between 10^5 and 10^7 ml^{-1}, assessed with a haemocytometer. The suspension was diluted with 10mM Tris-Cl, pH 7.20, if necessary, to give a final concentration of $2x10^4$ zoospores ml^{-1} for motility experiments, $5x10^5$ zoospores ml^{-1} for germination experiments and $5x10^5$ zoospores ml^{-1} for fluorimetry experiments.

The mineral salts solution comprised $Ca(NO_3)_2.4H_2O$ (0.308g), $MgSO_4.7H_2O$ (1.49g) and KNO_3 (0.51g) in 1l of HPLC water. To this was added 1ml of chelated iron solution composed of EDTA (0.6525g), KOH (0.375g) and $FeSO_4.7H_2O$ (1.245g) in 50ml HPLC water; this solution was filter sterilised (pore size 0.2μm, cellulose nitrate membrane, Whatman, Maidstone, England).

2.3 Culture of *Phytophthora infestans* and production of zoospores

Phytophthora infestans (Mont) de Bary was supplied by Dr. M. Chamberlain (Royal Botanic Gardens, Edinburgh, Scotland, UK.) and maintained at 17°C on rye grain agar (see below). For the production of zoospores 10-14 day-old cultures were used. These were flooded with 20ml of chilled (*c.* 12°C) sterile distilled water (SDW) and the sporangia dislodged by scraping with a sterile glass spreader. The resulting suspension, along with any displaced mycelial mat, was transferred to a sterile universal bottle and placed in a refrigerator (*c.* 4°C) for 90 minutes. After this time the zoospore numbers were typically around $5x10^4$ ml^{-1}. The suspension was diluted with SDW to give a final concentration of $2x10^4$ zoospores ml^{-1}, assessed with a haemocytometer.

For rye grain agar, 60g of fungicide-free organically-produced rye grain was soaked in 500ml of distilled water for 48h, boiled for 1h and then macerated in a food processor. Fifteen grams of Oxoid N°3 agar and 20g of sucrose where then added and made up to 1l in distilled water (protocol adapted from Caten & Jinks, 1968).

2.4 Culture of *Phytophthora palmivora* and production of zoospores

Phytophthora palmivora strain P3765 was supplied by Professor N. A. R. Gow (University of Aberdeen, Scotland, UK.) and maintained on V8 agar supplemented with 30mg of cholesterol and 3g of $CaCO_3$ l^{-1}. For zoospore production the cultures were grown at 27°C for 2-3 days in the dark before being transferred under lights in a 27°C incubator for a further 3-4 days to stimulate sporangia formation. After this time, sporangia were harvested by flooding the cultures with chilled SDW or 10mM phosphate buffer (pH 7.20) and gently scraping the surface with a sterile glass spreader. The resulting sporangial suspension was transferred to a fresh, sterile Petri dish and placed in a refrigerator at *c.* 4°C for 30 minutes (to trigger cleavage of the sporangial contents into zoospores) before being transferred to a laboratory bench (*c.* 16-20°C) for a further 30 minutes to ensure synchronous release of zoospores. Zoospore densities of $>10^6$ ml^{-1} were typically harvested after this time. The zoospore suspension was diluted with 10mM phosphate buffer (pH 7.20) to give a final concentration of $2x10^4$ zoospores ml^{-1}, assessed with a haemocytometer.

2.5 Culture of *Aphanomyces euteiches* and production of zoospores

Isolate GB33 of *A. euteiches*, obtained from C. R. Grau, University of Wisconsin, was isolated from diseased beans (*Phaseolus vulgaris*) and maintained on Difco cornmeal agar (CMA) at 24°C. Three blocks of agar, 5mmx5mm, were cut from the margin of a 6 day old colony on CMA and placed in a sterile 125ml conical flask containing 25ml peptone-glucose broth (20g Oxoid Bacteriological peptone, 5g glucose, 1l distilled water). The flasks were incubated static at 24°C for 2 days. The broth was then decanted and the mycelial mats were rinsed with 10ml sterile water (a mixture of lake water and distilled water, 1:1). This was decanted and the mats rinsed again with 10ml water and allowed to stand for 2h in 25ml water. After a further 2h the mats were rinsed with 25ml water, then 25ml water was added and the flasks were incubated overnight at 24°C. Primary zoospores were released during this time; they encysted immediately and produced secondary zoospores by the next morning. The zoospores were used for the experiments, typically at a concentration of about $2x10^4$ zoospores ml^{-1} water, assessed by a haemocytometer.

2.6 Induction of encystment

Motile zoospores were induced to encyst synchronously by transferring aliquots of the spore suspension to 1.5ml microcentrifuge tubes and holding them against a vortex mixer for 70 seconds. Preliminary tests were carried out to find other encystment methods for fluorimetry experiments (Chapter 5), so that zoospores could be encysted synchronously in the cuvette during fluorimetry. Potassium chloride at a final concentration of 67mM (S. W. Jones, 1989) caused synchronous encystment of *Ph. parasitica* zoospores; however, this concentration of KCl would change the ionic strength of the zoospore suspension and therefore have an unacceptable effect on the dissociation constant of fura-2 for Ca^{2+} during the experiment. It was also found that homogenising a zoospore suspension for 7 seconds using an Ultra-turrax T25 homogeniser (Janke & Kunkel GmbH & Co. KG, IKA-Labortechnic, Staufen) caused synchronous encystment but also caused many of the zoospores to lyse. Instead, the method of vortexing for 70 seconds was chosen as the most suitable encystment method for fluorimetry experiments.

2.7 Effects of cations, Ca^{2+}-modulators and nutrients on zoospore motility characteristics

A 50µl aliquot of zoospore suspension ($2x10^4$ zoospores ml^{-1}) was added to a microcentrifuge tube containing an equal volume of test solution. The contents were then withdrawn from the tube and added to the well of an acid-washed glass cavity slide. For acid-washing, slides were incubated for 48h in 2M HCl and washed thoroughly with HPLC water before use. Zoospore motility characteristics were observed, in the first 10 minutes following addition to cavity slides, by phase contrast microscopy using a Leitz Orthoplan microscope with a x4 or x10 objective and with a video camera attachment (Jones *et al.*, 1991; Donaldson & Deacon, 1993a). Video recordings of zoospore motility were made separately in the surface film of the zoospore suspension, within the body of the suspension and against the glass/suspension interface of the cavity slide. The swimming speeds and motility patterns of zoospores were analysed from video recordings, by tracing the paths of zoospores onto an acetate overlay on the video monitor. Swimming patterns were drawn on acetate overlays, by outlining the position of the spore. Fifty video frames represented each second of "real time". The number of frame intervals used for analysis of zoospore motility was varied in different treatments, the criterion being that zoospores had to have moved sufficiently between frame intervals for clear tracings to be made.

A range of Ca^{2+}-modulator supplements were compared to water controls for their effects on motility. The effects of selected amino acids were also examined.

Where applicable, the spores were assessed for swimming speed, amplitude and wavelength of the swimming helix and incidence of random turns.

2.8 Responses of encysted zoospores to Ca^{2+}-modulators, nutrients and cations

2.8.1 The effects of Ca^{2+}-modulators, nutrients and cations on cyst germination of *Pythium aphanidermatum*

A 1.0ml suspension of motile *Pythium aphanidermatum* zoospores ($2x10^4$ zoospores ml^{-1}), maintained in microcentrifuge tubes, was encysted by vortex agitation (70 sec) and transferred in 40μl aliquots either immediately (t_0) or after 5 min (t_5) to slides containing 40μl of test compounds at a range of compound concentrations. Sterile distilled water (SDW) was used as a control treatment and the other treatments included verapamil hydrochloride (ICN Biomedicals), caffeine (ICN Biomedicals), trifluoperazine hydrochloride (TFP) (ICN Biomedicals), 8-(diethyl-amino)-octyl-3,4,5,-trimethoxybenzoate hydrochloride (TMB-8) (ICN Biomedicals), 1,2-*bis*(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA, potassium salt) (ICN Biomedicals) and lanthanum chloride (La^{3+}) (ICN Biomedicals). Germination was assessed by microscopic examination (x10 objective) after 1.5h incubation on slides (*c* 16-20°C) maintained in humidity chambers. Five replicates were used and 100 spores were examined for each replicate. After microscopic examination to assess germination, a fluorescein diacetate (FDA)/propidium iodide solution was added to a final concentration of 100μg/ml (for each compound) and left for 5 min. This live/dead staining procedure allows the visualisation of the extent of cell viability. Propidium iodide penetrates the membranes of dead cells whereas FDA is passively taken up by all cells but requires activation by cytosolic esterases of live cells to fluoresce. Live cells (FDA-dyed) fluoresce green and dead cells (containing propidium iodide) fluoresce red with blue light excitation.

In other experiments, a suspension of motile *Py. aphanidermatum* zoospores (0.5ml), maintained in microcentrifuge tubes, was encysted by agitation (70 sec) and transferred in 40µl aliquots at 1 minute or at 2 minute intervals up to 9 minutes to acid-washed glass slides containing 40µl of the test compound. SDW was used as a control treatment and the other treatments included (final concentration shown): verapamil (100µM), caffeine (5mM), amiloride (200µM), BAPTA (5mM), TMB-8 (30µM), TFP (10µM), Mg^{2+} (10mM), Sr^{2+} (10mM), Ba^{2+} (10mM) and L-asparagine (10mM). Percentage germination was assessed after 1.5 hours by microscopic examination (x10 objective) of the first 100 spores observed for each of 5 replicates. Cell viability was assessed as above.

In a similar experiment 40µl aliquots of a cyst suspension were transferred 1 min after encystment to acid-washed glass slides containing increasing concentrations of test compounds. The compounds tested included: Ca^{2+}, Ba^{2+}, BAPTA, TMB-8 and the Ca^{2+} ionophore Br-A23187; SDW was used as a control.

All experiments were incubated in the dark because many of the Ca^{2+}-modulators are light-sensitive.

2.8.2 The effects of Ca^{2+}-modulators and Ca^{2+} on cyst germination of *Phytophthora parasitica*

Motile zoospores of *Ph. parasitica* ($5x10^5$ zoospores ml^{-1}) were incubated in microcentrifuge tubes for 1 min in the presence of the compound to be tested, using a range of compound concentrations. The zoospore suspension was then vortexed for 70 seconds. After vortexing, the spore suspension was incubated in the microcentrifuge tube for 1.5h. After this time, 50µl aliquots of the suspension were transferred to acid-washed glass slides and percentage germination assessed by microscopic examination (x10 objective). The calcium-modulating treatments included: Gd^{3+}, La^{3+}, verapamil, caffeine, TMB-8, TFP and BAPTA; SDW was used as a control. All treatments were supplied in 200µl aliquots to an equal volume of zoospore suspension. Cell viability was assessed as above.

In separate experiments to those above, zoospores were encysted by agitation and transferred immediately to microcentrifuge tubes containing either an equal volume of SDW (as the control treatment) or Ca^{2+} (50mM final concentration). Germination was assessed after 1.5 hours.

In further experiments, spores were encysted by agitation and transferred immediately or after 3 min or 15 min to glass slides with either distilled water or Ca^{2+}. Germination was assessed after 1.5 hours incubation in humidity chambers.

2.8.3 The effect of Ca^{2+}-modulators on cyst germination of *Pythium aphanidermatum* in the presence or absence of nutrients or cations

A 1ml suspension of motile *Py. aphanidermatum* zoospores was vortex-encysted and incubated in a microcentrifuge tube for 6 minutes. A 500μl aliquot was then incubated in a microcentrifuge tube for a further 10 minutes with either 500μl of SDW (as a control) or 500μl of one of the following treatments (final concentration given): BAPTA (3mM), verapamil (100μM), TFP (40μM) or calmidazolium (1μM). After this time (t_{16}) the cysts were subsequently treated with various sugars (10mM final concentration), amino acids (10mM final concentration), divalent cations (10mM final concentration), malt extract with peptone (each at 1% concentration) or amino acids or sugars (10mM final concentration) supplemented with divalent cations (10mM final concentration), by transferring 40μl aliquots of both the SDW pre-treated and the modulator pre-treated cysts to separate microcentrifuge tubes containing an equal volume of post-treatment solution. The spores were incubated in microcentrifuge tubes for 1.5h before being transferred to glass slides and percentage germination assessed by microscopic examination.

2.8.4 The effects of BAPTA, ions and nutrients on germ-tube growth of *Py. aphanidermatum* zoospores

Zoospores were encysted by agitation and maintained in suspension for 10 min before being transferred to slides with the treatments. In one experiment, cysts were treated with either an equal volume of SDW (as the control treatment) or with

Ca^{2+} (10mM final concentration); germ-tube length was measured from videotaped sequences, recorded at 4, 7 and 20 hours after incubation on slides. In another experiment, cysts were treated with Ca^{2+} (10mM), BAPTA at 5 or 10mM or BAPTA (5mM) plus Ca^{2+} (10mM); germ-tube length was measured from videotaped sequences, recorded at 3 and 20 hours after incubation on slides. In a further experiment, cysts were treated with either Ca^{2+}, Mg^{2+}, Sr^{2+}, Na^{2+}, Li^{2+}, L-aspartic acid or L-alanine (all 10mM final concentration) ; germ-tube length was measured from videotaped sequences, recorded at 16 hours after incubation on slides

2.9 Fluorimetry

2.9.1 Production of zoospores for fluorimetry experiments

A motile *Phytophthora parasitica* zoospore suspension was produced essentially as described in section 2.2. For fluorimetry experiments a concentration of 5×10^5 zoospores ml^{-1} was used, suspended in 10mM Tris-Cl (Ultra-Pure, ICN Biomedicals) made up in HPLC water (18.88MΩ.cm), pH 7.20.

2.9.2 Dye loading

Motile zoospores at a concentration of 5×10^5 zoospores ml^{-1} were incubated for 30 or 60 minutes in a range of fura-2 AM concentrations from 2.5μM to 30μM at 20°C. Fura-2 AM was obtained from Molecular Probes Inc. (Eugene, Oregon, USA) or Calbiochem-Novabiochem (U.K.) Ltd. (Nottingham, U.K.); a 10mM stock solution (in 40% dimethyl sulphoxide containing 0.01% pluronic F-127) was stored at -70°C. When required, the stock solution was diluted to 1mM in HPLC water and then diluted further by addition to the zoospore suspension.

2.9.3 Determination of the level of encystment

After incubation for 30 and 60 minutes in a range of fura-2 AM concentrations, an aliquot of the zoospore suspension was transferred to a glass cavity slide containing a formaldehyde/glutaraldehyde solution (2% and 1.5% final concentration, respectively, in distilled water) to fix the cells immediately. A cell was scored as being motile when it appeared ovoid in shape with flagella attached, whereas a cell was scored as encysted when it appeared rounded and without flagella. The cells were observed by microscopy using a x40 objective, the first 100 cells viewed being scored from each of four replicates.

2.9.4 Equipment used in fluorimetry experiments

Fluorimetry was performed with a Perkin Elmer LS50B luminescence spectrofluorimeter with a thermostatic temperature controller attached (Grant LTD 6) and a magnetic stirrer plate housed in the cuvette compartment. The fluorimeter was fitted with a Xenon lamp (50Hz beam chopper speed). Data was acquired by a Dell Optiplex 486 personal computer (8MB RAM, 66MHz processor speed) running FLDM software (Perkin Elmer).

2.9.5 *In vitro* calibration of fura-2 free acid response to Ca^{2+} using fluorimetry

An *in vitro* calibration of fura-2 to obtain the dissociation constant (K_d) of the indicator for Ca^{2+}, under known environmental conditions, was performed using a calibration buffer kit (Calcium Calibration Buffer Kit 1, C-3008, Molecular Probes Inc., Eugene, Oregon, USA.). A 1mM stock solution of fura-2 free acid was prepared in HPLC water (18.88MΩ.cm). All glassware was scrupulously cleaned by immersion in 2M HCl for 48 hours and then rinsed three times in excess HPLC water. The calibration was then performed at 20°C using a spectrofluorimeter and following the protocol provided with the calibration buffer kit.

2.9.6 Time drive experiments

A 2.5 ml aliquot of *Phytophthora parasitica* zoospore suspension (5×10^5 zoospores ml^{-1}) was transferred to a glass cuvette. The cuvette and contents were placed in the sample compartment of the fluorimeter and allowed to attain a temperature of 20°C before addition of fura-2 (free acid) to a final concentration of 1μM. A magnetic stirring-bar flea (PTFE-coated, 5x2mm) was then placed in the cuvette. An excitation scan was performed using the following instrument parameters.

Excitation wavelength range: 250-450nm.
Fluorescence emission collected at 511nm.
Excitation slits: 3.4nm.
Emission slit: 4.0nm.
Scan speed: 300.

Experiments in which an excitation scan indicated that background Ca^{2+} levels of the zoospore suspension had saturated the fura-2 were discarded. The Intracellular Biochemistry Application was then chosen from the computer programme menu to allow fura-2 fluorescence ratio data to be collected in real time. The following method parameters were chosen.

Ratio type: excitation.
Ratio speed: medium.
Excitation wavelengths: 340/380nm.
Emission wavelength: 511nm.
Excitation slits: 3.4nm.
Emission slit: 4.0nm.
Stirrer speed: low.
Emission filter: clear.

A 2 to 3 minute experiment was performed to detect the fluorescence ratio of fura-2 in the suspension surrounding a motile zoospore population. The experiment was paused and the suspension was rapidly pipetted into an acid washed glass HPLC bottle. The suspension was vortexed for 70 seconds to encyst the zoospore population synchronously and then the zoospores were transferred immediately back to the cuvette. The experiment was started again to run for 20 to 30 minutes to detect levels of Ca^{2+} in the medium surrounding the developing zoospore population.

2.9.7 Effects of Ca^{2+} channel-blockers

As above (section 2.9.6) a 3ml aliquot of a motile *Phytophthora parasitica* zoospore suspension (5×10^5 zoospores ml^{-1}) was transferred to a glass cuvette and fura-2 (1µM final concentration) was added. The cuvette was placed in the fluorimeter sample compartment, to allow the cuvette contents to reach 20°C, and a magnetic stirrer bar was placed in the cuvette. Either verapamil (10µM final concentration) or La^{3+} (1µM final concentration) was then added to the zoospore suspension, and after a further 1 minute, a time-drive experiment was initiated as described above (section 2.9.6).

2.9.8 Effects of intracellular Ca^{2+} antagonists

This experiment was carried out as above (section 2.9.6) with the addition of TMB-8 (5µM final concentration) to the zoospore suspension 1 minute before the start of the ratio data collection.

CHAPTER 3

Motility characteristics of fungal zoospores

3.1 Introduction

Motility studies involving *Phytophthora* spp. (eg., Bimpong & Clerk, 1970; Allen & Newhook, 1973) and *Pythium* spp. (Royle & Hickman, 1964a; Donaldson & Deacon, 1993a) have reported that zoospores swim in an extended helix with periodic abrupt changes of direction (random turns). Furthermore, zoospores of various fungi have been shown to be attracted to roots and more specifically towards amino acids (Royle & Hickman, 1964a; Khew & Zentmyer, 1973; Jones *et al.*, 1991), sugars (Orpin & Bountiff, 1978; Jones *et al.*, 1991) and volatile compounds (Allen & Newhook, 1973; Cameron & Carlile, 1978). Royle & Hickman (1964a) and Reid *et al.* (1995) described the responses of *Py. aphanidermatum* and *Ph. palmivora* zoospores to roots; zoospores initially orientate toward the stimulus and they then swim toward the source of attractant(s). Finally, when the zoospores arrive in the vicinity of the attractants, they show an excited activity with frequent changes in orientation prior to encystment. Reid *et al.* (1995) also describe this sequence of events for the response of *Ph. palmivora* zoospores to autoaggregates of cysts. Studies have shown that various ions and treatments which interfere with ion mediated effects can alter the speed or direction of swimming of zoospores (Allen & Harvey, 1974; Thomas & Butler, 1989; Ersek *et al.*, 1991; Donaldson & Deacon, 1993a). Studies on other cell types have implicated Ca^{2+} or other divalent cations in flagellar behaviour (Bloodgood, 1991; Boitano & Omoto, 1992) and bacterial chemotaxis (Ordal, 1977; Armitage & Lackie, 1990).

It has been suggested (Allen & Newhook, 1974) that random turning is suppressed when *Phytophthora cinnamomi* zoospores encounter a gradient of attractant and so zoospores may swim uninterrupted towards a root. In the present study, when initial experiments were performed to study the effects of ion-modulating treatments on zoospore chemotaxis it became apparent that zoospores of the three *Pythium* spp. made few random turns when swimming in a deep suspending medium of water or phosphate buffer. As a basis for understanding how these zoosporic fungi locate their hosts or other substrata, the effects of ion-modulating compounds and certain amino acids were tested for their effects on motility characteristics. The locomotory attributes of three *Pythium* spp., three *Phytophthora* spp. and *Aphanomyces euteiches* were compared in backgrounds of amino acids and Ca^{2+}-modulating compounds.

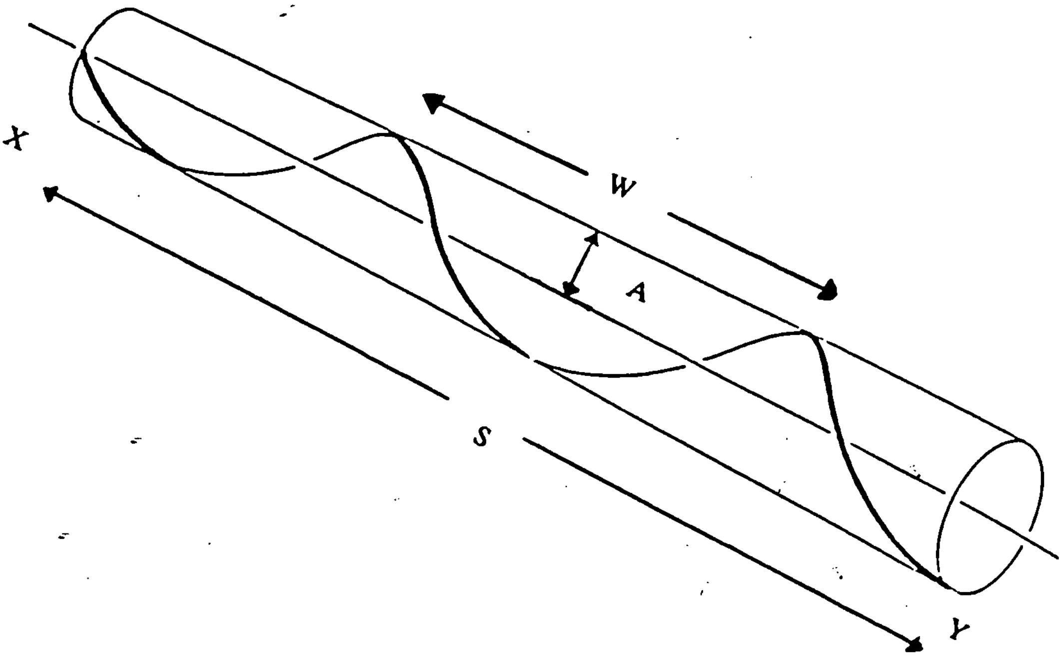
3.2 Normal motile behaviour of three *Pythium* spp and the effects of EGTA and BAPTA

Zoospore tracks of *Py. aphanidermatum*, *Py. dissotocum* and *Py. catenulatum* swimming in distilled water on cavity slides were observed using a x10 objective lens. The motility features examined are shown in Figure 3.1.

Zoospores of all *Pythium* spp. swam in a fashion typical of the Oomycota, with a helical (corkscrew-like) pattern (eg., Figure 3.2). However, each fungus differed in locomotory characteristics, measured by analysis of videotapes, as shown in Table 3.1. The amplitude of the helix was significantly lower for *Py. dissotocum* than for *Py. catenulatum* and *Py. aphanidermatum*. The three *Pythium* spp. differed in swimming speed, the order from fastest to slowest being *Py. catenulatum* = *Py. dissotocum* > *Py. aphanidermatum*. It was observed that zoospores of the three *Pythium* spp., swimming in the middle of a deep suspension of distilled water did not show periodic abrupt changes of direction.

In this study, it was observed that *Pythium* zoospores swimming in the centre of a deep suspension swam in a typical extended helix (Figure 3.2). When zoospores were swimming at the water/air interface, a circular motility pattern was observed (Figure 3.3), whereas spores swimming against the glass slide showed periodic changes of direction (Figure 3.4). Thirty one zoospore tracks in distilled water were analysed (x10 objective) at random by focusing at the mid depth of the zoospore suspension where zoospores did not contact the liquid surface or the glass slide; these represented a total of over 100 seconds of uninterrupted swimming paths for each of the three *Pythium* spp. in SDW. If, for example,

Figure 3.1 Diagrammatic representation showing the typical helical path of movement of a zoospore of the Oomycota, such as when *Pythium aphanidermatum* swims in water.



Speed (S) = distance between two points, X and Y, per unit time

Amplitude (A)

Wavelength (W)

Table 3.1 Locomotory attributes[1] of three *Pythium* species in backgrounds of sterile distilled water and the effect of EGTA (500μM) on *Pythium aphanidermatum*.

Attributes of locomotion	*Pythium aphanidermatum*	*Pythium dissotocum*	*Pythium catenulatum*	[2]*Pythium aphanidermatum* + EGTA (500μM)
Amplitude (μm)	28 ± 2.0	14 ± 1.9	28 ± 2.2	9 ± 1.3
Wavelength (μm)	142 ± 4.3	372 ± 12.8	390 ± 14.6	64 ± 8.0
Speed ($\mu m\ s^{-1}$)	167 ± 5.5	297 ± 8.4	277 ± 6.4	75 ± 5.1
Mean time between turns (s)	> 100^{+}	> 100^{+}	> 100^{+}	>100^{+}
Number of replicate zoospore tracks analysed	31	31	31	31

[1] Means ± s.e. for the number of replicates shown.

[2] Zoospore tracks were analysed 5 minutes after addition of EGTA.

[+] Total time covered by 31 zoospores was 134 s for *Py. aphanidermatum*, 108 s for *Py. dissotocum*, 111 s for *Py. catenulatum* and 144 s for *Py. aphanidermatum* in EGTA.

Table 3.2 Locomotory attributes[1] of three *Pythium* species in backgrounds of 10mM phosphate buffer, pH 7.0.

Attributes of locomotion	*Pythium aphanidermatum*	*Pythium dissotocum*	*Pythium catenulatum*
Amplitude (µm)	26 ± 3.1	14 ± 0.8	26 ± 2.3
Wavelength (µm)	136 ± 4.0	380 ± 10.2	387 ± 10.5
Speed ($\mu m\ s^{-1}$)	171 ± 5.0	290 ± 7.6	286 ± 7.4
Mean time between turns (s)	> 50[+]	> 50[+]	> 25[+]
Number of replicate zoospore tracks analysed	15	15	15

[1] Means ± s.e. for the number of replicates shown.

[+] Total time covered by 15 zoospores was 72 s for *Py. aphanidermatum*, 51 s for *Py. dissotocum* and 56 s for *Py. catenulatum*.

zoospores of *Py. aphanidermatum* undergo random turns every 2 seconds (Donaldson & Deacon, 1993a), then over 50 turns should have been observed. However, the three *Pythium* spp. tested did not make frequent random changes in direction when swimming in the mid depth of the zoospore suspension. *Py. catenulatum* was the only *Pythium* spp. to make a random turn and only one was noted, this occurring in phosphate buffer (Table 3.2).

In separate experiments, zoospore tracks of *Py. aphanidermatum*, *Py. dissotocum* and *Py. catenulatum* swimming in 10mM phosphate buffer (pH 7.0) on cavity slides were observed using a x10 objective lens. No significant difference in locomotory attributes was found when each of the three *Pythium* spp. was swimming in phosphate buffer (Table 3.2) compared with distilled water (Table 3.1).

In tests on the effects of calcium-chelating agents on zoospore motility both EGTA (500μM) and BAPTA (100μM) caused zoospores of *Py. aphanidermatum* to swim in continuous straight paths both within the centre of the suspension and against the bottom of the welled slides (Figures 3.5 & 3.6). EGTA and BAPTA caused zoospores of all fungi tested to swim at the bottom of the suspension so motility characteristics could not be compared with control experiments where zoospores swam in the surface film. The amplitude of the swimming helix was reduced compared with that of control spores and the swimming speed was also significantly reduced (Table 3.1). When tested on *Py. aphanidermatum*, EGTA and BAPTA caused a complete suppression of turns against the glass slide. After 20 minutes EGTA caused immobilisation of a significant number of zoospores so motility was examined 5-10 minutes after addition of the treatment.

3.3 Normal motile behaviour of three *Phytophthora* spp and the effects of EGTA and BAPTA

Zoospore tracks of *Ph. palmivora* and *Ph. parasitica* in 10mM phosphate buffer (pH 7.0) and *Ph. infestans* swimming in distilled water on cavity slides were observed using a x10 objective lens.

Table 3.3 Locomotory attributes[1] of three *Phytophthora* species in backgrounds of 10mM phosphate buffer, pH 7.0 or phosphate buffer with EGTA[2] (in parentheses)

Attributes of locomotion	*Phytophthora palmivora*	*Phytophthora parasitica*	*Phytophthora infestans*
Amplitude (μm)	5 ± 0.8	6.2 ± 0.7	5.2 ± 1.2
Wavelength (μm)	89 ± 5.7	210 ± 22.9	94 ± 6.6
Speed ($\mu m\ s^{-1}$)	159 ± 4.8 (118 ± 12.8)	185 ± 6.1 (86 ± 11.1)	77 ± 7.3 (55 ± 3.4)
Mean time between turns (s)	4.0 ± 0.2 (> 40^{+})	6.3 ± 1.1 (> 40^{+})	2.5 ± 0.5 (> 20^{+})
Number of replicate zoospore tracks analysed	31 (15)	31 (15)	31 (15)

[1] Means ± s.e. for the number of replicates shown.

[2] Zoospore tracks were analysed 5 minutes after the addition of 250μM EGTA to *Ph. palmivora*, 500μM EGTA to *Ph. parasitica* and *Ph. infestans*.

$^{+}$ Total time covered by 15 zoospores was 41 s for *Ph. palmivora*, 47 s for *Ph. parasitica* and 57 s for *Ph. infestans*.

The three *Phytophthora* spp. tested, *Ph. palmivora*, *Ph. parasitica* and *Ph. infestans*, exhibited the helical motility pattern typical of zoospores of the Oomycota (Figures 3.7 & 3.9). The amplitude of the helical path, however, was significantly lower for all three *Phytophthora* spp. (Table 3.3) than for any of the *Pythium* spp. studied (Tables 3.1 & 3.2). Another major difference between the *Pythium* and *Phytophthora* zoospores was that *Phytophthora* zoospores made frequent, abrupt changes of direction when swimming in the mid depth of a deep zoospore suspension (Figures 3.7 & 3.9) where zoospores did not make contact with the suspension surface or the glass slide; these observations prompted a more thorough examination of turning behaviour as presented in Section 3.5. When tested on the three *Phytophthora* spp., EGTA and BAPTA completely suppressed random turning (eg. Figure 3.8 & 3.10). In addition, EGTA also caused a significant reduction in the swimming speed of *Phytophthora* zoospores (Table 3.3).

3.4 Normal motile behaviour of *Aphanomyces euteiches* zoospores and the effects of EGTA

Zoospore tracks of *A. euteiches* swimming in distilled water on cavity slides were observed using a x10 objective lens.

Aphanomyces euteiches exhibited the helical motility pattern typical of zoospores of the Oomycota. However, in similarity to *Phytophthora* zoospores and in contrast to *Pythium* zoospores, *A. euteiches* zoospores made frequent, abrupt turns (Table 3.4) when swimming in the mid depth of a zoospore suspension. At 3mM, EGTA caused *A. euteiches* zoospores to swim in continous straight paths, significantly reduced the amplitude and wavelength of the helical path and the speed at which the zoospores swam (Table 3.4). EGTA (3mM) also prevented random turning of *A. euteiches* zoospores (Table 3.5).

Table 3.4 Locomotory attributes[1] of *Aphanomyces euteiches* in backgrounds of distilled water or EGTA[2] (in parentheses)

Attributes of locomotion	
Amplitude (μm)	30.8 ± 2.3 (7.3 ± 0.8)
Wavelength (μm)	332.9 ± 8.2 (92.0 ± 5.3)
Speed ($\mu m\ s^{-1}$)	200.4 ± 4.1 (136.0 ± 7.0)
Mean time between turns (s)	8.9 ± 2.2 (> 45⁺)
Number of replicate zoospore tracks analysed	30 (15)

[1] Means ± s.e. for the number of replicates shown.

[2] Zoospore tracks were analysed 5 minutes after the addition of 3mM EGTA.

⁺ Total time covered by 15 zoospores of *A. euteiches* was 46 s

3.5 Further analysis of turning behaviour of *Phytophthora* and *Aphanomyces* zoospores

To determine the turning frequency of *Ph. palmivora*, *Ph. parasitica*, *Ph. infestans* and *A. euteiches* zoospores swimming in the mid depth of a spore suspension, zoospore tracks were again examined from videotapes. Zoospores were studied in distilled water or 10mM phosphate buffer (pH 7.0) or in 10mM phosphate buffer containing EGTA (pH 7.0). From ten replicate experiments, ten tracks were chosen at random from a single field of view; the mean turning frequency of 100 spores for each fungus was thus obtained (Table 3.5). Addition of EGTA completely prevented random turning when added to *Ph. palmivora* at 250µM, *Ph. parasitica* at 500µM and *A. euteiches* at 3mM; EGTA also significantly reduced the number of random turns made by *Ph. infestans* at 500µM. These concentrations of EGTA had previously been determined as the minimum levels that would significantly affect turning behaviour without causing rapid encystment or lysis of the zoospores

3.6 Effects of amino acids on zoospore motility

Py. aphanidermatum, *Ph. palmivora*, *Ph. parasitica* and *Ph. infestans* were tested for responses to amino acids. In chemotaxis tests, several L-amino acids have previously been reported to be attractive to *Py. aphanidermatum*; they include L-aspartic acid (10mM), L-glutamic acid (10mM), L-glutamine (10mM) and L-alanine (10mM) (Jones *et al.*, 1991). When these amino acids were tested for their effects on zoospore motility, L-aspartic acid (15mM) and L-glutamic acid (15mM) were found to cause the zoospores to swim in irregular helices and to exhibit jerky movements throughout the suspension (Table 3.6). L-glutamine and L-alanine were found to be less attractive than L-aspartic acid and L-glutamic acid in chemotaxis assays (S.P.Donaldson,1992). Here, these two amino acids did not cause zoospores to elicit aberrant swimming patterns (Table 3.6) even when tested at up to 25mM final concentration (eg. Figure 3.12); instead, the zoospores swam in three distinct patterns as in control experiments, depending on their locations in

Table 3.5 Turning behaviour of zoospores of *Phytophthora palmivora* and *Ph. parasitica* in backgrounds of 10mM phosphate buffer (pH 7.0) or EGTA[1], and of *Ph. infestans* and *Aphanomyces euteiches* in backgrounds of distilled water or EGTA[1].

	Phytophthora palmivora		*Phytophthora parasitica*	
Turning behaviour	[2]Control	[3]EGTA (250μM)	[2]Control	[3]EGTA (500μM)
Mean distance between turns (μm)	612 ± 94	> 26000*	972 ± 133	> 30000*
Mean time between turns (sec)	4.0 ± 0.5	> 170+	6.3 ± 0.8	> 200+

	Phytophthora infestans		*Aphanomyces euteiches*	
Turning behaviour	[2]Control	[3]EGTA (500μM)	[2]Control	[3]EGTA (3mM)
Mean distance between turns (μm)	279 ± 71	1265 ± 205	1943 ± 474	> 20000*
Mean time between turns (sec)	2.7 ± 1.0	22.3 ± 3.8	9.1 ± 2.1	> 150+

[1] Zoospore tracks were analysed 5 minutes after the addition of 250μM EGTA to *Ph. palmivora*, 500μM EGTA to *Ph. parasitica* and *Ph. infestans* and 3mM EGTA to *A. euteiches*.

[2] Means ± s.e. of 10 replicate experiments based on measurements of 10 randomly chosen zoospore tracks in each replicate.

[3] Means ± s.e. of 5 replicate experiments based on measurements of 10 randomly chosen zoospore tracks in each replicate.

[*] Total distance covered by 50 zoospores was 26011μm for *Ph. palmivora*, 30166μm for *Ph. parasitica* and 20090μm for *A. euteiches*. [+] Total time covered by 50 zoospores was 177 s for *Ph. palmivora*, 208 s for *Ph. parasitica* and 152 s for *A. euteiches*.

the depth of the suspension. L-glutamic acid (15mM) and L-asparagine (15mM) caused *Ph. palmivora* zoospores to exhibit jerky movements (eg., Figure 3.11), whereas, L-alanine (25mM) did not do so (Table 3.6). L-glutamic acid (15mM), L-glutamine (15mM) and L-asparagine (15mM) all caused *Ph. parasitica* zoospores to swim in a jerky fashion whereas a normal motility pattern with random turns was observed with 25mM L-alanine (Table 3.6). L-aspartic acid and L-glutamic acid caused *Ph. infestans* zoospores to exhibit a jerky motility pattern whereas L-asparagine and L-alanine did not do so (Table 3.6). When the chemotactically non-attractive D-isomers (Donaldson & Deacon 1993b) were tested it was found that D-glutamic acid (15mM) and to a lesser extent D-aspartic acid (15mM) caused *Py. aphanidermatum* and *Ph. parasitica* zoospores to exhibit a jerky motility pattern. Where amino acids caused irregular swimming patterns, a large proportion of zoospores encysted after 20-25 minutes. The irregular swimming occurred immediately after addition of the amino acid. Zoospore swimming paths were therefore videotaped between 2 and 5 minutes after the addition of the test compound.

When EGTA was added to zoospores which had been swimming in the presence of an amino acid for 10 minutes, motility changed to the typical EGTA mode of straight swimming (Table 3.6); *Ph. palmivora*, *Ph. parasitica* and *Ph. infestans* failed to make random turns, *Py. aphanidermatum* failed to make turns against the glass slide and the amplitude of the swimming helix was significantly reduced. The effect on *Ph. palmivora* zoospores could be achieved at 250μM EGTA whereas 500μM EGTA was required to produce the same effect with *Py. aphanidermatum*, *Ph. parasitica* and *Ph. infestans* zoospores.

In separate experiments on *Ph. parasitica* zoospores (Figure 3.13) the addition of 15mM L-asparagine caused zoospores to swim in a jerky, irregular pattern. With the subsequent addition of 500μM EGTA the motility pattern changed to one of straight swimming with the suppression of random turning. When 2.5mM Ca^{2+} was then added the swimming pattern reverted to the jerky mode seen upon addition of the amino acid and the zoospores started to encyst after 2 minutes.

Table 3.6 The effects of amino acids on the swimming pattern of zoospores of *Pythium aphanidermatum* and three *Phytophthora* spp., and effects of the subsequent addition of EGTA (in parentheses)

Amino acid	*Pythium aphanidermatum*	*Phytophthora palmivora*	*Phytophthora parasitica*	*Phytophthora infestans*
L-aspartic acid (15mM)	jerky (straight)	-*	-*	jerky (straight)
L-asparagine (15mM)	-*	jerky (straight)	jerky (straight)	normal helix (straight)
L-glutamic acid (15mM)	jerky (straight)	jerky (straight)	jerky (straight)	jerky (straight)
L-glutamine (25mM)	normal helix (straight)	-*	jerky (straight)	-*
L-alanine (25mM)	normal helix (straight)	normal helix (straight)	normal helix (straight)	normal helix (straight)
D-glutamic acid (15mM)	jerky (straight)	-*	jerky (straight)	-*
D-aspartic acid (15mM)	jerky (straight)	-*	jerky (straight)	-*

* - = not tested

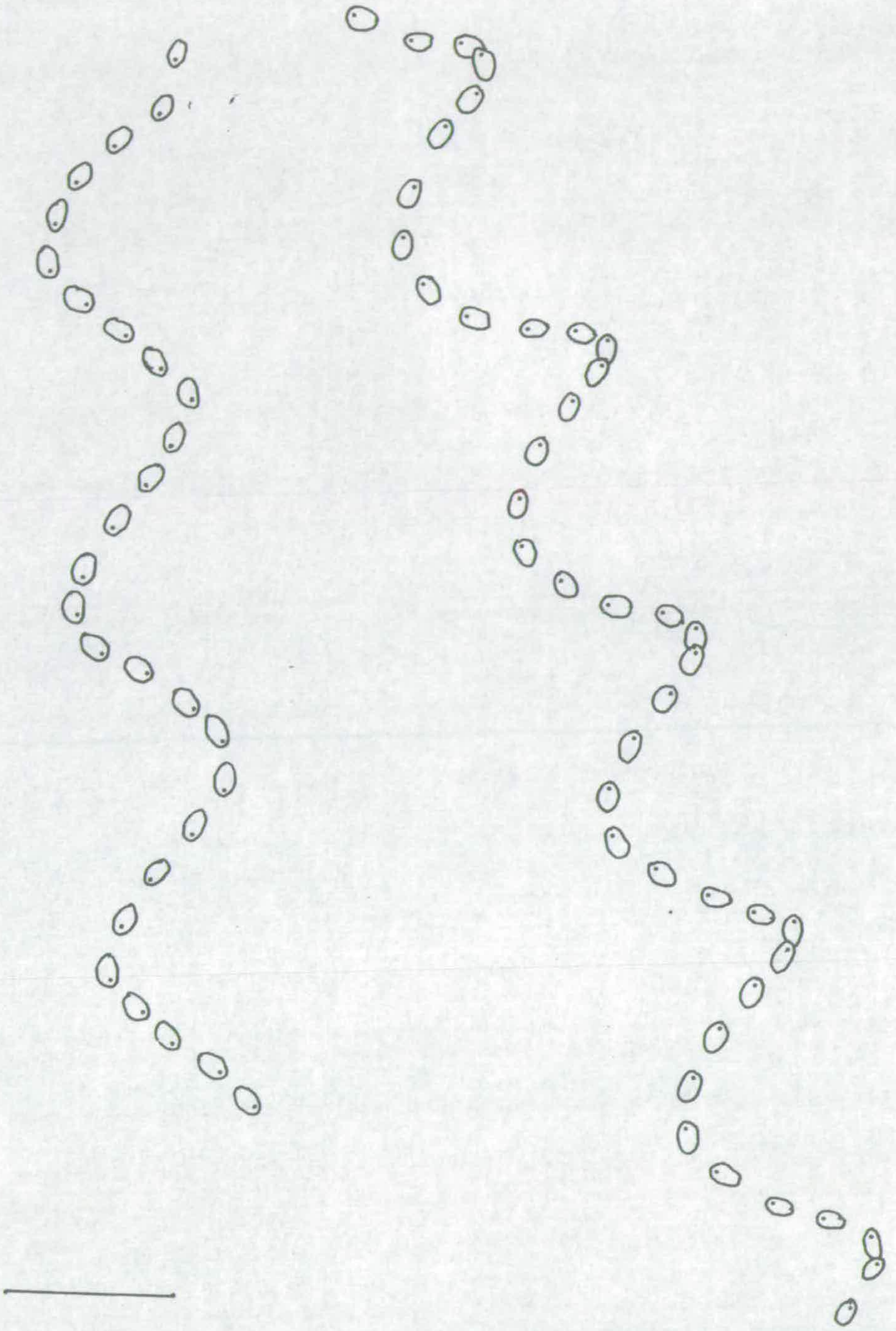


Figure 3.2 Representative tracings of single zoospores of *Pythium aphanidermatum* at 0.1 second intervals, exhibiting normal helical swimming in the mid depth of a zoospore suspension of distilled water background. The front of each spore is marked by a dot. (Bar = 50μm).

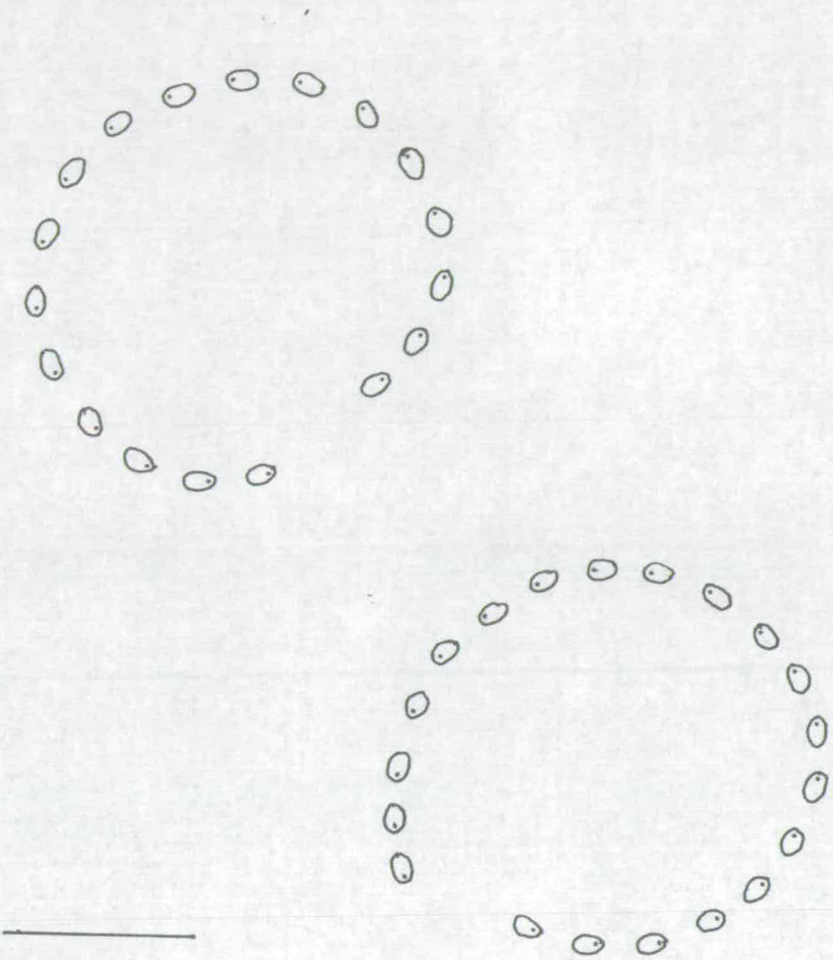


Figure 3.3 Representative tracings of single zoospores of *Pythium aphanidermatum* at 0.1 second intervals, exhibiting circular swimming at the top of a zoospore suspension of distilled water background. The front of each spore is marked by a dot. (Bar = 50μm).

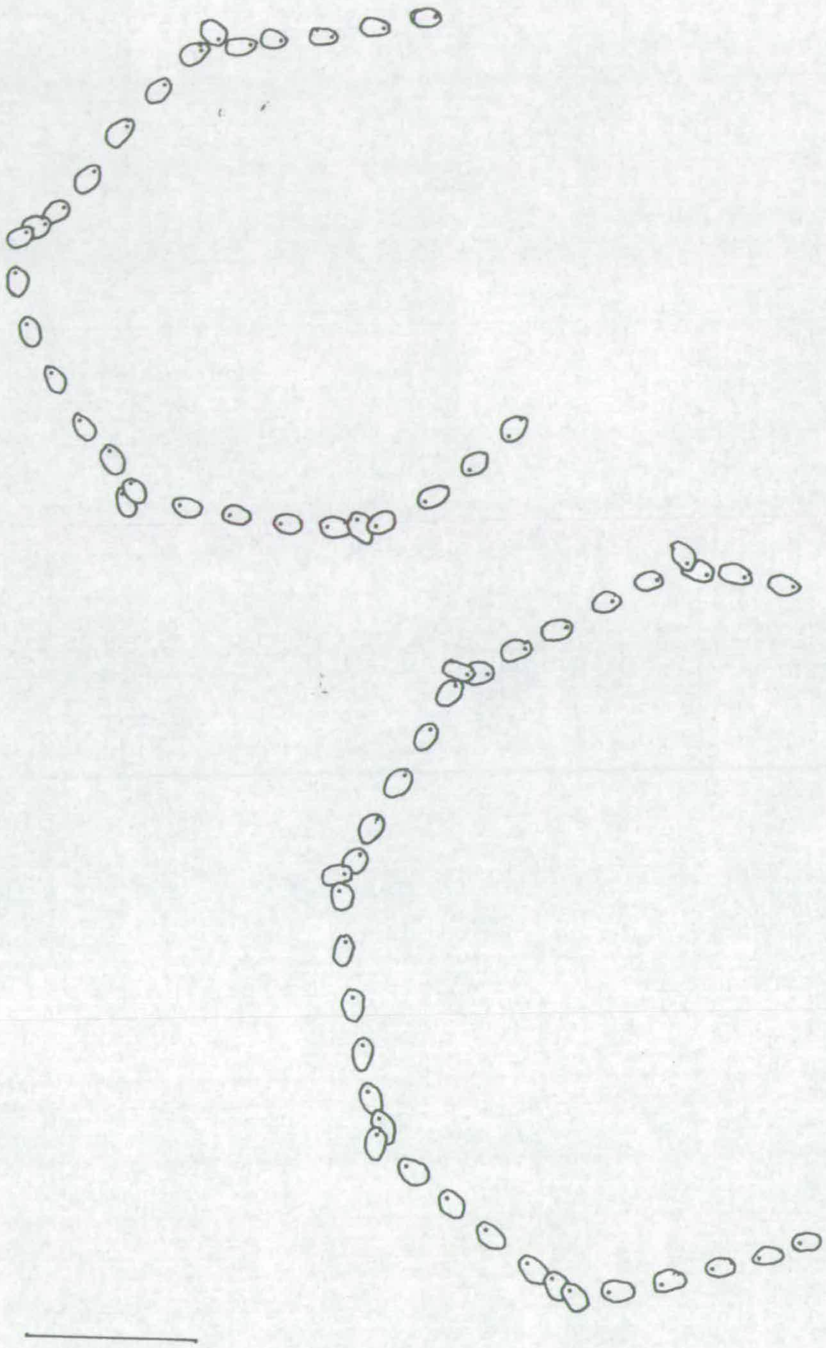


Figure 3.4 Representative tracings of single zoospores of *Pythium aphanidermatum* at 0.1 second intervals, exhibiting turns against the slide when swimming at the bottom of a zoospore suspension of distilled water background. The front of each spore is marked by a dot. (Bar = 50µm).

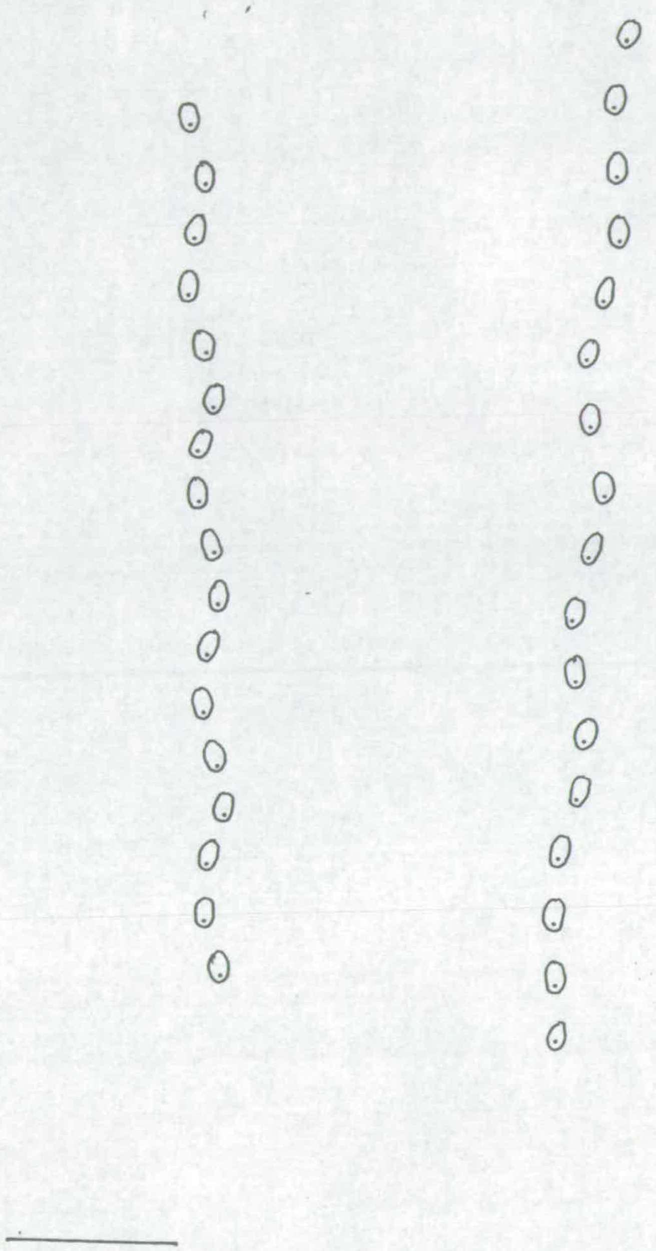


Figure 3.5 Representative tracings of single zoospores of *Pythium aphanidermatum* at 0.2 second intervals in the presence of 500μM EGTA showing straight swimming with a reduced amplitude. The front of each spore is marked by a dot. (Bar = 50μm).

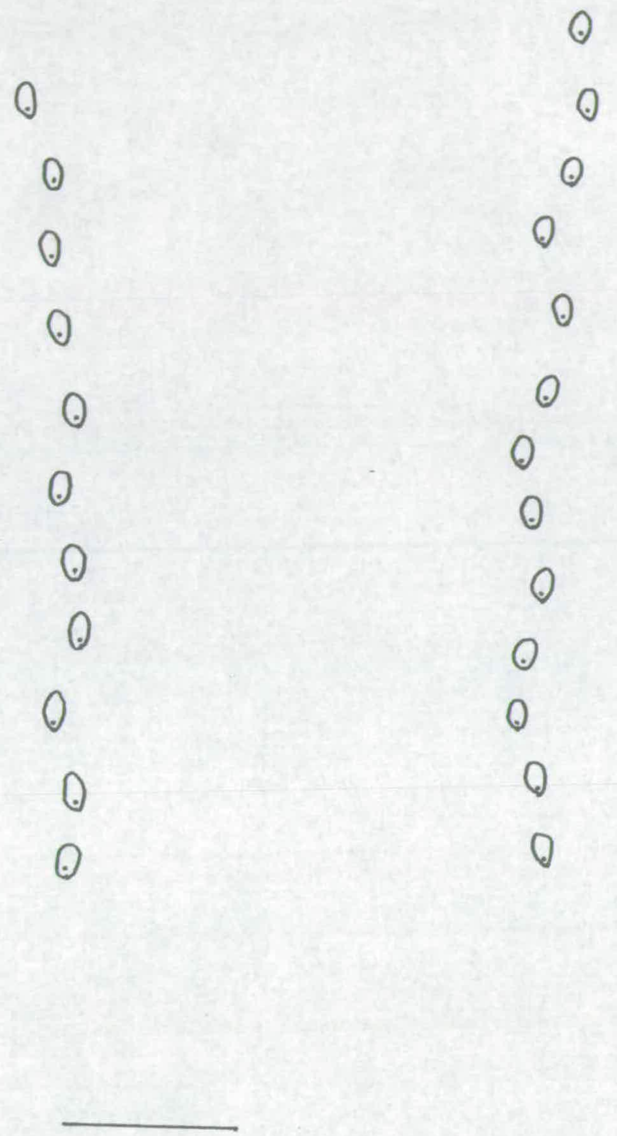


Figure 3.6 Representative tracings of single zoospores of *Pythium aphanidermatum* at 0.2 second intervals in the presence of 100μM BAPTA showing straight swimming with a reduced amplitude. The front of each spore is marked by a dot. (Bar = 50μm).

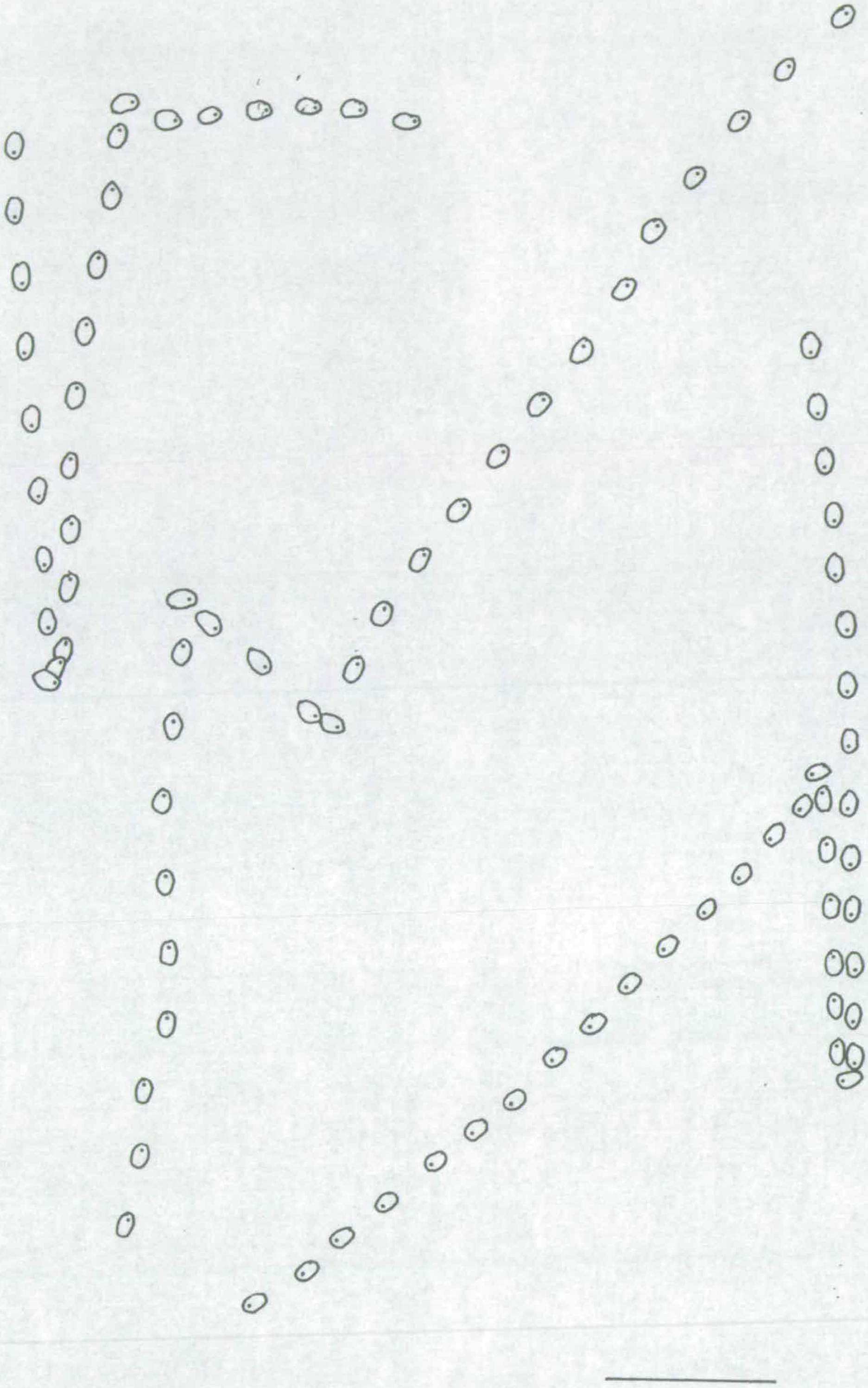


Figure 3.7 Representative tracings of single zoospores of *Phytophthora parasitica* at 0.2 second intervals showing helical swimming with random changes of direction in the mid depth of a zoospore suspension of phosphate buffer background. The front of each spore is marked by a dot. (Bar = 100μm).

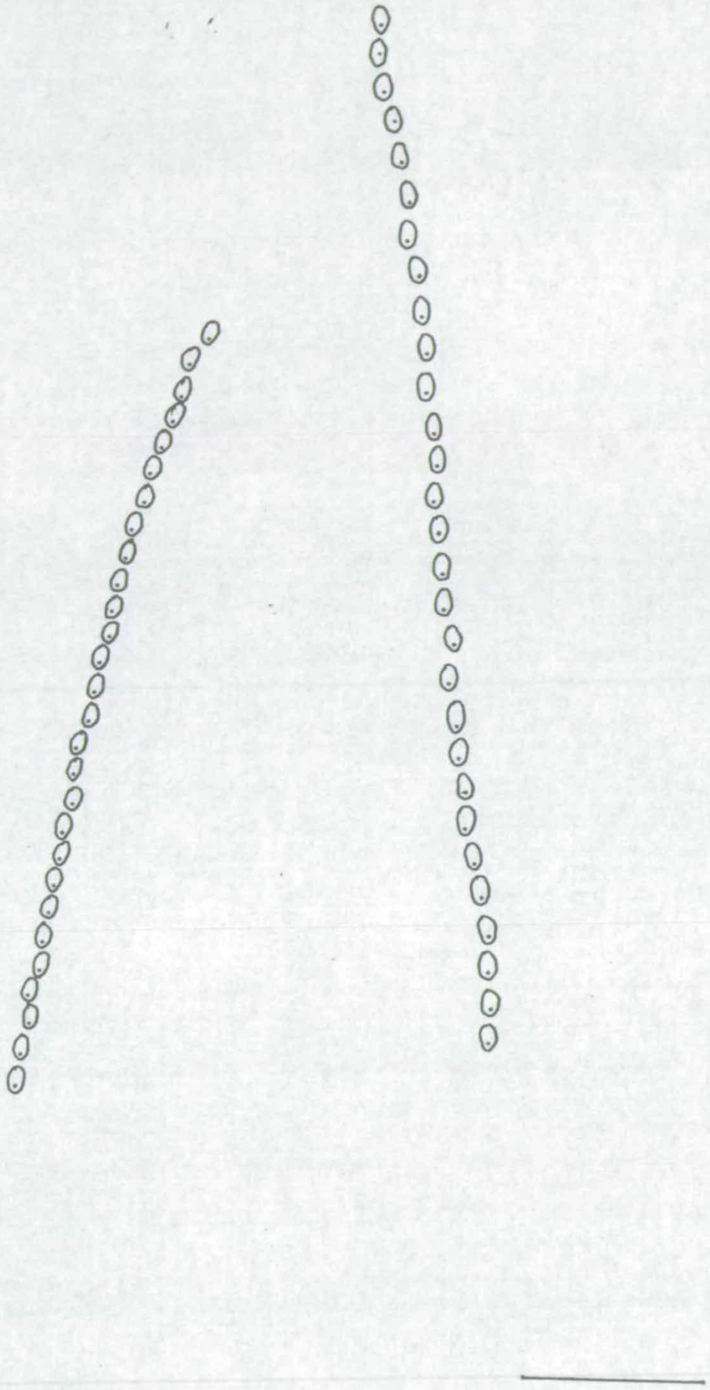


Figure 3.8 Representative tracings of single zoospores of *Phytophthora parasitica* at 0.2 second intervals in the presence of 500μM EGTA showing suppression of random changes of direction. The front of each spore is marked by a dot. (Bar = 100μm).

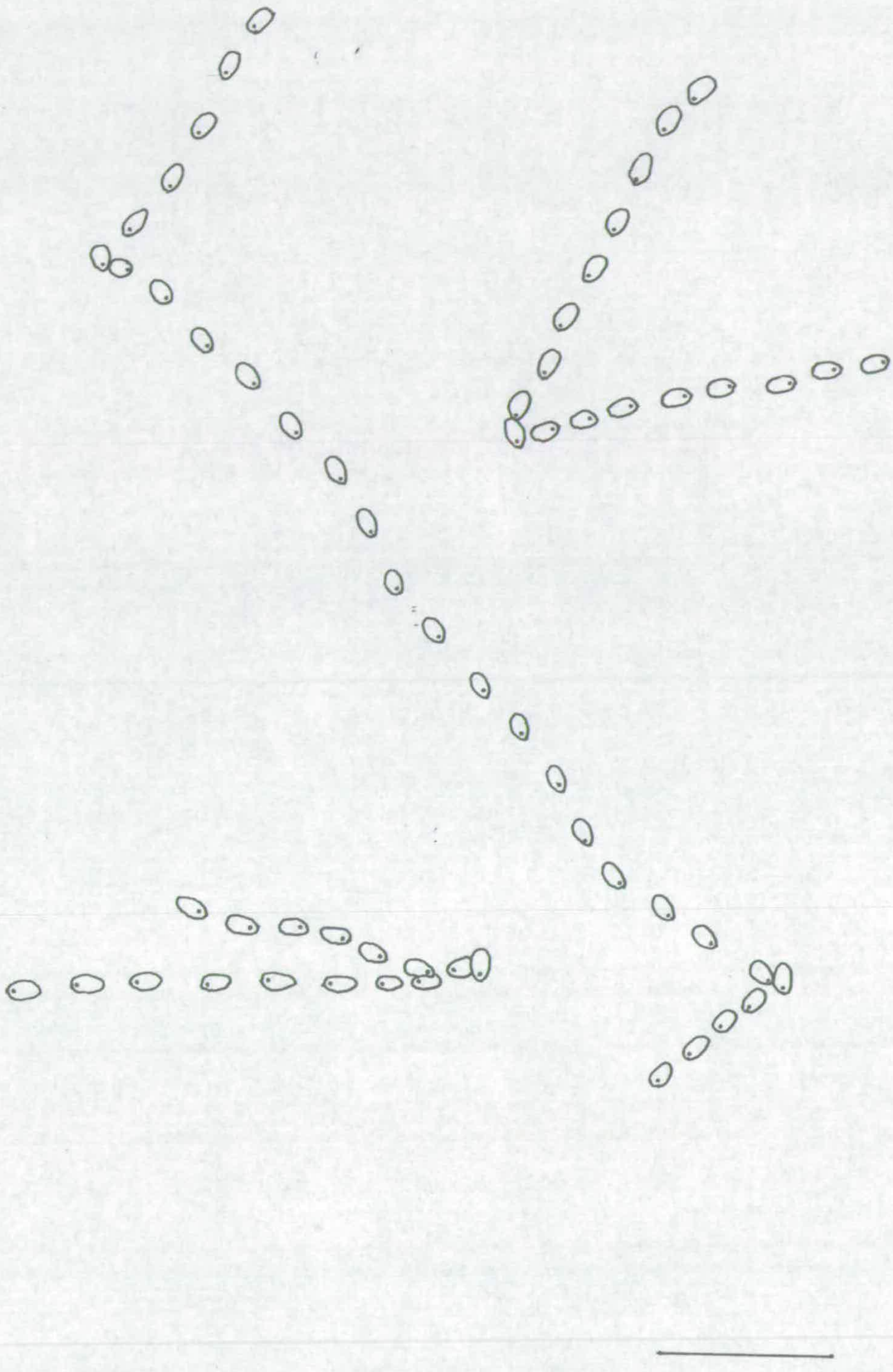


Figure 3.9 Representative tracings of single zoospores of *Phytophthora palmivora* at 0.2 second intervals showing helical swimming with random changes of direction in the mid depth of a zoospore suspension of phosphate buffer background. The front of each spore is marked by a dot. (Bar = 100μm).

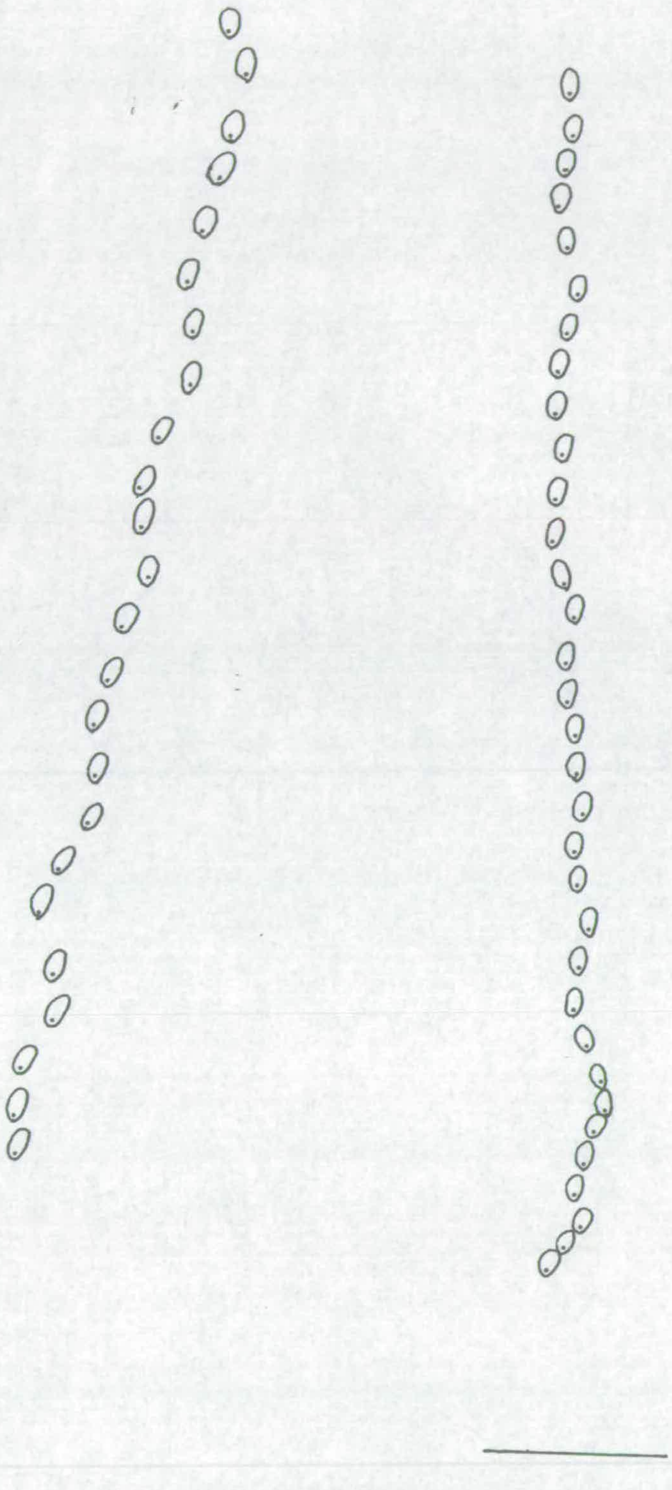


Figure 3.10 Representative tracings of single zoospores of *Phytophthora palmivora* at 0.2 second intervals in the presence of 250μM EGTA showing suppression of random changes of direction. The front of each spore is marked by a dot. (Bar = 100μm).

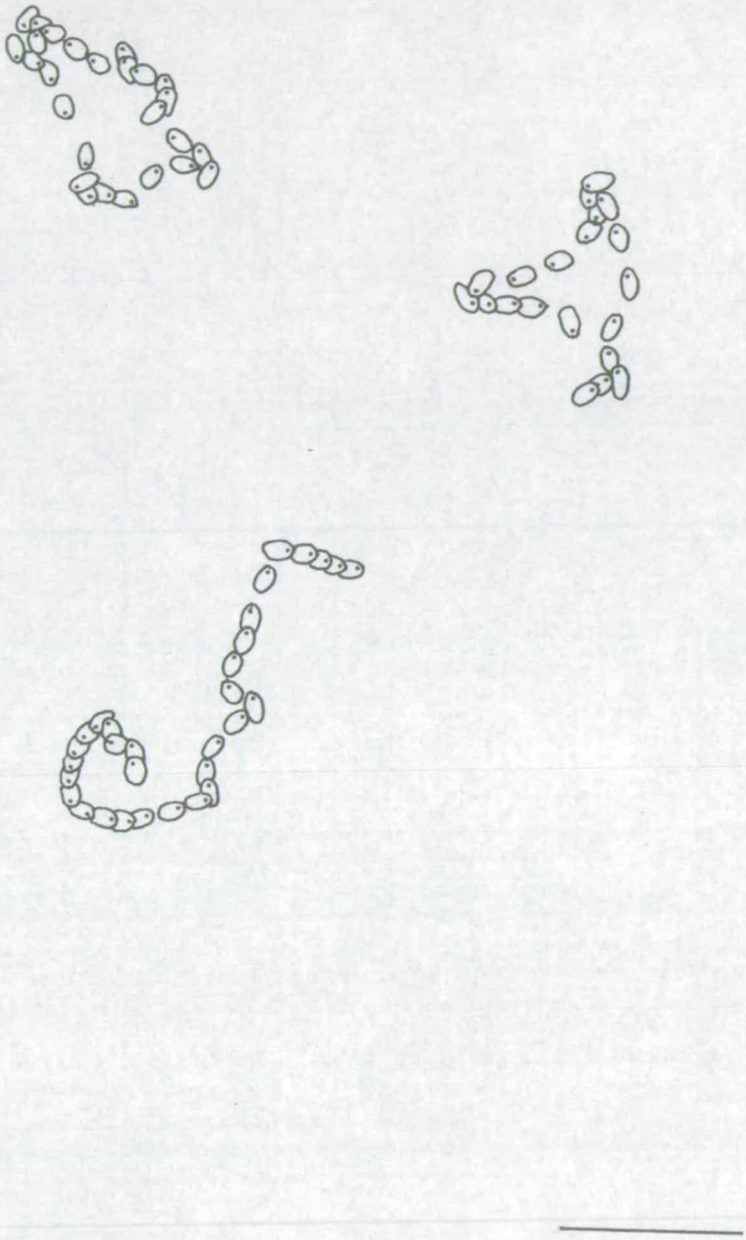


Figure 3.11 Representative tracings of single zoospores of *Phytophthora palmivora* at 0.2 second intervals in the presence of 15mM L-glutamic acid showing irregular, jerky motility in the mid depth of a zoospore suspension. The front of each spore is marked by a dot. (Bar = 50μm).

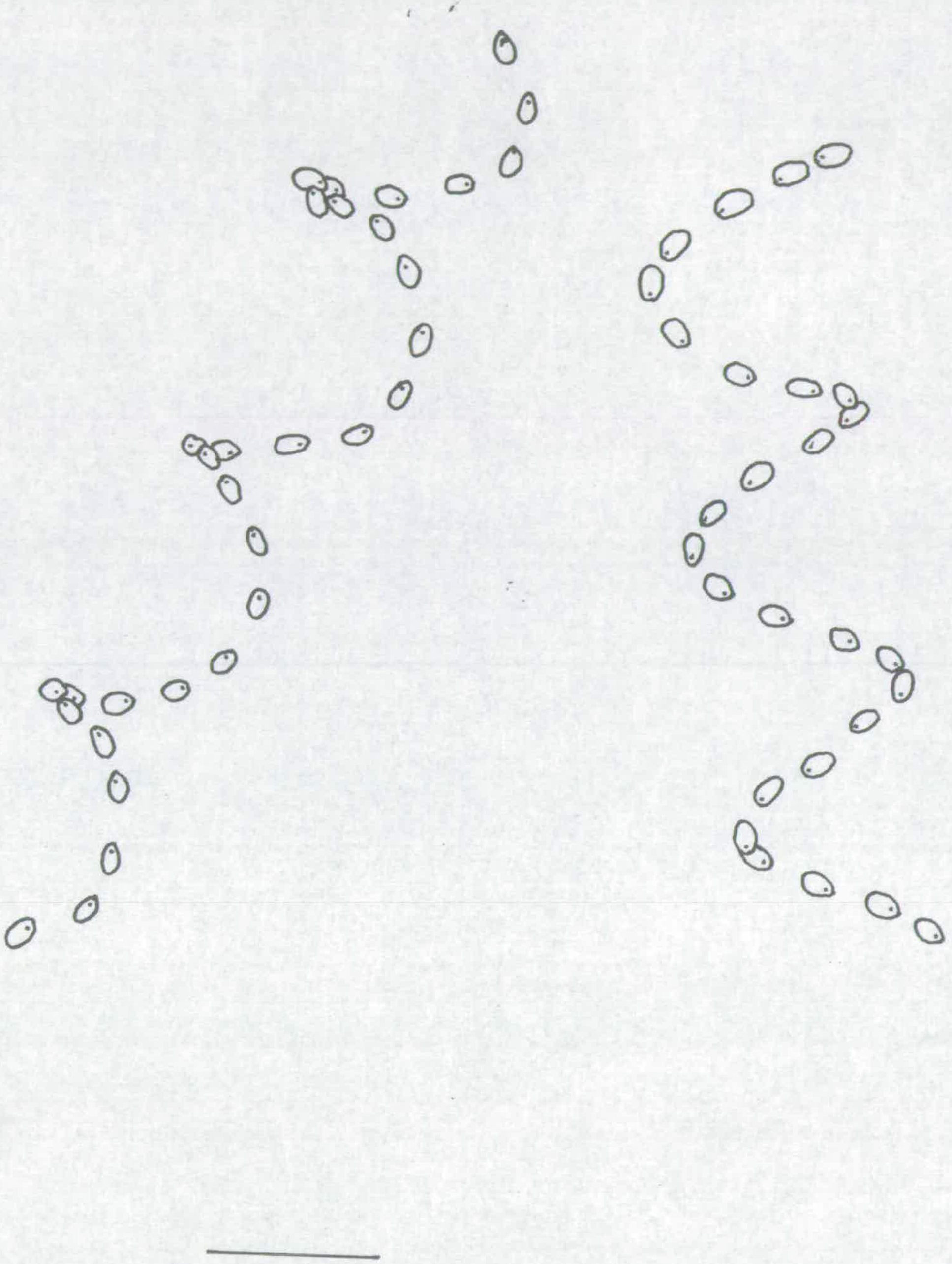


Figure 3.12 Representative tracings of single zoospores of *Pythium aphanidermatum* at 0.1 second intervals in the presence of 25mM L-alanine showing normal helical swimming in the mid depth of a zoospore suspension. The front of each spore is marked by a dot. (Bar = 50μm).

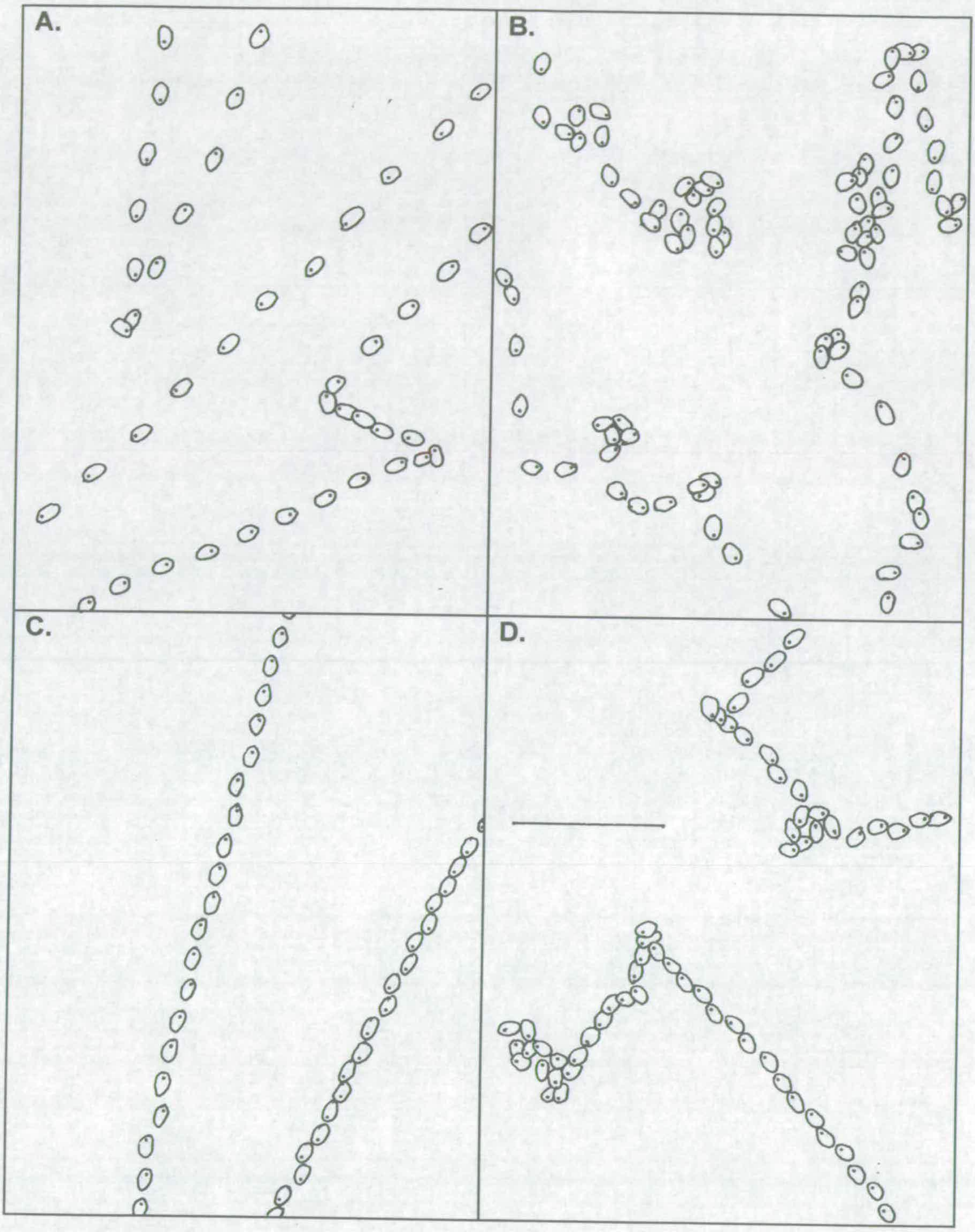


Figure 3.13 Representative tracings of single zoospores of *Phytophthora parasitica* at 0.2 second intervals exhibiting normal helical swimming with random changes of direction (A). Then after subsequent additions of: (B) 15mM L-asparagine, (C) 500μM EGTA and (D) 2.5mM Ca^{2+}. The front of each spore is marked by a dot. (Bar =100μm).

3.7 Geotaxis of fungal zoospores

Studies with *Phytophthora* spp. in an aqueous suspension have indicated that zoospores accumulate just below the surface (Ho & Hickman, 1967; Cameron & Carlile, 1977); it was concluded that this effect occurs due to negative geotaxis. Preliminary experiments here using *Ph. parasitica* and *Ph. infestans* indicated that zoospores from these species also tended to accumulate just below the surface of the zoospore suspension, but that *Py. aphanidermatum* zoospores did not do so. To examine this effect zoospore suspensions were diluted, if necessary, to 2×10^5 zoospores ml^{-1}. A 100μl aliquot of spore suspension was transferred to a glass cavity slide and after a period of 5 min in darkness the number of zoospores present at the surface of the suspension compared with the bottom of the suspension was assessed microscopically (x4 objective) by focusing at the top and bottom of the suspension respectively, and a short 30 sec period at each position was videotaped. During playback of the video sequences, the videotape was paused and the number of zoospores in a 10cmx10cm square drawn on the video monitor was counted. The mean number of zoospores at each position was then calculated from three replicate experiments.

It was found that zoospores of *Phytophthora* spp. predominated at the surface of the suspension whereas *Py. aphanidermatum* zoospores accumulated at the bottom of the suspension (Table 3.7). Addition of 500μM EGTA prevented *Phytophthora* zoospores from accumulating at the surface, and caused them to swim at the bottom of the suspension. Because the suspension was maintained in darkness for 5 min it is unlikely that phototaxis caused the observed distribution of zoospores within the suspension.

Table 3.7 Distribution of zoospores within a suspension and in the presence of 500μM EGTA.

	Mean[1] number of zoospores counted at the top and bottom of a zoospore suspension.	
Fungus	Top	Bottom
Ph. parasitica		
Control	79.0 ± 4.4	7.0 ± 0.6
EGTA	7.7 ± 0.7	85.0 ± 2.1
Ph. infestans		
Control	28.0 ± 2.0	3.7 ± 0.3
EGTA	4.7 ± 0.3	29.0 ± 1.0
Py. aphanidermatum		
Control	18.3 ± 3.5	89.0 ± 4.0
EGTA	3.3 ± 0.9	90.0 ± 4.2

[1] Data are means ± s.e. for three replicate experiments. Zoospores were added (100μl) to glass cavity slides and examined microscopically after 5 min. The number of zoospores at the top and bottom of the suspension was assessed by analysis of videotaped fields of view.

3.8 Discussion

Results in this chapter have revealed several new features of zoospore behaviour. Notably, it has been shown that, in the conditions of these experiments, zoospores of three *Pythium* spp. differ from those of three *Phytophthora* spp. and of *A. euteiches* because the *Pythium* spp. do not exhibit random turns unless they encounter an obstacle such as the surface of a glass slide. This is of interest because it has been assumed that the motility behaviour of all these fungi is broadly similar (Deacon & Donaldson, 1993). The zoospores of *Pythium* did, however, make abrupt changes of direction when they contacted a surface. So these zoospores must have a mechanosensory capacity, whereas the zoospores of the *Phytophthora* spp. studied here evidently have an endogenously regulated capacity for abrupt changes of direction in addition to a mechanosensory ability. The implications of these differences for site-selection behaviour of zoospores of the two genera merit further study.

It has also been shown that zoospores of *Phytophthora* spp. accumulate at the top of a liquid suspension, whereas zoospores of *Pythium* spp. accumulate near the bottom of a suspension - another difference between the two genera that could have significance in dispersal or site-location by zoospores. However, zoospores of the two genera behaved identically in the presence of EGTA, indicating that Ca^{2+}-mediated control of swimming behaviour could be essentially similar in all these fungi.

Another notable finding was that amino acids can influence motility attributes in a similar way for all the species examined, although the different species sometimes responded differently to the amino acids. For example, L-asparagine and L-glutamine caused irregular, jerky swimming with repeated changes of direction by zoospores of some species but had no effect on other species. Such differences might be interpreted in terms of the presence of different chemoreceptors on the zoospores of different species. But two points argue against this. First, *Ph. infestans* is not known to show chemotaxis to any of the tested amino acids (D. E. Grayson, personal communication) and yet it responded to some of them by a change in swimming pattern. Second, D-forms of some of the amino acids also elicited the effects whereas, at least for *Py. aphanidermatum*, D-amino acids do not elicit chemotaxis (Donaldson & Deacon, 1993b). Nevertheless, the findings here are of interest in possibly explaining a previous report that some D-amino acids can block the chemotactic responses of zoospores to the strong attractant, L-glutamic acid (Donaldson & Deacon, 1993b).

Finally, it has been shown here that zoospores can be subjected to a succession of different treatments which reverse the effects of previous treatments by making zoospores exhibit a characteristically different swimming mode. Some of these features are now discussed in relation to other studies on motility of microbial cells.

Previous studies on the locomotory attributes of zoospores of various *Pythium* (Donaldson & Deacon, 1993a) and *Phytophthora* (Allen & Newhook, 1974) species have revealed that the motile spores exhibit a corkscrew-like swimming pattern. Although the actual swimming speed and wavelength may differ between the two genera and even between species, the wave-like path in which the zoospores move is similar. Similarly, the results in this chapter demonstrate that *Ph. parasitica* and *Ph. infestans* zoospores also follow a helical path. *Pythium* and *Phytophthora* zoospores have been reported to undergo frequent, periodic, random changes of direction (Donaldson & Deacon, 1993a; Allen & Newhook, 1974). However, reanalysis of the motility pattern of three *Pythium* spp. indicates that *Py. aphanidermatum*, *Py. dissotocum* and *Py. catenulatum* do not undergo random changes of direction when swimming in the mid depth of a zoospore suspension away from any obstacles. This new finding was supported by inspecting S. P. Donaldson's original videotaped sequences of swimming behaviour of *Py. aphanidermatum*. It was observed that the zoospores were in fact behaving in an identical manner to that described in this chapter: zoospores swimming in the surface film moved in a circular manner, where zoospores showed the helical pattern in the mid depth of the suspension and made occasional turns when they collided with the glass slide at the bottom of the suspension. *Ph. palmivora*, *Ph. parasitica* and *Ph. infestans* zoospores, however, do make random turns.

Similarly, bacteria alternately swim smoothly and tumble; this results in random orientation for the next swim (Berg & Brown, 1972). When chemotaxis toward a favourable environment occurs, bacteria tend to swim smoothly; however, when swimming towards an unfavourable environment they tend to tumble. Swimming of bacteria occurs by a counterclockwise rotation of the flagella whereas tumbling is caused by a clockwise rotation (Larsen *et al.*, 1974). The mechanism by which fungal zoospores locate favourable environments and move away from unfavourable ones is largely unknown; however, it has been suggested by Deacon & Donaldson (1993) that zoospore taxis is analagous to the biased random walk of bacterial cells.

Several studies have implicated a role for Ca^{2+} in mediating the motile behaviour of zoospores. Ca^{2+} reportedly caused zoospores of *Achlya* to swim in straight paths, whereas K^+ caused slow, circular swimming and this effect could be reversed by subsequent addition of Ca^{2+} (Thomas & Butler, 1989). The initiation of trout sperm motility involves a circular swimming mode which can be induced by addition of Ca^{2+} or Mg^{2+} (Boitano & Omoto, 1992). Ersek *et al.* (1991) found that Li^+ caused immobilisation of *Ph. infestans* zoospores and that this could be counteracted with subsequent addition of Ca^{2+}.

Several findings indicate that the intracellular free $[Ca^{2+}]$ has an important role in the regulation of ciliary and flagellar activity. For example, Ca^{2+} has been found to control a reversal in wave propagation in the flagellated trypanasomid, *Crithidia* (Holwill & McGregor, 1975). Additionally, the reversal of ciliary beating seen in the avoidance response of *Paramecium* cells *in vivo* can be simulated by addition of external Ca^{2+} to cells that have been treated with Triton X-100 and reactivated with Mg^{2+} and ATP (Naitoh & Kaneko, 1972). Furthermore, Schmidt & Eckert (1976) provide good evidence that different flagellar beating patterns in *Chlamydomonas reinhardtii* are all governed by the intracellular Ca^{2+} concentration.

Donaldson & Deacon (1993a) showed that treatments which interfere with Ca^{2+}-mediated processes caused *Py. aphanidermatum* zoospores to swim in several distinct patterns. The calcium-chelating agent EGTA reduced the amplitude of the swimming helix and reduced the velocity at which zoospores moved; random changes of direction were also reported to be suppressed, but it can now be suggested that this was a suppression of mechanosensory responses rather than suppression of random changes of direction. Addition of Ca^{2+}, or other divalent cations, caused perpetual circular swimming. The Ca^{2+} ionophore A23187 and the Na^{+}/Ca^{2+} membrane flux inhibitor amiloride caused an irregular swimming pattern with repeated changes of direction and greatly reduced the motile speed. Similarly, A23187 has been shown to affect the motile behaviour of bacteria, causing *B. subtilis* to repeatedly tumble (Ordal, 1977). Both of these compounds were suggested to act by altering the internal to external Ca^{2+} ratio across the cell membrane, thereby having similar effects to each other on zoospore motility (Donaldson & Deacon, 1993a).

Other swimming perturbations were caused by the calmodulin antagonists dibucaine and trifluoperazine (TFP), which significantly reduced swimming speed and caused zoospores to swim in spirals with posterior drift, indicating an effect on the anterior flagellum (Donaldson & Deacon, 1993a). In contrast, TFP has been reported to completely arrest motility of *A. heterosexualis* (Thomas & Butler, 1989).

Results presented in this chapter show that, in addition to EGTA, BAPTA buffers can alter the motile behaviour of *Py. aphanidermatum*, causing the zoospores to swim in straighter paths by reducing the amplitude of the swimming helix and also reducing the swimming speed. Random changes of direction were not observed when zoospores of *Py. aphanidermatum* were moving in the mid depth of the suspension and so measurements on the ability of these compounds to suppress turning behaviour were not possible. However, turns which occur in response to collisions with the glass slide were completely suppressed, presumably resulting from override of mechanosensory perception. These calcium-chelating agents did, however, completely suppress rapid, random changes of direction by zoospores of the three *Phytophthora* spp. swimming in the mid depth of a zoospore suspension. It is possible that these chelating agents, alone or in combination with divalent cations, have toxic effects on cells (Youatt, 1994), although little evidence of toxicity was found in this study at appropriate concentrations that affected swimming patterns. The alternative explanation is that at least some external Ca^{2+} is necessary for normal flagellar functions and locomotory responses of the cells, and that the chelating agents affect swimming patterns and responses by removal of this external Ca^{2+}.

Several observations of zoospore swimming behaviour have suggested that the anterior flagellum, bearing tubular tripartate hairs, provides most of the thrust for forward swimming; the posterior flagellum is proposed to be responsible for changes in the direction of swimming (Holwill, 1982; Carlile, 1983; Cahill *et al.*, 1996). Morris *et al.* (1995) suggest, however, that the posterior flagellum is responsible for stabilising the zoospores during swimming rather than having a steering function. It is possible, therefore, that treatments which alter swimming speed or direction exert their effect upon the flagella. For instance, compounds such as EGTA, which reduce the swimming speed of zoospores, probably have an effect on the anterior flagellum. Indeed, it has recently been shown that monoclonal antibodies specific for tubular tripartate hairs on the anterior flagellum of *Ph. cinnamomi* caused zoospores to swim more slowly and also reduced the wavelength of the swimming helix; the amplitude of the swimming helix was unaffected (Cahill *et al.*, 1996).

The random changes of direction of *Phytophthora* zoospores and the "avoidance response" which occurs when *Pythium* and *Phytophthora* zoospores encounter mechanical obstructions, such as a glass slide, were overcome by reducing Ca^{2+} levels of the medium using Ca^{2+}-chelating agents (EGTA and BAPTA).

Many findings provide compelling evidence that membrane-regulated changes in the intracellular Ca^{2+} concentration control ciliary and flagellar activity (eg., Eckert, 1972; Naitoh & Kaneko, 1973; Holwill & McGregor, 1975; Schmidt & Eckert, 1976; Hyams & Borisy, 1978; Beck & Uhl, 1994; Tamm & Terasaki, 1994; Yoshimura, 1996; Yoshimura *et al.*, 1997). Evidently, Ca^{2+} has a major role in regulating zoospore motility. In studies involving zoospores, the effects of cations and other compounds in eliciting changes in motility have been explained in terms of membrane polarisation (Allen & Harvey, 1974; Cameron & Carlile, 1980). It is suggested that during chemotaxis to ethanol, the plasma membrane of *Ph. cinnamomi* zoospores hyperpolarises such that the depolarisation which accompanies flagellar re-orientation during random 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 on the cell surface. This in turn changes the transmembrane potential, altering flagellar 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 *Paramecium* (Eckert, 1972), *Chlamydomonas* (Schmidt & Eckert, 1976) and *Crithidia* (Holwill & McGregor, 1975). The central idea, well characterised for *Paramecium*, is that during the avoidance response the calcium influx produced by an increase in the Ca^{2+}-conductance of the surface membrane results in ciliary reversal (Eckert, 1972). Furthermore, divalent cations can also have direct effects on motility and sliding disintegration of demembranated macrocilia of the ctenophore *Beroe* (Tamm, 1989). The changes in motility of *Pythium* and *Phytophthora* zoospores seen upon addition of EGTA or BAPTA strongly suggest a specific role of Ca^{2+} (or other substitute divalent cations) in determining motility and turning behaviour in general.

Donaldson & Deacon (1993a) reported that EGTA prevented the chemotactic response of *Py. aphanidermatum* zoospores to L-glutamic acid. Although there seems to be a broad measure of agreement on the types of amino acids that commonly elicit taxis by *Pythium* and *Phytophthora* spp., different reports seldom give completely consistent results, even for the same species. For example, Royle & Hickman (1964b) and Jones *et al.* (1991), using different isolates of *Py. aphanidermatum*, found strong attraction of zoospores towards glutamate whereas Chang Ho & Hickman (1970), working with a different isolate, found only weak attraction to this amino acid. None of the three *Pythium* spp. tested showed attraction to any D-amino acid (Jones *et al.*, 1991). When isotropic backgrounds of amino acids, known to be attractive to zoospores of the *Py. aphanidermatum* isolate used in this study, were examined for their effects on the motile behaviour of *Py. aphanidermatum*, *Ph. palmivora*, *Ph. parasitica* and *Ph. infestans* zoospores, 15mM L-glutamic acid caused all fungi to swim in a jerky fashion and 15mM L-aspartic acid caused zoospores of *Py. aphanidermatum* and *Ph. infestans* to exhibit an irregular, jerky swimming pattern similar to the "milling" behaviour described by Royle & Hickman (1964a) when zoospores are close to attractive regions of roots. L-alanine had no obvious effect on motility of zoospores of any of the fungi tested, L-glutamine had no effect on *Py. aphanidermatum* zoospores but caused milling with *Ph. parasitica* zoospores and L-asparagine had no effect on *Ph. infestans* zoospore motility.

Where an amino acid had an effect on motility, the addition of EGTA overcame this and zoospores swam in the straight "EGTA mode" with complete suppression of random turning. In a similar test on *Ph. parasitica* zoospores, addition of L-asparagine caused irregular, jerky motility, EGTA overcame this leading to straight swimming and upon subsequent addition of Ca^{2+} the irregular, jerky motility re-occurred and the zoospores began to encyst within 2 minutes. These findings suggest a role for external Ca^{2+} is a prerequisite for the turning behaviour of zoospores in response to attractive compounds.

Cameron & Carlile (1977) and Ho & Hickman (1967) found that zoospores of *Phytophthora* spp. tended to accumulate at the surface of a zoospore suspension; it was concluded that this effect was not the result of phototaxis or aerotaxis, but was due to "negative geotaxis". The results presented in this chapter demonstrate that zoospores of *Ph. parasitica* and *Ph. infestans* also accumulate at the surface of a suspension but that those of *Py. aphanidermatum* accumulate at the bottom. Accumulation of zoospores at the surface of a suspension is thought to occur due to the shape of the zoospore soma and the asymmetrical position of the flagella, causing the zoospore to tilt upwards as it swims (Carlile, 1983). Whether small differences in the morphology or nature of flagellation of *Pythium* zoospores compared to those of *Phytophthora* are responsible for the accumulation of *Py. aphanidermatum* at the bottom of a suspension requires further study. As shown in this chapter, EGTA affected the swimming pattern and distribution of

Phytophthora zoospores within the zoospore suspension, and led to their accumulation at the bottom. It is possible, therefore, that the maintenance of a normal motility pattern is necessary for accumulation at the surface.

Royle & Hickman (1964a) described a sequence of events that occurs when *Py. aphanidermatum* zoospores respond to an attractive compound. After initial interruption of the original smooth, helical path, zoospores then reorientate towards the source of attractant by topochemotaxis. This is followed by trapping close to the source whereby the zoospore shows repeated changes in direction with a reduction in net linear displacement (phobochemotaxis). Reid *et al.* (1995) observed a similar sequence of events when *Ph. palmivora* zoospores responded to roots and to zoospore aggregates. In studies on electrotaxis, Morris & Gow (1993) found that the response of *Py. aphanidermatum* zoospores was toward the cathode, whereas *Ph. palmivora* zoospores moved toward the anode. Application of an electrical field to *Ph. palmivora* zoospores caused an increase in both the swimming speed and the frequency with which zoospores made turns, compared to spores swimming in the absence of an electrical field. However, there was no difference in the rate of turning when zoospores swam toward the anode compared to those swimming toward the cathode. In studies on the tactic response of *Ph. palmivora* zoospores to solutions of different pH, Morris *et al.* (1995) found that zoospores were attracted to buffers of pH 6.0 and repelled at pH 8.0; however, zoospores turned more frequently at pH 6.0 than at pH 8.0. Royle & Hickman (1964a) gave no mention of random turning behaviour when zoospores were swimming up a gradient of attractant or when swimming away from the attractive source; therefore it is difficult to draw analogies with the biased random walk of bacteria proposed for *Phytophthora* zoospores. However, the data presented here indicate that, unlike the zoospores of *Phytophthora*, *Pythium* zoospores do not make frequent, random changes of direction and so an alternative explanation must be sought for the mechanism of accumulation in response to attractant compounds.

Bacterial chemosensors are well characterised - especially those detecting amino acids (Mesibov & Adler, 1972). With regard to chemotaxis of zoospores it has been suggested that fungal spores have two or more spacially separated membrane 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 greater stimulation until the receptors are stimulated equally with respect to the longitudinal axis of the zoospore. The zoospore then turns directly towards the attractant source. An alternative hypothesis is that the zoospore senses a change in chemoattractant concentration by sensory adaptation to an existing level and can then respond to an increased level.

Increasing evidence indicates a strong relationship between transmembrane signalling in cilia and flagella and motility and taxis. To explain this relationship, Carlile (1980b) 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. A change in intracellular Ca^{2+} concentration may then alter the motor activity of cilia and flagella to bring about a change in direction and a tactic response. In the *Phytophthora* and *Pythium* zoospores examined it is likely that the stimuli eliciting taxis *in vivo* will include amino acids rather than Ca^{2+} *per se*. Ca^{2+} would then serve as part of a signal transduction sequence that links the binding of an attractant to an external receptor with changes in motility. Treatments such as EGTA, which alter the pattern of motility, may act by perturbing the normal Ca^{2+}-mediated process, thereby locking the zoospores into different perpetual swimming modes. This would account for the observation that spores in a perturbed swimming mode, due to calcium-chelating agents, failed to respond to attractive amino acids (Donaldson & Deacon, 1993a). It would also explain why the altered pattern of zoospore motility caused by addition of some amino acids was negated when EGTA was added subsequently.

CHAPTER 4

Effects of nutrients, cations and calcium modulators on germination of zoospore cysts

4.1 Introduction

Zoospores of *Ph. palmivora* are reported to release up to one third of their intracellular Ca^{2+} at an early stage during encystment (Irving *et al.*, 1984). They also begin to absorb exogenously supplied radiolabelled Ca^{2+} within the first few minutes of encystment (Irving *et al.*, 1984). A release of Ca^{2+} has been implicated in adhesion of newly encysting zoospores of *Phytophthora* (Gubler *et* al., 1989) and *Pythium* spp. (Donaldson & Deacon, 1992) because the normal adhesiveness of encysting zoospores is abolished by calcium-chelating compounds, whereas the normal loss of adhesiveness of spores that are kept away from a surface can be restored by addition of calcium. Donaldson & Deacon (1992) linked these effects to cyst germination, because naturally adhered zoospore cysts of *Pythium* germinated readily whereas non-adhered cysts germinated only when supplied with exogenous calcium supplements. Several studies have investigated the effects of Ca^{2+}-modulating treatments on zoospore encystment and germination (Irving *et al*, 1984; Grant *et al.*, 1986; Donaldson & Deacon, 1992; von Broembsen & Deacon, 1996; Deacon & Saxena, 1997b). All the findings from these studies have suggested a central role for Ca^{2+}, not only as a component of signal transduction but also as an ion that potentially is released and then reabsorbed during encystment and that might act as an autonomous cyst germination trigger (Donaldson & Deacon, 1992). In the experiments in this chapter, the effects of Ca^{2+}-modulating treatments, divalent cations and certain nutrients on germination of pre-encysted zoospores were studied, in particular to determine the specific time at which these compounds were most effective. In addition, certain ions and nutrients were tested for their ability to counteract the effects of Ca^{2+}-modulators on cyst germination. The aim was to further the work by Donaldson & Deacon (1992) for *Py. aphanidermatum* but also to extend it to another fungus, *Ph. parasitica.*

4.2 Effects of Ca^{2+}-modulator concentration on cyst germination of *Pythium aphanidermatum*

As described in Materials and Methods (section 2.8.1), zoospores of *Py. aphanidermatum* were vortexed for 70s to induce encystment, then transferred to glass slides immediately (t_0 series) or after being held in suspension in microcentrifuge tubes for 5 min (t_5 series). For controls, aliquots of the vortexed suspension (40µl) were added to an equal volume of sterile distilled water. In other cases the aliquots were added to an equal volume of Ca^{2+}-modulator treatment to give the final concentration shown in Table 4.1. Germination in all cases was assessed microscopically 1.5h after the cells were placed on the slides.

Owing to the large number of treatments, the experiment was performed over several days, but every treatment with a Ca^{2+}-modulator had a corresponding water control. Also, the replicates were sometimes performed on separate days, to accumulate the data for five replicates, but t_0 and corresponding t_5 treatments were always done in parallel. To ensure that this experimental design did not influence interpretation of the results, comparisons were made within each of the modulator treatments, by 2-way analysis of variance in which the replicates were treated as one factor in the analysis. At the end of each experiment, viability was determined using a live/dead staining test described in the Materials and Methods (section 2.8.1).

In controls, vortexed spores that were transferred immediately to drops of water on slides typically germinated to a level of over 50% after 1.5h incubation. A 5 min delay in transfer of the cells to the slides (t_5 treatment) did not significantly reduce germination compared with that for cysts transferred immediately (t_0).

Chelation of Ca^{2+} with BAPTA significantly lowered germination levels compared to those for cysts in the water controls (Table 4.1). This was true for both the t_0 and t_5 cysts: germination was reduced to 50% of that in controls by addition of BAPTA to a final concentration between 4mM and 5mM. As shown in Table 4.2, the highest concentration of BAPTA (5mM) marginally reduced the viability of cysts as evidenced by their staining properties, but only in the t_0 treatment, not in the t_5 treatment.

Lanthanum chloride, an inorganic inhibitor of plasma membrane Ca^{2+} channels, reduced germination of t_0 cysts to approximately half of that in controls at a concentration between 40μM and 60μM (Table 4.1). In contrast, it had much less effect on the t_5 cysts, only reducing their germination significantly at above 60μM concentration. Verapamil hydrochloride, an organic Ca^{2+} channel inhibitor, had less effect than La^{3+} in suppressing germination (Table 4.1). This effect was not significant overall ($P > 0.9$ by 2-way ANOVA) due to the high variance within the experiment but 80 and 100μM verapamil did significantly reduce germination of t_0 cysts when compared with that in the water controls by Student's t-test ($P = 0.02$ and 0.005 respectively). Verapamil at 100μM also significantly reduced germination of the t_5 cysts ($P = 0.01$). Staining reactions indicated that verapamil (100μM) did not reduce cyst viability compared with SDW treated controls, even when added to encysted spores at 0 min (Table 4.2).

TMB-8, an intracellular Ca^{2+} antagonist, significantly inhibited germination of *Py. aphanidermatum* cysts but only when the cysts were treated immediately after encystment and not when added to older (5 min) cysts. A concentration of 40μM TMB-8 reduced germination to about 50% of the level of water treated cysts (Table 4.1). TMB-8 (50μM) did not affect cell viability, evidenced by staining, compared with the water treated control (Table 4.2).

Caffeine, an organic compound which exerts its effect by reducing the level of Ca^{2+} stored within intracellular organelles, significantly suppressed germination of cells treated at t_0 or at t_5 (Table 4.1). It was effective at even 200μM concentration with t_0 cells, but a concentration of over 2.5mM was required significantly to reduce the germination of t_5 cells. Caffeine had a much greater effect on germination than on cyst viability as assessed by live/dead staining (Table 4.2); even at 5mM concentration it slightly, but significantly, reduced viability of the newly-formed cysts but not when added to cysts pre-incubated for 5 min.

Trifluoperazine (TFP), a calmodulin antagonist, significantly reduced germination of t_0 cysts at 20μM concentration or above (Table 4.1). Older (t_5) cysts were less sensitive, requiring 30μM TFP for strong inhibition of germination. Treatment of cysts with 20μM TFP reduced cyst viability compared with water treated controls (Table 4.2). Increasing the TFP concentration to 30μM reduced cell

Table 4.1 Effects of Ca^{2+}-modulators on percentage germination[*] of *Pythium aphanidermatum* when zoospores were encysted by agitation and transferred immediately (0 min) or after 5 min to slides containing the treatment or distilled water (control).

Treatment	**Time**	**Treatment final concentration**											
		Control	**0.5mM**	**1mM**	**2mM**	**3mM**	**4mM**	**5mM**	5% LSD				
BAPTA	0 min	56.3	42.7	46.7	43.0	42.3	32.0	9.0	4.0				
	5 min	46.0	47.3	44.7	45.0	44.0	36.3	13.3	3.6				
		Control	**5μM**	**10μM**	**20μM**	**40μM**	**60μM**	**100μM**	5% LSD				
La^{3+}	0 min	58.3	46.7	51.0	33.7	33.0	23.7	5.7	6.3				
	5 min	57.7	55.0	55.7	59.0	55.3	57.0	33.0					
		Control	**5μM**	**10μM**	**20μM**	**30μM**	**40μM**	**50μM**	**60μM**	**80μM**	**100μM**	(SED)	
Verapamil	0 min	62.4	50.4	43.4	42.0	39.6	51.4	53.4	50.0	45.2^+	32.0^+	(24.0)	
	5 min	52.0	52.2	52.4	57.0	48.0	49.2	45.4	48.2	44.2	39.0^+		

Table 4.1 continued.

		Control	10μM	20μM	30μM	40μM	50μM	5% LSD	(SED)
TMB-8	0 min	64.2	28.6	24.6	34.4	29.2	23.6	10.1	
	5 min	61.0	59.0	56.6	57.0	55.6	53.2		(13.5)

		Control	200μM	500μM	800μM	1mM	2.5mM	5mM	5% LSD
Caffeine	0 min	55.6	45.4	38.2	34.2	31.0	17.0	2.0	8.3
	5 min	48.0	52.4	48.8	49.4	39.8	37.6	6.6	11.5

		Control	1μM	2μM	3μM	5μM	10μM	20μM	30μM	5% LSD
TFP	0 min	77.6	77.8	78.4	78.0	72.0	65.8	10.2	2.6	32.2
	5 min	82.5	80.8	84.8	80.8	81.0	82.0	50.8	2.0	

[*] Data are means for 5 replicates, assessed after 1.5h incubation on slides, based on 100 spores in each replicate. Zoospores were encysted by agitation for 70 sec and then either added to glass slides immediately (0 min) or after being kept in microcentrifuge tubes for 5 min. In all cases, 40μl of cyst suspension was added to 40μl of water or treatment solution on a slide. The results were analysed initially by two-way ANOVA (time against treatments) to calculate 5% LSD, and then by one-way ANOVA if there was no significant interaction between treatments and times; SED is given where no significant difference was found by ANOVA. [+]Mean value differs significantly from the control by Student's t-test.

Table 4.2 Effects of Ca^{2+}-modulating treatments on the percentage of *Pythium aphanidermatum* cysts* remaining viable at 1.5h; investigated after the germination assay shown in Table 4.1. Viability was assessed by live/dead staining with Fluorescein diacetate (FDA) and propidium iodide.

Treatment	Time	Control	Treatment concentration		5% LSD (SED)
			5mM		
BAPTA	0 min	94.0	86.0		5.7
	5 min	91.4	93.0		(2.8)
			100μM		
La^{3+}	0 min	93.0	63.6		5.6
	5 min	93.2	88.0		(1.9)
			100μM		
Verapamil	0 min	93.0	94.4		(1.9)
	5 min	94.0	91.0		(2.3)
			50μM		
TMB-8	0 min	91.2	93.4		(2.2)
	5 min	93.4	90.8		(1.4)
			2.5mM	5mM	
Caffeine	0 min	91.2	93.0	78.4	4.4
	5 min	93.6	90.8	88.4	(1.6)
			20μM	30μM	
TFP	0 min	95.2	82.4	68.4	4.2
	5 min	95.6	84.8	87.0	2.6

*Data are means for 5 replicates, assessed immediately after recording percentage germination for Table 4.1, based on counts of 100 spores in each replicate. Viable cysts showed strong green fluorescence in the presence of FDA, whereas non-viable cysts stained red with propidium iodide. One-way analysis of variance was used to calculate 5% LSD; SED (in parentheses) is given where no significant difference was found.

viability still further, the effect being more pronounced for cysts treated immediately with the compound compared with older (5 min) cysts (Table 4.2).

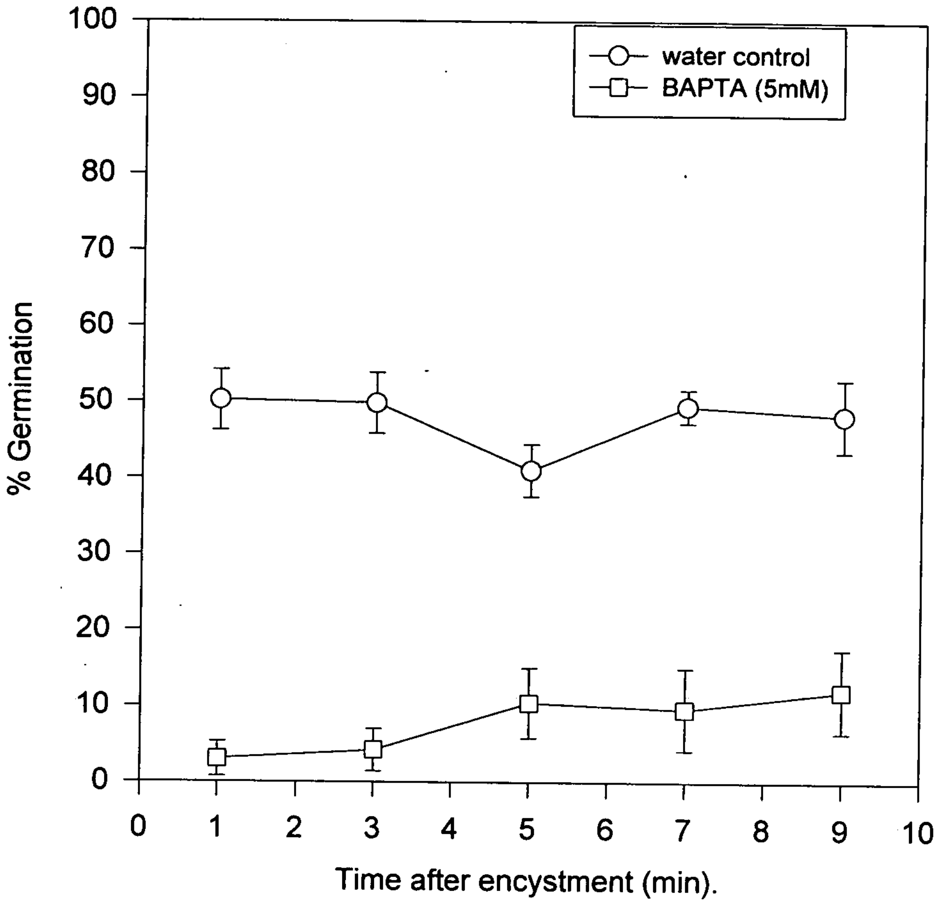
4.3 Effects of time of Ca^{2+}-modulator treatment on cyst germination of *Pythium aphanidermatum*

In separate experiments to those above, calcium modulating treatments, certain cations and an amino acid were tested for their effects on germination when applied at increasing times after encystment. Zoospores were encysted by agitation, maintained in suspension and transferred to the treatments on glass slides at 1 min or at 2 min intervals up to 9 min after vortex treatment.

BAPTA (5mM) markedly reduced germination levels below those of water controls even when added to cysts that were 9 min old (Figure 4.1). Treatment of cysts with 5mM BAPTA at 1 min after vortex encystment caused a significant reduction in cell viability, assessed by staining, compared with cysts treated with water (Table 4.3) but this effect was marginal compared with the strong effect on germination. BAPTA (5mM) did not cause a reduction in cell viability when the cysts had been left for 5 or 9 min before treatment (Table 4.3). Verapamil (100μM) reduced germination levels of 1 min old cysts only (Figure 4.2) but had no significant effect when added at 3 min or later, and it had no obvious effect on cell viability assessed by staining (Table 4.3). TMB-8 (30μM) had its most marked effect on cyst germination when the cysts were treated 1 min after encystment (Figure 4.3); it had lesser effect when cysts were left for over 5 min before treatment. TMB-8 (30μM) had no apparent effect on cyst viability, assessed by staining (Table 4.3). Caffeine (5mM) strongly inhibited germination when added to cysts at any stage, even when the cysts were 9 min old when treated (Figure 4.4). As assessed by staining, caffeine (5mM) significantly reduced viability of cysts treated 1 min after encystment, but had no effect on older cysts (Table 4.3).

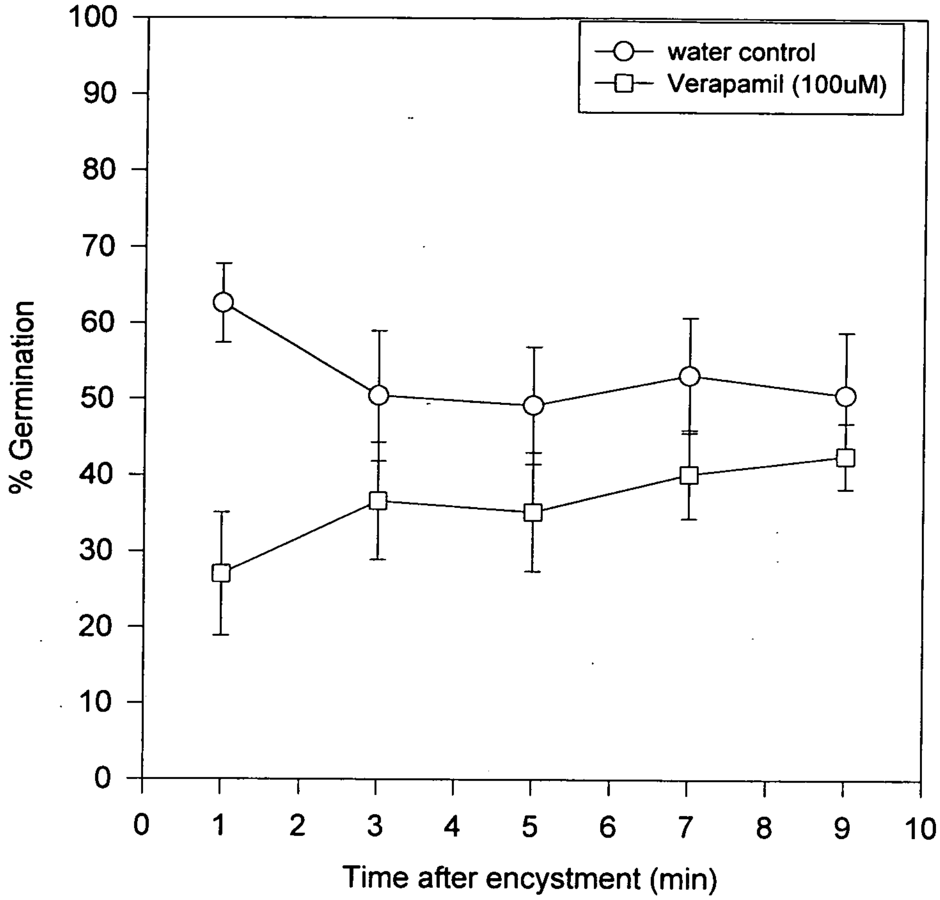
The amino acid L-asparagine (10mM) was similarly tested for its effect on germination of *Py. aphanidermatum* when added at various times after encystment (Figure 4.5). L-asparagine significantly elevated the germination level above that of water controls, irrespective of the time of treatment, and it did not reduce the

Figure 4.1 Effect of BAPTA on germination* of *Pythium aphanidermatum*, when zoospores were induced to encyst by agitation and transferred to glass slides with either BAPTA (5mM) or distilled water (control) at different times (1-9 min).



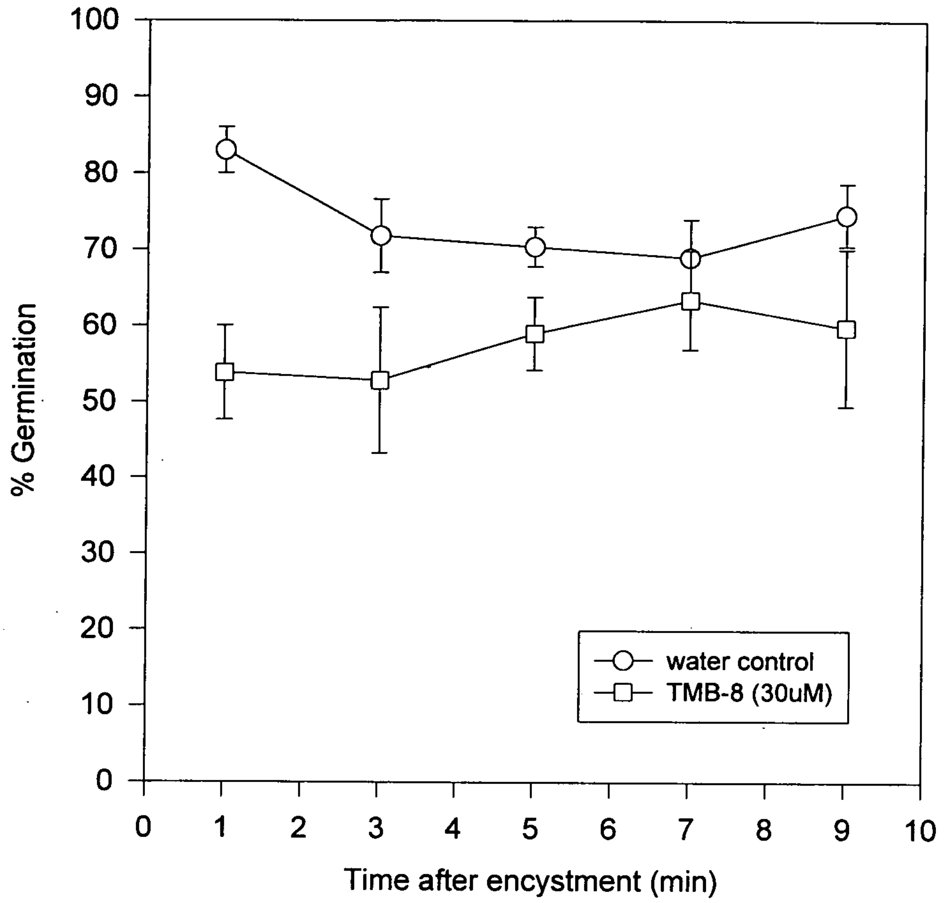
*Data are means ± s.e. for 5 replicates, assessed after 1.5h, based on counts of 100 spores for each replicate.

Figure 4.2 Effect of verapamil on germination* of *Pythium aphanidermatum*, when zoospores were induced to encyst by agitation and transferred to glass slides with either verapamil (100μM) or distilled water (control) at different times (1-9 min).



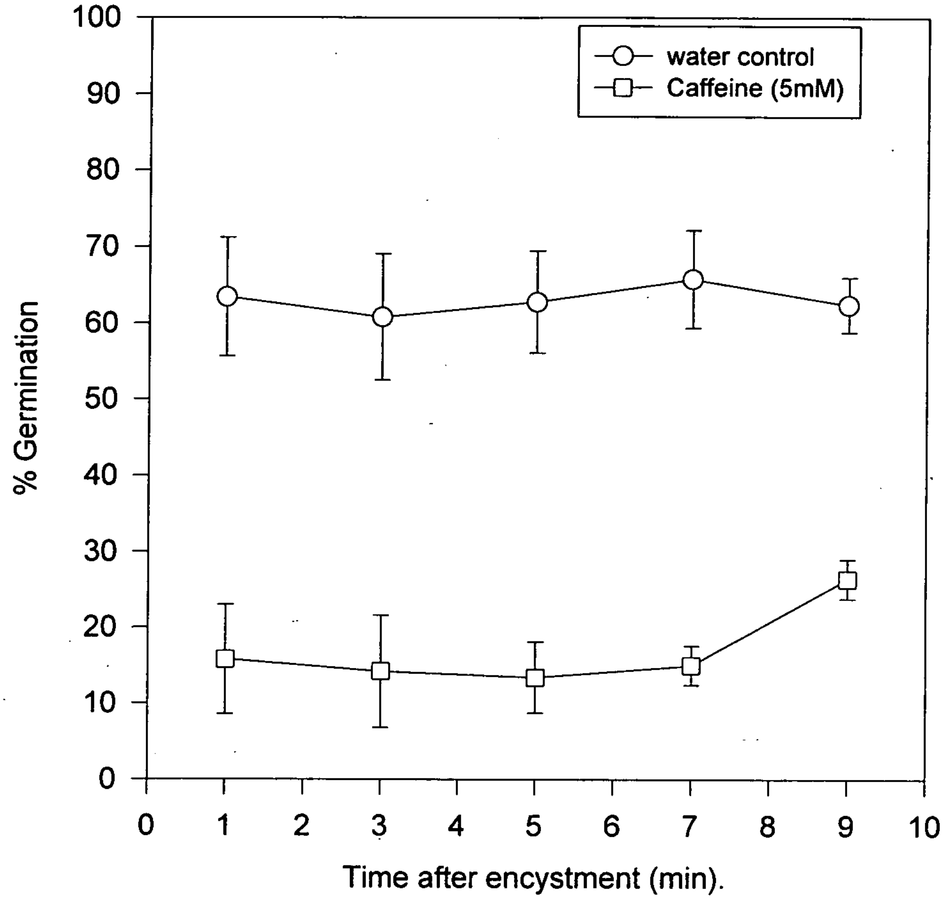
*Data are means ± s.e. for 5 replicates, assessed after 1.5h, based on counts of 100 spores for each replicate.

Figure 4.3 Effect of TMB-8 on germination* of *Pythium aphanidermatum*, when zoospores were induced to encyst by agitation and transferred to glass slides with either TMB-8 (30µM) or distilled water (control) at different times (1-9 min).



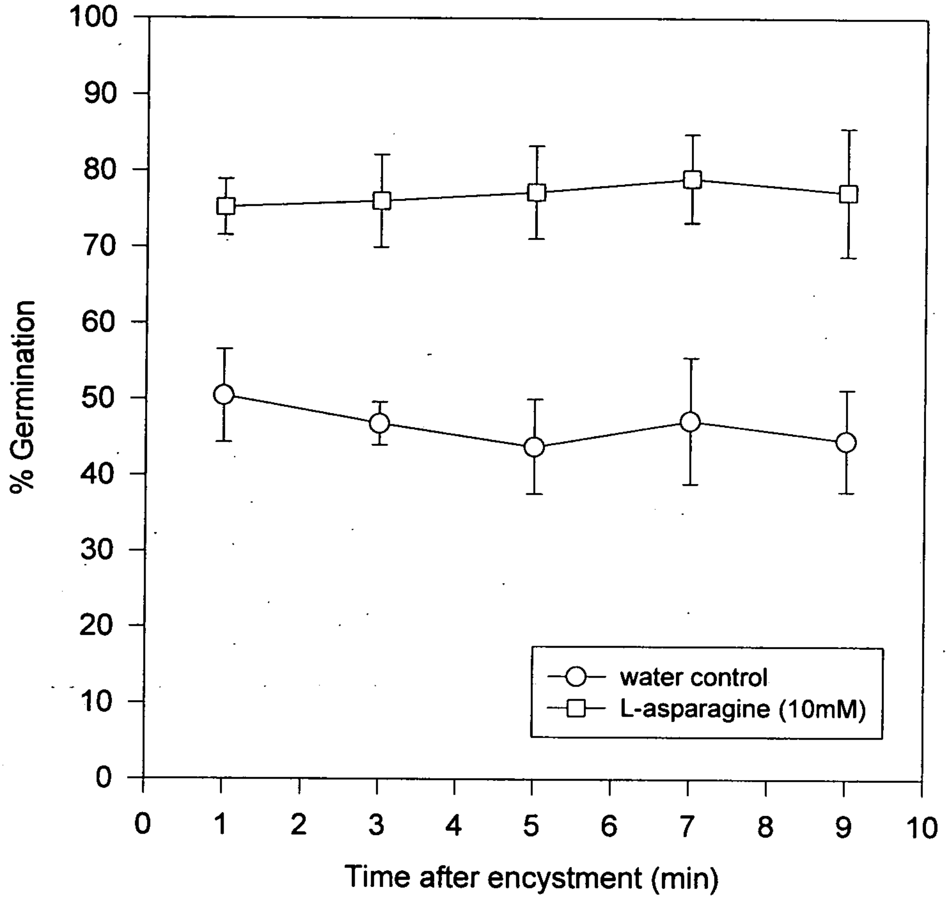
*Data are means ± s.e. for 5 replicates, assessed after 1.5h, based on counts of 100 spores for each replicate.

Figure 4.4 Effect of caffeine on germination* of *Pythium aphanidermatum*, when zoospores were induced to encyst by agitation and transferred to glass slides with either caffeine (5mM) or distilled water (control) at different times (1-9 min).



*Data are means ± s.e. for 5 replicates, assessed after 1.5h, based on counts of 100 spores for each replicate.

Figure 4.5 Effect of L-asparagine on germination* of *Pythium aphanidermatum*, when spores were induced to encyst by agitation and transferred to glass slides with either L-asparagine (10mM) or distilled water (control) at different times (1-9 min).



*Data are means ± s.e. for 5 replicates, assessed after 1.5h, based on counts of 100 spores for each replicate.

Table 4.3 Effects of calcium-modulators and L-asparagine on the percentage of *Pythium aphanidermatum* cysts remaining viable to 1.5h. Viability assessed by live/dead staining with fluorescein diacetate (FDA) and propidium iodide. (Means of 5 replicates, counts of 100 spores each).

Treatment	Time after encystment (min)			5% LSD (SED)
	1	5	9	
Control	93.8	92.4	94.2	3.0
BAPTA (5mM)	87.2	93.4	93.0	
Control	91.0		93.6	(2.9)
Verapamil (100μM)	93.0		91.0	
Control	93.0		95.6	(2.1)
TMB-8 (30μM)	95.8		94.6	
Control	93.0	90.8	94.4	6.4
Caffeine (5mM)	78.4	93.0	95.6	
Control	96.2		96.8	(1.5)
L-asparagine (10mM)	96.6		97.2	

Two-way analysis of variance was used to calculate 5% LSD; SED is given (in parentheses) where no significant difference was found with two-way ANOVA.

Table 4.4 Effects of amiloride (200μM) and TFP (10μM) on percentage germination* of *Pythium aphanidermatum*, when zoospores were induced to encyst by agitation and transferred to glass slides with either the Ca^{2+}-modulator treatment or distilled water (control) at different times (1-9 min).

Time (min)	Water	Amiloride	Water	TFP
1	61.0 ± 9.7	55.0 ± 11.0	68.0 ± 9.0	52.6 ± 10.3
3	61.2 ± 7.7	54.2 ± 10.1	66.6 ± 10.1	73.0 ± 6.1
5	52.8 ± 10.9	48.2 ± 11.0	66.4 ± 10.5	71.8 ± 9.2
7	55.2 ± 10.5	45.4 ± 9.2	63.6 ± 7.4	74.6 ± 7.4
9	54.8 ± 11.0	51.4 ± 10.0	60.2 ± 10.4	71.6 ± 8.7

*Data are means ± s.e. for 5 replicates, assessed after 1.5h, based on counts of 100 spores for each replicate.

Table 4.5 Effects of divalent cations (10mM) on percentage germination* of *Pythium aphanidermatum*, when spores were induced to encyst by agitation and transferred to glass slides with either the cation or distilled water (control) at different times after encystment (1-9 min).

Time (min)	**Water**	**Mg^{2+}**
1	58.2 ± 8.1	67.0 ± 1.7
3	52.8 ± 6.2	60.2 ± 5.6
5	45.6 ± 6.0	57.6 ± 5.2
7	52.2 ± 7.1	52.6 ± 6.9
9	53.0 ± 8.2	59.6 ± 8.0

Time (min)	**Water**	**Sr^{2+}**
1	81.0 ± 4.9	70.2 ± 2.1
3	72.0 ± 3.5	71.2 ± 4.1
5	76.0 ± 1.6	72.0 ± 4.9
7	75.6 ± 4.0	75.4 ± 3.1
9	72.4 ± 1.7	74.8 ± 2.6

Time (min)	**Water**	**Ba^{2+}**
1	64.4 ± 4.8	0.6 ± 0.6
3	59.6 ± 3.9	1.5 ± 0.9
5	63.6 ± 6.4	0.8 ± 0.6
7	64.2 ± 3.8	0.8 ± 0.4
9	64.0 ± 3.9	0.4 ± 0.2

*Data are means ± s.e. for 5 replicates, assessed after 1.5h, based on counts of 100 spores for each replicate.

number of viable cysts below that in the control (Table 4.3). The calmodulin antagonist, TFP (10μM) and the membrane ion flux inhibitor amiloride (200μM) had no effect on germination of either newly formed cysts or older cysts (Table 4.4) and had no apparent effect on cyst viability (not shown).

Of the 3 cations tested (Mg^{2+}, Sr^{2+} and Ba^{2+}), 10mM Ba^{2+} had the greatest effect on cyst germination compared to that in controls; in all cases, Ba^{2+} significantly reduced germination, whether the cysts were treated 1 min or up to 9 min after encystment (Table 4.5); however, extensive cell lysis was evident when 1- and 3- min-old cysts were treated with Ba^{2+} (10mM). Sr^{2+} only reduced germination below control levels when added to 1 min old cysts; Mg^{2+} had no effect when added at any time.

In a further experiment *Py. aphanidermatum* cysts were transferred to slides with treatment solutions 1 min after encystment. BAPTA, TMB-8 and Ba^{2+} were used at a range of concentrations for comparison with other experiments and Br-A23187 and Ca^{2+} were included to test their effects on cyst germination (Table 4.6). Previously, Ba^{2+} at 10mM was shown to inhibit cyst germination (Table 4.5). Findings from this experiment indicate that the effects of Ba^{2+} are dose-dependent, a concentration between 2.5mM and 5mM being required to reduce germination levels to 50% of that of the water control. TMB-8 acted in a fashion similar to when it was used previously (Table 4.1), by tending to reduce germination of cysts at 50μM although it did not attain significance.

Table 4.6 Effect of Ca^{2+}-modulator concentrations on percentage germination[*] of *Pythium aphanidermatum* when zoospores were encysted by agitation and transferred after 1 min to glass slides with treatment solutions or distilled water as a control.

Treatment	Treatment final concentration							5% LSD	1% LSD	0.1% LSD	SED
Ba^{2+}	500μM	1mM	2.5mM	5mM	10mM						
	63.6	64.8	49.4	14.6	4.4			12.5	16.8	22.3	
TMB-8	10μM	20μM	30μM	50μM	100μM						
	71.4	68.0	70.6	52.2	46.0[+]						10.7
BAPTA	500μM	1mM	2.5mM	5mM							
	68.4	69.0	67.8	2.0				17.6	23.9	32.0	
Ca^{2+}	500μM	1mM	2mM	4mM	6mM	8mM	10mM				
	64.8	66.2	70.2	70.2	70.8	64.0	62.8				10.2
Br-A23187	1μM	2.5μM	5μM	7.5μM	10μM						
	70.0	70.0	67.6	71.2	68.0						6.3
Water (control)	68.3										

[*] Data are means for 5 replicates (each performed on a different day), assessed after 1.5h, based on counts of 100 spores for each replicate. Data were analysed by 1-way ANOVA with the water treatment as the experimental control; SED is given where there was no significant difference.

[+] Paired samples t-test showed no significant difference between this treatment and the water control.

BAPTA at 5mM concentration almost completely suppressed germination (Table 4.6) but BAPTA had no effect at 2.5mM concentration.

The addition of up to 10mM Ca^{2+} had no effect on germination when added to *Py. aphanidermatum* cysts 1 min after encystment (Table 4.6). The calcium ionophore, Br-A23187 (1μM-10μM), also had no effect on germination (Table 4.6).

4.4 Effects of Ca^{2+}-modulator concentrations on cyst germination of *Phytophthora parasitica*

Ph. parasitica was used in a similar range of experiments to those for *Py. aphanidermatum* above.

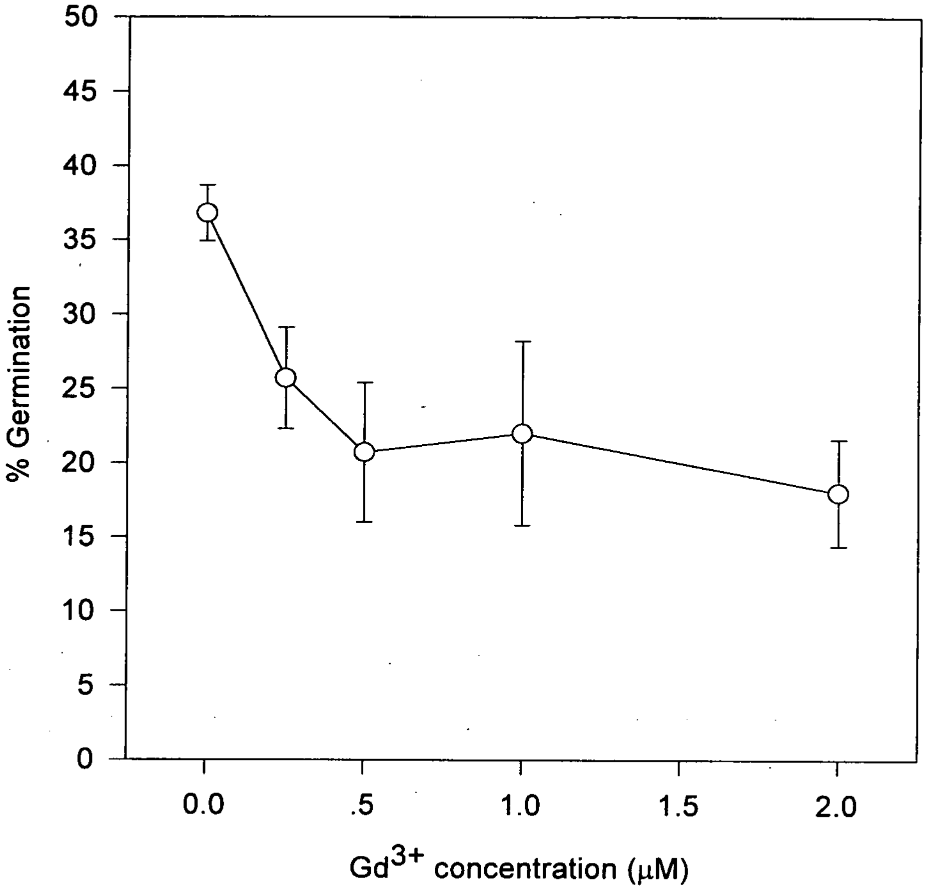
Motile zoospores of *Phytophthora parasitica* were incubated in microcentrifuge tubes for 1 min in the presence of various treatments, then the zoospore suspension was vortexed for 70s to encyst the zoospores. After vortexing, the cyst suspension was incubated in the microcentrifuge tubes for 1.5h, before aliquots of cyst suspension were transferred to glass slides and percentage germination was recordeded microscopically.

In water controls, *Ph. parasitica* cysts typically germinated to a level of between 35 and 40%. Gd^{3+} significantly reduced cyst germination at concentrations as low as 250nM (Figure 4.6). Higher Gd^{3+} concentrations caused further reduction of germination, to about half the control level at 2μM Gd^{3+}. Even at this concentration there was no apparent effect on cyst viability, as assessed by staining (Table 4.7). However, Gd^{3+} at 4μM concentration caused many cells to lyse (not shown). La^{3+} caused significant reduction of germination at 1μM concentration, and further reductions at concentrations up to 2μM (Figure 4.7) but had no effect on cell viability at this concentration (Table 4.7). The organic Ca^{2+} channel antagonist, verapamil, significantly reduced germination at 500nM, and more than halved the level of germination at a verapamil concentration of 10μM, compared with the water control (Figure 4.8). Verapamil at 10μM had no effect on cell viability (Table 4.7).

The intracellular Ca^{2+} antagonist TMB-8 reduced germination to below 50% of that in the control at concentrations of 15μM or more, and had a significant effect at even 2.5μM (Figure 4.9); it had no apparent effect on cyst viability at 15μM concentration but did reduce the apparent cyst viability at 25μM concentration (Table 4.7). Caffeine also reduced germination to below 50% of the control level at 1.25mM concentration (Figure 4.10) but had no apparent effect on cyst viability at this level (Table 4.7).

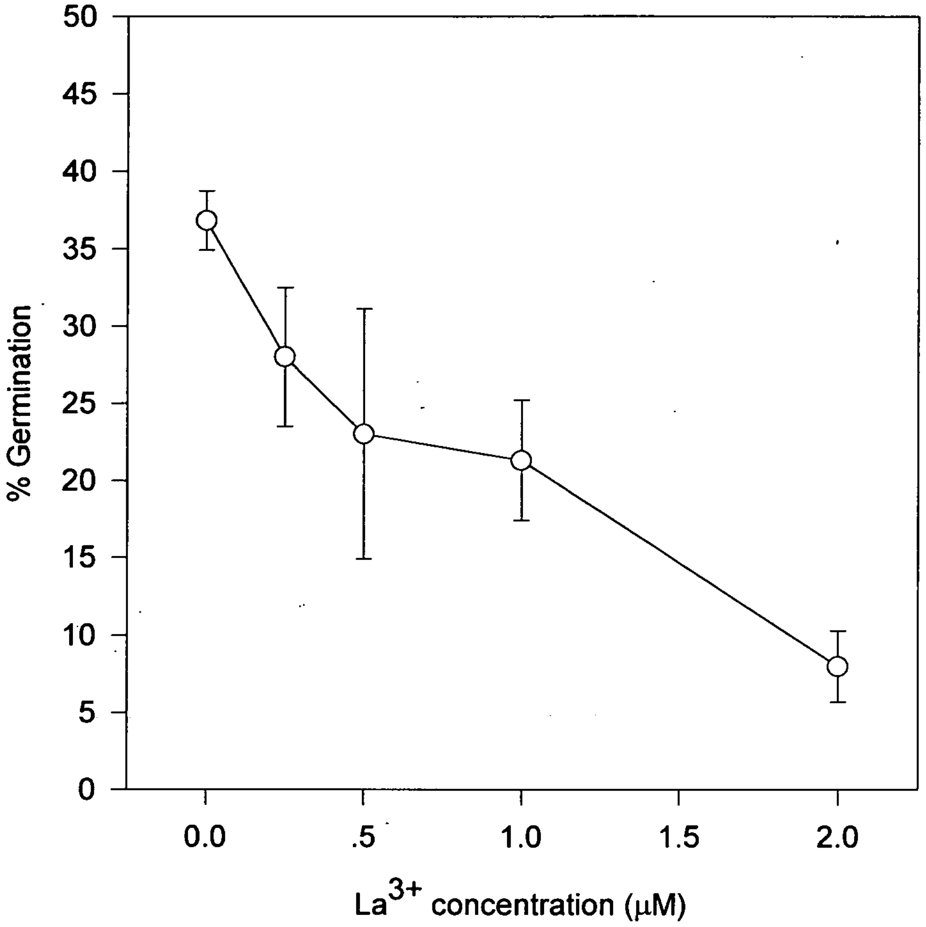
The calmodulin antagonist TFP reduced germination to almost 50% of that in the control at a concentration of 2.5μM (Figure 4.11) with no apparent effect on cyst viability (Table 4.7); suppression was also seen with only 250nM TFP.

Figure 4.6 Effect of Gd^{3+} concentration on germination* of *Phytophthora parasitica*, when spores were induced to encyst by agitation in the presence of Gd^{3+} or water (control) and then maintained in suspension for 1.5h.



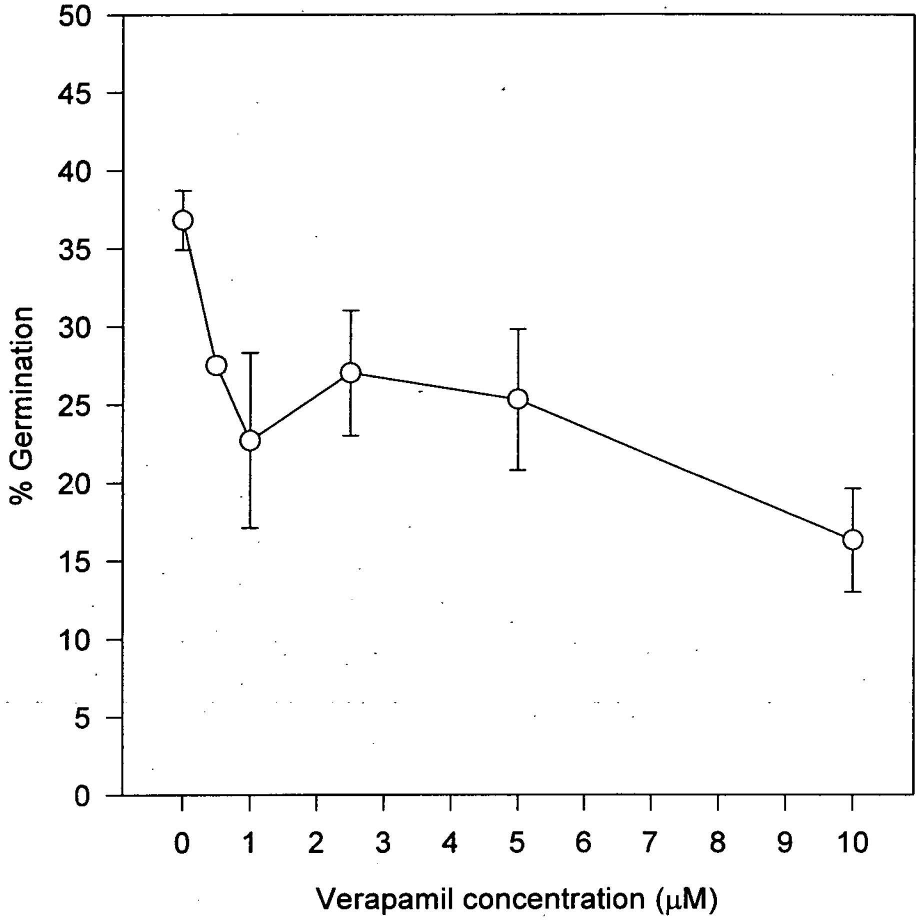
*Means ± s.e. for 3 replicates, assessed after 1.5h, based on counts of 100 spores for each replicate.

Figure 4.7 Effect of La^{3+} concentration on germination* of *Phytophthora parasitica*, when spores were induced to encyst by agitation in the presence of La^{3+} or water (control) and then maintained in suspension for 1.5h.



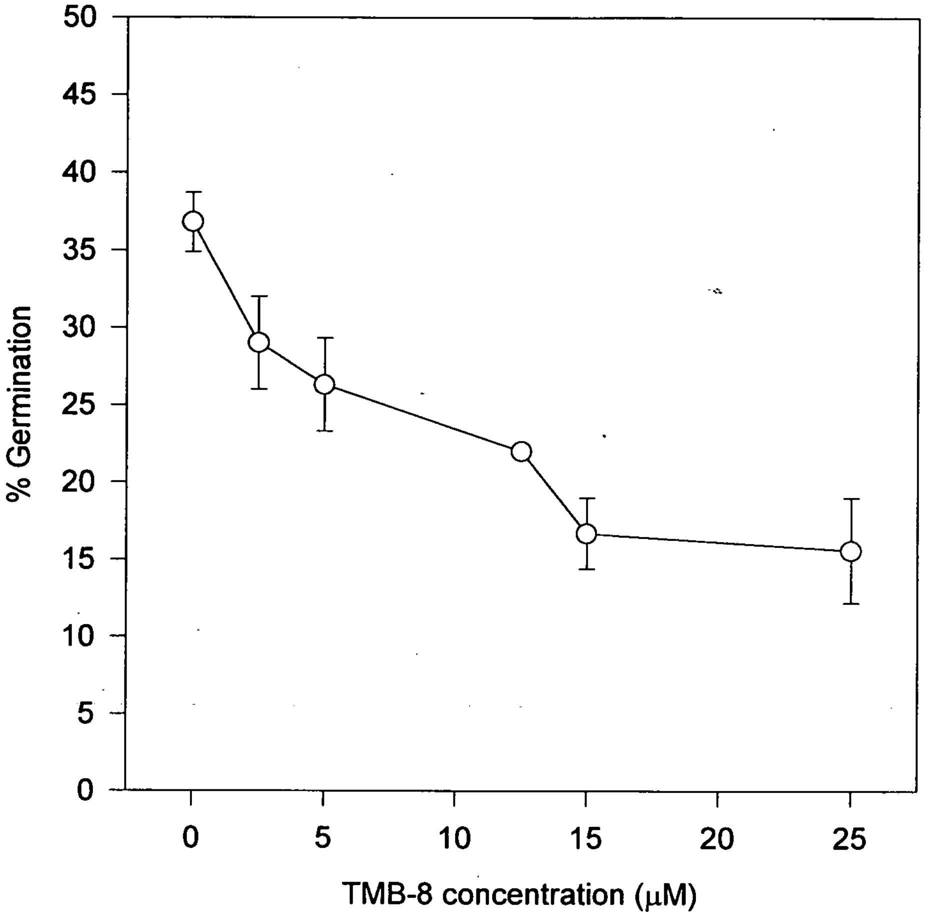
*Means ± s.e. for 3 replicates, assessed after 1.5h, based on counts of 100 spores for each replicate.

Figure 4.8 Effect of verapamil concentration on germination* of *Phytophthora parasitica*, when spores were induced to encyst by agitation in the presence of verapamil or water (control) and then maintained in suspension for 1.5h.



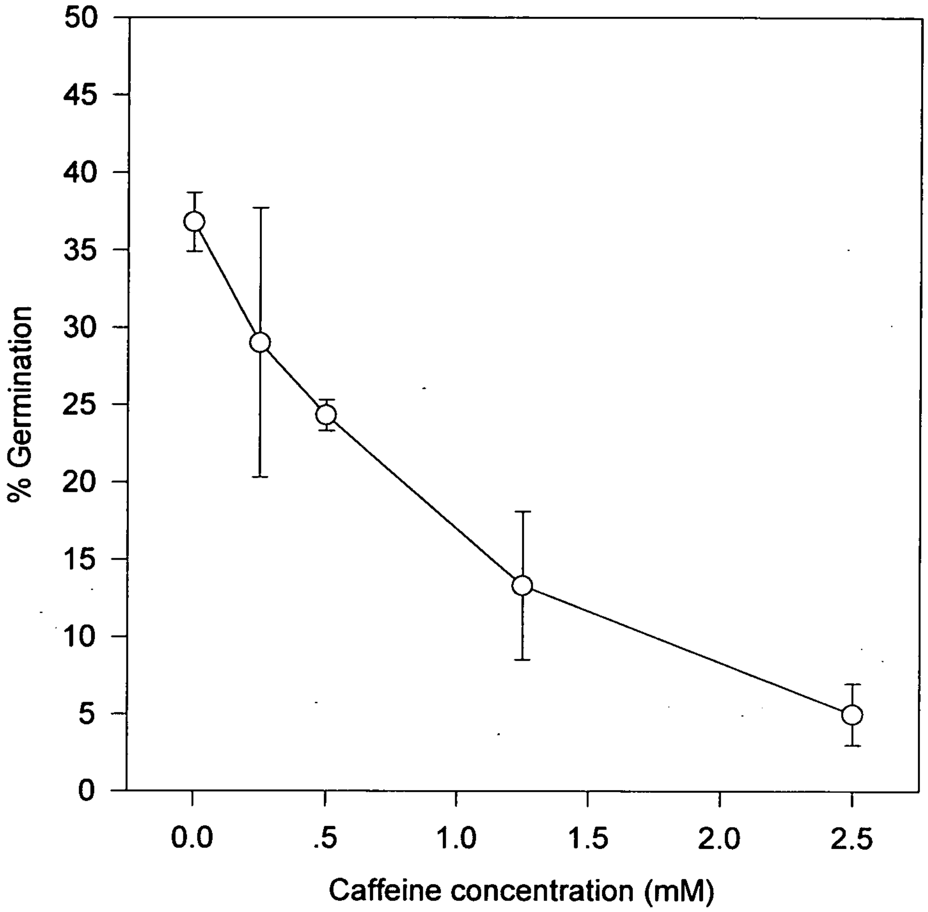
*Means ± s.e. for 3 replicates, assessed after 1.5h, based on counts of 100 spores for each replicate.

Figure 4.9 Effect of TMB-8 concentration on germination* of *Phytophthora parasitica*, when spores were induced to encyst by agitation in the presence of TMB-8 or water (control) and then maintained in suspension for 1.5h.



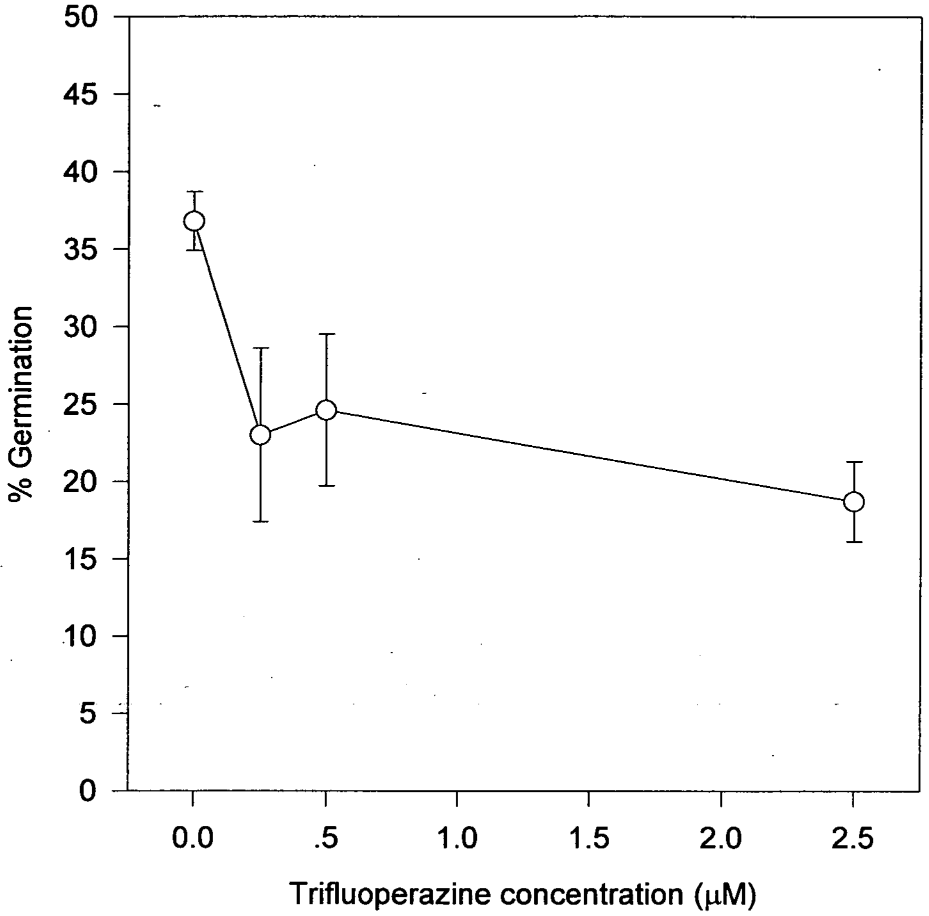
*Means ± s.e. for 3 replicates, assessed after 1.5h, based on counts of 100 spores for each replicate.

Figure 4.10 Effect of caffeine concentration on germination* of *Phytophthora parasitica*, when spores were induced to encyst by agitation in the presence of caffeine or water (control) and then maintained in suspension for 1.5h.



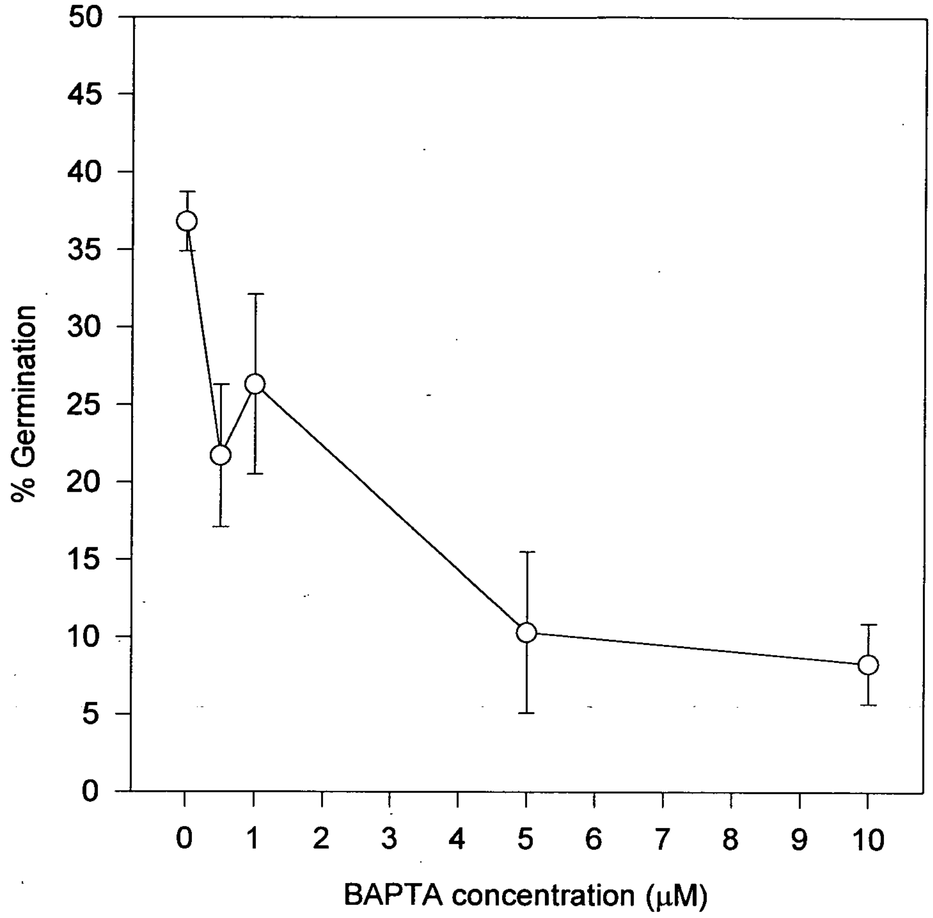
*Means ± s.e. for 3 replicates, assessed after 1.5h, based on counts of 100 spores for each replicate.

Figure 4.11 Effect of TFP concentration on germination* of *Phytophthora parasitica*, when spores were induced to encyst by agitation in the presence of TFP or water (control) and then maintained in suspension for 1.5h.



*Means ± s.e. for 3 replicates, assessed after 1.5h, based on counts of 100 spores for each replicate.

Figure 4.12 Effect of BAPTA concentration on germination* of *Phytophthora parasitica*, when spores were induced to encyst by agitation in the presence of BAPTA or water (control) and then maintained in suspension for 1.5h.



*Means ± s.e. for 3 replicates, assessed after 1.5h, based on counts of 100 spores for each replicate.

Table 4.7 Effects of Ca^{2+}-modulating treatments on the percentage of *Phytophthora parasitica* cysts[*] remaining viable at 1.5h; investigated after the germination assays shown in Figures 4.6-4.12. Viability was assessed by live/dead staining with fluorescein diacetate (FDA) and propidium iodide.

Treatment	Control	Treatment concentration		*P* value[+]
		2μM		
Gd^{3+}	97.6	98.0		n.s.
		2μM		
La^{3+}	97.6	98.2		n.s.
		10μM		
Verapamil	97.6	97.4		n.s.
		15μM	25μM	
TMB-8	97.6	96.8	78.8[+]	0.0053
		2.5mM		
Caffeine	97.6	97.6		n.s.
		2.5μM		
TFP	97.6	96.4		n.s.
		10μM		
BAPTA	97.6	96.6		n.s.

[*] Data are means for 5 replicates, assessed immediately after recording percentage germination for Figures 4.6-4.12, based on counts of 100 spores in each replicate. Viable cysts showed strong green fluorescence in the presence of FDA, whereas non-viable cysts stained red with propidium iodide. Significant difference was assessed by paired samples t-tests

[+] Significantly different from water control by paired samples t-test; n.s. = not significant.

BAPTA (10μM) reduced cyst germination to below 50% of that in the controls (Figure 4.12), and a significant reduction in germination was obtained when the BAPTA concentration was only 500nM. BAPTA at a concentration of 10μM did not reduce apparent cyst viability (Table 4.7).

4.5 Effects of Ca^{2+} on cyst germination of *Phytophthora parasitica*

In separate experiments to those above, Ca^{2+} was tested for its effects on germination of *Ph. parasitica*. Zoospores were encysted by agitation and transferred immediately to microcentrifuge tubes containing distilled water or Ca^{2+}, and incubated for 1.5h. Three experiments of this type were done at different times but in similar experimental conditions (Table 4.8). In all three cases the presence of Ca^{2+} (50mM) enhanced germination over that in the water controls, so the effect of Ca^{2+} overall was very highly significant when the experiments were analysed by 2-way ANOVA. However, only the results of the first experiment were significant individually.

In further experiments to test the effects of Ca^{2+} on germination of *Ph. parasitica*, spores were encysted by agitation and transferred immediately (t_0) or after 3 min (t_3) or 15 min (t_{15}) to slides with either distilled water or Ca^{2+}, and incubated for 1.5h. In the first of these experiments, cysts which were diluted immediately with water germinated at a lower level compared with cysts diluted with water after 3 min or 15 min (Table 4.9). Treatment of cysts immediately after encystment with 50mM Ca^{2+} triggered germination compared with the corresponding water-treated control, but did not elevate germination above the level of cysts treated with distilled water after 15 min. However, treatment of cysts with 50mM Ca^{2+} at later times after encystment (3 and 15 min) resulted in a significant inhibition of germination compared with the corresponding water control. The experiment was repeated later the same day, but using an older zoospore population (experiment 2). The levels of cyst germination in this repeat experiment were much lower than in the first. There was no significant difference between the control (water) and Ca^{2+} treatments, but even a 3 min delay in transferring the cysts to slides caused a reduction in germination compared with the zero minute transfer.

Table 4.8 Effects of Ca^{2+} (50mM final concentration) on germination* of *Ph. parasitica*, when spores were induced to encyst by agitation then treated immediately with an equal volume of either distilled water (control) or $CaCl_2$ solution, and maintained in microcentrifuge tubes for 1.5h.

	Control	Ca^{2+} (50mM)
Expt1		
Mean	**14.3**	**55.5**
Expt2		
Mean	**79.5**	**89.5**
Expt3		
Mean	**31.8**	**40.8**
5%LSD	**19.1**	

ANOVA

Source of variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	12780.25	2	6390.125	38.47863	3.16E-07	3.554561
Columns	2420.042	1	2420.042	14.57247	0.001262	4.413863
Interaction	1345.083	2	672.5417	4.049762	0.035299	3.554561
Within	2989.25	18	166.0694			
Total	19534.63	23				

*Data are percentage germination based on counts of 100 spores from 3 experiments (4 replicates in each experiment). 5% LSD was calculated using 2-way ANOVA in Microsoft Excel 5.

Table 4.9 Effects of Ca^{2+} (50mM final concentration) on germination[*] of *Ph. parasitica*, when spores were induced to encyst by agitation then transferred to slides immediately (t_0) or after 3 min (t_3) or 15 min (t_{15}) with either distilled water (control) or Ca^{2+}, and incubated for 1.5h.

Expt1	Control	Ca^{2+} (50mM)
t_0		
Mean	**19.8**	**32.5**
t_3		
Mean	**28.8**	**12**
t_{15}		
Mean	**34.3**	**5**
5%LSD	**13.8**	

Expt2	Control	Ca^{2+} (50mM)
t_0		
Mean	**14.5**	**15.5**
t_3		
Mean	**5**	**4.3**
t_{15}		
Mean	**4**	**4.3**
5%LSD	**10.4**	

ANOVA of Expt 1

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	202.3333	2	101.1667	1.167869	0.333513	3.554561
Columns	737.0417	1	737.0417	8.508418	0.009203	4.413863
Interaction	1860.333	2	930.1667	10.73785	0.000852	3.554561
Within	1559.25	18	86.625			

ANOVA of Expt 2

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	603.0833	2	301.5417	6.102024	0.009481	3.554561
Columns	0.166667	1	0.166667	0.003373	0.954329	4.413863
Interaction	3.083333	2	1.541667	0.031197	0.969337	3.554561
Within	889.5	18	49.41667			

[*] Data are percentage germination based on counts of 100 spores from 2 experiments (4 replicates in each experiment). 5% LSD was calculated using 2-way ANOVA using Microsoft Excel 5.

4.6 Effects of nutrients on germination of *Pythium aphanidermatum* zoospore cysts which had been pre-treated with Ca^{2+}-modulators

BAPTA, TFP and verapamil had been demonstrated to reduce germination of *Py. aphanidermatum* zoospore cysts (Tables 4.1, 4.6; Figures 4.1, 4.2) whereas Ca^{2+} is reported to enhance germination of cysts of both *Pythium* (Donaldson & Deacon, 1992) and *Phytophthora* spp. (Byrt *et al.*, 1982). The aim of the following experiments was to see whether the effects of verapamil, BAPTA, TFP and calmidazolium (a calmodulin antagonist) on germination of cysts of *Py. aphanidermatum* could be overcome by the subsequent addition of cations and organic nutrients (Tables 4.10-4.13). Either SDW (control) or a calcium-modulating compound was added to microcentrifuge tubes 6 minutes after vortex-induction of encystment; the tubes were then incubated and ion or organic nutrient post-treatments were applied 10 minutes later (16 min after encystment). Germination was assessed microscopically 1.5h after post-treatment by removing aliquots of cyst suspension to slides.

Germination of control cysts (pre-treated with SDW) was significantly enhanced by post-treatment with Ca^{2+} (10mM), glucose (10mM) plus Ca^{2+} (10mM) or L-glutamine (10mM) (Table 4.10). In the same experiment, spores initially treated with BAPTA (3mM) germinated below the level of water controls and this suppression was not overcome by any of the organic nutrients alone apart from glucose (10mM) which partly relieved the suppression. The effect of pre-treatment with BAPTA was also partly relieved by post-treatment with Ca^{2+} (10mM) or any of glucose (10mM), L-asparagine (10mM) or MEP (1%) when supplied with Ca^{2+} (10mM). Of these organic nutrient supplements, only glucose (10mM) plus Ca^{2+} (10mM) enhanced the percentage germination over that caused by Ca^{2+} (10mM)

Table 4.10 Effects of post-treatments with ions or organic nutrients on cysts of *Pythium aphanidermatum* pre-incubated for 6 min with water or 3mM BAPTA[*]. All post-treatments were 10mM unless stated.

	Percentage germination[a].	
	Pre-treatment	Pre-treatment
Post treatment	SDW	BAPTA (3mM)
SDW	27.6	2.6 [+]
Glucose	41.8	12.8 [+]
Xylose	29.4	8.6 [+]
L-asparagine	45.0	3.0 [++]
L-glutamine	51.0	5.8 [++]
Ca^{2+}	48.2	19.0 [+]
L-asparagine + Ca^{2+}	46.6	22.6
Glucose + Ca^{2+}	53.0	33.6 [++]
MEP (1%) + Ca^{2+}	44.2	17.0 [+]
	5% LSD = 19.5	5% LSD = 8.7 1% LSD = 12.2 0.1% LSD = 18.1

[*] Zoospores encysted by agitation were treated 6 min later with the pre-treatment and post-treated after a further 10 min. MEP = malt extract with peptone (each at 1% concentration).
[a] Data are means for 5 replicates (performed on different days), assessed after 1.5h, based on counts of 100 spores in each replicate. One-way analysis of variance was used to calculate significance of difference between post-treatments for the same pre-treatment. Significance of difference from matching controls pre-treated with distilled water by paired samples t-tests: [+], 0.05; [++], 0.01; [+++], 0.001.

Table 4.11 Effects of post-treatments with ions or organic nutrients on cysts of *Pythium aphanidermatum* pre-incubated for 6 min with water or 100µM verapamil[*]. All post-treatments were 10mM unless stated.

	Percentage germination[a].	
	Pre-treatment	Pre-treatment
Post-treatment		
	SDW	Verapamil (100µM)
SDW	39.2	23.8 $^+$
Glucose	75.2	63.0
Sucrose	59.4	54.4
Xylose	33.2	21.2
L-asparagine	72.8	70.8
L-glutamine	49.6	29.3 $^+$
MEP (1%)	93.0	83.3
Ca^{2+}	55.8	57.4
L-asparagine + Ca^{2+}	76.8	79.2
Glucose + Ca^{2+}	83.2	84.0
	5% LSD = 21.6	5% LSD = 26.9
	1% LSD = 28.8	1% LSD = 36.0
	0.1% LSD = 37.9	0.1% LSD = 47.3

[*] Zoospores encysted by agitation were treated 6 min later with the pre-treatment and post-treated after a further 10 min. MEP = malt extract with peptone (each at 1% concentration).

[a] Data are means for 5 replicates (performed on different days), assessed after 1.5h, based on counts of 100 spores in each replicate. One-way analysis of variance was used to calculate significance of difference between post-treatments for the same pre-treatment. Significance of difference from matching controls pre-treated with distilled water by paired samples t-tests: $^+$, 0.05; $^{++}$, 0.01; $^{+++}$, 0.001.

Table 4.12 Effects of post-treatments with ions or organic nutrients on cysts of *Pythium aphanidermatum* pre-incubated for 6 min with water or 40μM TFP*. All post-treatments were 10mM unless stated.

	Percentage germination[a].	
	Pre-treatment	Pre-treatment
Post-treatment	SDW	TFP (40μM)
SDW	66.8	1.2 $^{+++}$
Glucose	91.4	5.2 $^{+++}$
Sucrose	86.4	3.8 $^{+++}$
Xylose	56.6	1.4^{+++}
L-asparagine	87.6	1.4 $^{+++}$
L-glutamine	55.4	0.8 $^{+++}$
MEP (1%)	88.0	37.6 $^{+++}$
Ca^{2+}	79.8	19.4 $^{+++}$
L-asparagine + Ca^{2+}	86.8	15.8 $^{+++}$
Glucose + Ca^{2+}	94.2	36.6 $^{+++}$
Glucose + Mg^{2+}	92.5	33.0 $^{+++}$
	5% LSD = 14.3	5% LSD = 8.1
	1% LSD = 19.1	1% LSD = 10.8
	0.1% LSD = 25.1	0.1% LSD = 14.2

*Zoospores encysted by agitation were treated 6 min later with the pre-treatment and post-treated after a further 10 min. MEP = malt extract with peptone (each at 1% concentration).

[a]Data are means for 5 replicates (performed on different days), assessed after 1.5h, based on counts of 100 spores in each replicate. One-way analysis of variance was used to calculate significance of difference between post-treatments for the same pre-treatment.
Significance of difference from matching controls pre-treated with distilled water by paired samples t-tests: $^{+}$, 0.05; $^{++}$, 0.01; $^{+++}$, 0.001.

Table 4.13 Effects of post-treatments with ions or organic nutrients on cysts of *Pythium aphanidermatum* pre-incubated for 6 min with water or 1μM Calmidazolium[*]. All post-treatments were 10mM unless stated.

	Percentage germination[a].	
	Pre-treatment	Pre-treatment
Post-treatment	SDW	Calmidazolium (1μM)
SDW	36.2	9.4^{+}
Glucose	57.2	16.2^{++}
Sucrose	47.8	14.4^{+}
Xylose	50.2	8.6^{+}
L-asparagine	56.6	13.8^{++}
L-glutamine	56.6	10.2^{++}
Ca^{2+}	60.8	27.0^{+}
L-aparagine + Ca^{2+}	64.2	23.0^{+}
Glucose + Ca^{2+}	66.6	35.6
Glucose + Mg^{2+}	63.2	36.2
MEP (1%) + Ca^{2+}	82.5	49.8^{++}
Mg^{2+}	56.6	21.6
	5% LSD = 20.5	5% LSD = 9.8
	1% LSD = 27.4	1% LSD = 13.2
	0.1% LSD = 36.0	0.1% LSD = 17.3

[*] Zoospores encysted by agitation were treated 6 min later with the pre-treatment and post-treated after a further 10 min. MEP = malt extract with peptone (each at 1% concentration).

[a] Data are means for 5 replicates (performed on different days), assessed after 1.5h, based on counts of 100 spores in each replicate. One-way analysis of variance was used to calculate significance of difference between post-treatments for the same pre-treatment. Significance of difference from matching controls pre-treated with distilled water by paired samples t-tests: $^{+}$, 0.05; $^{++}$, 0.01; $^{+++}$, 0.001.

alone. Also, none of these treatments that partly relieved the suppression of BAPTA restored the level of germination to that which the corresponding treatment caused with the water pre-treatment (Table 4.10).

In a separate experiment with its corresponding water control, verapamil (100µM) as a pre-treatment significantly reduced germination compared to the water control (Table 4.11). Most post-treatments which stimulated germination of cysts pre-treated with water also stimulated germination of verapamil pre-treated cysts; although Ca^+ (10mM) just failed to significantly enhance germination of water pre-treated cysts, it significantly enhanced germination of verapamil treated spores. L-glutamine (10mM) and xylose (10mM), however, did not stimulate germination of either the water- or verapamil-pre-treated cysts.

In a third experiment, TFP (40µM) significantly reduced germination compared to the SDW control (Table 4.12). This suppression could be relieved partly by MEP (1%), Ca^{2+} (10mM) or mixtures of Ca^{2+} (10mM) with glucose (10mM) or L-asparagine (10mM). It was also relieved partly by Mg^{2+} (10mM) combined with glucose (10mM). Rescue of germination by the above post-treatments was never to the level of that of the corresponding post-treatment with SDW pre-treatment (Table 4.12). Post-treatment with glucose (10mM) plus Mg^{2+} (10mM) enhanced germination of both SDW and TFP pre-treated spores, to a similar level as did glucose with Ca^{2+}.

In a fourth experiment, calmidazolium (1µM) was similar to TFP in its effects on germination, which was significantly lower compared to the SDW control (Table 4.13). This suppression was partly relieved by Ca^{2+} (10mM) or Ca^{2+} (10mM) together with L-asparagine (10mM), glucose (10mM) or MEP (1%). Suppression of germination was also relieved by Mg^{2+} (10mM) or Mg^{2+} (10mM) with glucose. These treatments were as effective as Ca^{2+} (10mM) or Ca^{2+} (10mM) plus glucose (10mM) respectively. However, rescue of germination with any of these compounds was never to the level of that in the corresponding post-treatment after SDW pre-treatment.

4.7 Effects of ions, chelation of Ca^{2+}, and amino acids on germ-tube growth of *Pythium aphanidermatum*

Calcium has been implicated in hyphal growth of fungi of the Oomycota. In short, findings from experiments in which an exogenous Ca^{2+} influx was prevented, either by chelating external Ca^{2+} or by blocking Ca^{2+} channels in the plasma membrane using agonists, have been interpreted as showing that movement of Ca^{2+} into the fungal hypha is required for normal growth. For example, Garrill *et al.* (1993) used Gd^{3+} to block stretch-activated Ca^{2+} channels (and not K^+ channels) in *Saprolegnia* protoplasts and found that the tip-high cytoplasmic free Ca^{2+} gradient was rapidly dissipated and the hyphae stopped growing. Jackson & Heath (1989) also found that increasing the external Ca^{2+} concentration caused an increase in hyphal growth rate of *Saprolegnia* and Ca^{2+} was required for normal hyphal morphology; higher concentrations (above 50mM) inhibited tip extension. Experiments in this chapter have shown that exogenous ions such as Ca^{2+} and Mg^{2+} can trigger germination and that chelation of these ions with BAPTA can result in reduced levels of cyst germination.

Certain nutrients such as L-asparagine and glucose induced germination of *Py. aphanidermatum* cysts, as shown in the experiments in this chapter. This is of interest because zoospores and zoospore cysts of *Ph. 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 effect on germination might represent a receptor-mediated event rather than a direct nutritional stimulus.

Donaldson & Deacon (1993b) showed for *Py. aphanidermatum* that both L-alanine and L-aspartic acid trigger germination and that L-alanine enhances germ-tube growth; L-aspartic acid was not tested on germ-tube growth. The effect of L-aspartic acid on germ-tube growth of *Py. aphanidermatum* cysts was therefore tested in direct comparison with the effects of L-alanine. Furthermore, Ca^{2+} and BAPTA were tested for their effects on germ-tube growth; other divalent cations,

Table 4.14 Effects of Ca^{2+} (10mM) on germ-tube growth of germlings of *Pythium aphanidermatum*; values shown are means for 2 replicates, 11 cysts per replicate, with matched water-treated controls after 4h, 7h and 20h*.

	Mean germ-tube length (μm)	
Incubation time (hours)	Water control	Ca^{2+} (10mM)
4	118	135
7	149	268
20	162	323

1% LSD⁺ = 102

*Zoospores were encysted by agitation, maintained in microcentrifuge tubes for 10 min, and then transferred to slides with the treatment. The germ-tubes of the same 11 cysts in each replicate field of view were measured microscopically (x10 objective) at each time.

⁺Significance was assessed by 2-way analysis of variance.

Table 4.15 Effects of Ca^{2+} alone, BAPTA and BAPTA in the presence of Ca^{2+} on germ-tube growth of germlings of *Pythium aphanidermatum*; values are means for 2 replicates, 5 cysts per replicate, with matched water-treated controls after 3h and 20h*.

	Mean germ-tube length (μm).				
Incubation time (hours)	Water control	Ca^{2+} (5mM)	BAPTA (5mM)	BAPTA (10mM)	BAPTA (5mM)/Ca^{2+} (10mM)
3	34	84	26	21	70
20	67	96	59	26	151

5% LSD+ = 34, 1% LSD+ = 44

*Zoospores were encysted by agitation, maintained in microcentrifuge tubes for 10 min, and then transferred to slides with the treatment. The germ-tubes of the same 5 cysts in each replicate field of view were measured microscopically (x10 objective) at each time.

+Significance was assessed by 2-way analysis of variance.

Table 4.16 Effects of ions and nutrients on germ-tube growth of germlings of *Pythium aphanidermatum*; values shown are means for 2 replicates, 5 cysts per replicate, with matched water-treated controls after 16h[*].

Treatment (all 10mM final)	Mean germ-tube length (μm)
Water control	145
Ca^{2+}	238
Mg^{2+}	162
Sr^{2+}	189
Na^{+}	128
Li^{+}	55
L-aspartic acid	225
L-alanine	170

5% LSD[+] = 39
1% LSD[+] = 51

[*] Zoospores were encysted by agitation, maintained in microcentrifuge tubes for 10 min, and then transferred to slides with the treatment. The germ-tubes were measured microscopically (x10 objective) after 16h.

[+] Significance was assessed by 2-way analysis of variance.

Mg^{2+} and Sr^{2+}, were also tested for their effects, as were Na^{+} and Li^{+}. Zoospore cyst suspensions were added to glass slides with the treatments, 10 min after agitation by vortex for 70s.

In one experiment, the effect of 10mM Ca^{2+} addition was tested in comparison with water treated controls. Germ-tube growth was assessed microscopically (x10 objective) at 4h, 7h and 20h. The slide was placed on the microscope stage, which had been fastened using adhesive tape to enable the same cysts to be found for analysis at each time and videotaped for later measurements of germ-tube length. The slides were maintained in moist chambers between time point measurements. It was found that addition of Ca^{2+} (10mM) to cysts significantly enhanced germ-tube growth compared with water controls after 7h incubation (Table 4.14). Germ-tubes of water-treated cysts did not grow significantly ($P = 0.01$) after 4h incubation on slides, whereas Ca^{2+} promoted growth up to 20h.

In a separate experiment the effects of Ca^{2+} (10mM), BAPTA at 5 and 10mM and BAPTA (5mM) in the presence of Ca^{2+} (10mM) were tested. Germ-tube growth was assessed after 3h and 20h incubation. It was found that addition of 5mM Ca^{2+} significantly enhanced germ-tube growth compared with water controls after 3h incubation (Table 4.15) but the effect was not significant after 20h because the Ca^{2+}-treated spores made little further growth than at 3h. BAPTA at 5mM had no significant effect on germ-tube growth compared with the water controls, but BAPTA at 10mM did significantly reduce germ-tube growth after 20h. Germ-tubes of cysts treated with BAPTA (5mM) in the presence of Ca^{2+} (10mM) had not grown significantly longer than those of control cysts by 3h; however, at 20h the germ-tubes were significantly longer ($P = 0.01$); although experiments using light-sensitive compounds were performed under darkened conditions, a slight damaging effect on BAPTA over 20h can not be ruled out.

In a further experiment Ca^{2+}, Mg^{2+}, Sr^{2+}, Na^{+}, Li^{+}, L-aspartic acid and L-alanine (all at 10mM final concentration) were tested for their effect on germ-tube growth. Germ-tube length was assessed after 16h incubation. Of the cations tested, it was found that germ-tubes of cysts treated with 10mM Ca^{2+} and 10mM Sr^{2+} had grown significantly longer (P = 0.01 and P = 0.05, respectively) than those of control cysts (Table 4.16), Mg^{2+} (10mM) and Na^{+} (10mM) had no significant effect, and Li^{+} (10mM) caused a significant reduction (P = 0.01) in germ-tube growth. L-aspartic acid caused a significant increase in germ-tube growth (P = 0.01) whereas L-alanine did not do so.

4.8 Discussion

The experiments in this chapter investigated the effects of Ca^{2+}-modulating treatments on germination of pre-encysted zoospores of *Py. aphanidermatum* and *Ph. parasitica*. The results in this chapter demonstrate that several pharmacological treatments, which interfere with Ca^{2+} ion mediated events in other systems, could affect cyst germination in these different fungi. It has been shown that certain cations and nutrients elicit a range of responses in *Py. aphanidermatum*. The importance of the time of treatment was also demonstrated.

Of the cations tested here for their effects on germination of pre-encysted *Py. aphanidermatum* spores, Ca^{2+}, Sr^{2+} and Mg^{2+} were ineffective compared to distilled water controls whereas Ba^{2+} inhibited germination. The effects of Ba^{2+} have been reported elsewhere. Donaldson and Deacon (1992) found that, when added directly to motile *Py. aphanidermatum* spores, Ba^{2+} caused many cells to lyse, and germination of the remaining intact cells was significantly reduced. Byrt *et al.* (1982), working with *Ph. cinnamomi*, and Grant *et al.* (1986) with *Ph. palmivora*, have shown that Ba^{2+} elicits encystment of motile spores, but also led to slow lysis of the encysting *Ph. palmivora* cells.

Ca^{2+}, Mg^{2+} and Sr^{2+} have previously been reported to elicit germination when added to both motile and pre-encysted *Py. aphanidermatum* zoospores (Donaldson & Deacon, 1992); Ca^{2+} caused encystment and subsequent germination when added to motile spores of *Ph. cinnamomi* (Byrt *et al.*, 1982) and *Ph. palmivora* (Grant *et al.*, 1986). Sr^{2+} also caused encystment in both of these fungi; *Ph. palmivora* subsequently germinated whereas *Ph. cinnamomi* cysts only went on to germinate when supplied with further nutrients.

von Broembsen & Deacon (1996) reported that vortex-induced cysts of *Ph. parasitica* behave in a similar way to those of *Py. aphanidermatum* (Donaldson & Deacon, 1992) in that they germinate poorly when suspended cysts are incubated in microcentrifuge tubes, but significantly better on glass slides. The ability of *Ph. parasitica* cysts to respond to applied treatments was lost within the first 9-12 min of encystment. Furthermore, von Broembsen & Deacon (1996) found that dilution of a *Ph. parasitica* cyst suspension with distilled water significantly reduced germination, whereas dilution of the cyst suspension with the supernatent from a centrifuged cyst suspension restored germination to the control level. These workers suggested that during vortex-induced encystment, zoospores release a substance necessary for cyst germination. Dilution of this substance reduced germination, but Ca^{2+} (50mM) could substitute for this in restoring germination to control levels. Results presented in this chapter also suggest that dilution of *Ph. parasitica* spores, immediately after vortex-induced encystment, reduces the germinability of cysts from a young zoospore population (Table 4.9) above that of water controls, but only when the control level of germination is low. For cysts that show higher control levels of germination (approximately 30% or above), Ca^{2+} has no effect on germination (Table 4.8) or may even be inhibitory (Table 4.9). Cerenius & Soderhall (1985) found that 50mM Ca^{2+} induced germination in several *Aphanomyces* spp., and Donaldson & Deacon (1992) found that 7-10mM Ca^{2+} was sufficient to restore germination of *Pythium* cysts that had been suspended in water. Secondary zoospore release from ungerminated cysts of the parasitic Oomycota has been studied for *Pythium* spp. (Jones *et al.*, 1991), *Phytophthora* spp. (eg., von Broembsen & Deacon, 1996) and *Aphanomyces* spp. (Cerenius & Soderhall, 1985). This phenomenon is believed to represent a default option when the zoospores fail to find a suitable host (von Broembsen & Deacon, 1996), and can be prevented by addition of Ca^{2+} in *Ph. parasitica* (von Broembsen & Deacon, 1996) and the parasitic *Aphanomyces* spp. (Cerenius & Soderhall, 1985).

As shown in the results presented here, germination of *Py. aphanidermatum* in controls treated with water consistently showed higher levels of germination in comparison to those obtained in experiments previously performed in this laboratory (Donaldson & Deacon, 1992). Approximately 35-50% of pre-encysted zoospores failed to germinate when treated with distilled water and could not be triggered to germinate with exogenously applied divalent cations. However, when supplied with an amino acid, L-asparagine (previously reported to enhance germination of *Py. aphanidermatum* cysts by Donaldson & Deacon, 1993b), germination levels were elevated above those of cysts treated with distilled water.

Sing & Bartnicki-Garcia (1975a, b) found that newly formed cysts are naturally adhesive, whereas cysts that do not come into contact with a surface soon lose their adhesiveness. A glycoprotein released early during encystment is thought to confer, this adhesive property on the cysts. Gubler *et al.* (1989) showed that the natural adhesiveness of young *Ph. cinnamomi* cysts is abolished by adding EGTA, whereas adhesiveness can be restored to older cysts by addition of Ca^{2+}. Experiments on *Pythium* (Donaldson & Deacon, 1992) have confirmed these findings and suggest that adhesion is Ca^{2+}-dependent. Adding further to this, Donaldson & Deacon (1992) demonstrated that there was a close correlation between adhesion and germination of *Pythium* cysts, both of these factors being influenced in the same way by age of the cysts, Ca^{2+} and EGTA. It is proposed that the calcium which facilitates this adhesion is released from the cells early during encystment. Irving *et al.* (1984) reported that spores of *Ph. palmivora* release up to 30% of their intracellular Ca^{2+} within 2 min of the induction of encystment. Dispersion of Ca^{2+} from cysts maintained in suspension could result in loss of adhesiveness of these cells.

Donaldson & Deacon (1992) found that adhesion was not an absolute requirement for germination (in *Pythium*) because, for example, Ca^{2+} stimulated germination of suspended cysts to levels comparable with those of firmly attached cysts. *Pythium* cells adhering early during encystment seemed independent of exogenously supplied Ca^{2+} whereas cells not adhering early require a Ca^{2+} supplement for adhesion and germination. The critical time for this change seemed to be 5-7 min (Donaldson & Deacon, 1992), which coincides with the period when the cyst wall of *Py. aphanidermatum* is proposed to have developed fully (Deacon & Mitchell, 1985).

The high control levels of germination of *Py. aphanidermatum* cysts seen here (Tables 4.1, 4.4-4.6, 4.12; Figures 4.1-4.5) and the lack of stimulation of germination when supplied with divalent cations, such as Ca^{2+}, could be due to a high level of contaminating ions already surrounding the cysts, thus stimulating them to germinate maximally under these conditions. In further support of this, when BAPTA was used to chelate divalent cations, mainly Ca^{2+}, germination was suppressed (Tables 4.1, 4.10; Figure 4.1). Iser *et al.* (1989) suggested that Ca^{2+} is rapidly reabsorbed by *Ph. palmivora* cells after it has been released and this reuptake then stimulates germination. Thus, in the case of suspended cysts, BAPTA suppressed germination in the experiments described in this chapter by sequestering Ca^{2+} released from the spores or from the surrounding medium. Verapamil also suppressed germination, presumably by acting as a Ca^{2+}-channel blocker preventing Ca^{2+} uptake. From experiments here it would appear that germination of spores in water controls is also dependent on Ca^{2+} flux. Further tests served to support a role for Ca^{2+}. Pharmacological drugs with Ca^{2+}-modulating actions were found to suppress germination of suspended cysts below even the control levels in water, the exception being amiloride, although this was reported previously by Donaldson & Deacon (1992).

Ca^{2+} involvement in germination of zoospores of the Oomycota was supported by similar experiments using Ca^{2+}-modulating treatments on *Phytophthora parasitica*. These experiments were designed so that the motile zoospores were induced to encyst **in the presence of the drug to be used**, to test for its effects on germination. Not surprisingly, and in comparison with the findings for *Py. aphanidermatum*, all of the pharmacological drugs had the effect of suppressing germination of *Ph. parasitica*. Interestingly, all of the modulators led to, approximately, a 50% reduction in *Ph. parasitica* cyst germination, at more physiologically relevent concentrations than those used for vortex-induced cysts of *Py. aphanidermatum*.

Lowering of extracellular Ca^{2+} levels by chelation with BAPTA had the same effect in lowering germination as did blocking of Ca^{2+}-specific uptake mechanisms with the three membrane channel-blockers, Gd^{3+}, La^{3+} and verapamil. These experiments implied a requirement for extracellular Ca^{2+} and, in particular, the involvement of Ca^{2+}-channels for the movement of Ca^{2+} across the membrane in the differentiation of *Ph. parasitica* from motile zoospore to the germling stage. Two other drugs used which antagonise intracellular Ca^{2+} homeostasis, TMB-8 and caffeine, also reduced germination compared to cells treated with water. This effect could be explained on the assumption that intracellular antagonism of Ca^{2+}-mediated processes prevented development from motile zoospore to germling when Ca^{2+} was not directly prevented from entering the cells.

The Ca^{2+}-binding protein calmodulin is known to link Ca^{2+}-signals with the activities of numerous proteins and enzymes in animal, plant and fungal cells. Calmodulin is known to be present in spores of *Ph. cinnamomi* (Gubler *et al.*, 1990) and is almost certainly present in *Ph. parasitica*. A relationship between Ca^{2+} and calmodulin in the developmental stages leading to cyst germination in *Ph. parasitica* is shown by the effect of the calmodulin antagonist TFP. At physiologically relevant concentrations, this compound is likely to act on the Ca^{2+}/calmodulin complex to perturb the events leading to germination. Many Ca^{2+}/calmodulin antagonists are known to have secondary effects; for example, TFP has been reported to inhibit calmodulin-insensitive Mg^{2+}-ATPase in animal cells. In spite of this, calmodulin antagonists have been used to obtain initial evidence for Ca^{2+}/calmodulin dependent processes in a variety of animal, plant and fungal cells (Hepler & Wayne, 1985).

Addition of L-aspartic acid was found to promote germ-tube growth of germinating *Py. aphanidermatum* cysts, whereas L-alanine did not do so. This finding is in contrast to that reported by Donaldson & Deacon (1993b) who found L-alanine to promote germ-tube growth of *Py. aphanidermatum* cysts but had no effect on germination. Donaldson & Deacon (1993b) found no absolute correlation between compounds that stimulate germination and those which promote germ-tube growth. Similarly, Barash *et al.* (1965) reported differences between the ability of nutrients to stimulate germination and to stimulate germ-tube growth. An explanation would be that some nutrient transport mechanisms may be present or active in germinated cysts, thus enabling nutrient uptake and metabolism, but absent or inactive in ungerminated cysts. In support of this, Penington *et al.* (1989) reported that uptake of glucose and glutamate, into zoospores of *Ph. palmivora*, only occurs after germ-tube emergence.

Donaldson & Deacon (1992) found that Ca^{2+}, Mg^{2+} and Sr^{2+} at 7-10mM significantly increased germination of suspended cysts of three *Pythium* spp. whereas Li^{+} and Na^{+} had no effect up to 25mM. These workers suggested a central role for Ca^{2+} ions in adhesion and germination of zoospore cysts of *Pythium* spp., and found that Mg^{2+} and Sr^{2+} could substitute for Ca^{2+}. In support of this, results presented in this chapter show that Ca^{2+} can trigger cyst germination of *Py. aphanidermatum* and *Ph. parasitica* and that Mg^{2+} can substitute for Ca^{2+}. Ca^{2+} ions promoted germ-tube growth and could be substituted for by Sr^{2+} ions but not Mg^{2+}; Sr^{2+} were not as effective at promoting germ-tube growth as was Ca^{2+}. In agreement with S. P. Donaldson (1992), findings from this study have shown that Na^{+} ions do not promote germ-tube growth; however, Li^{+} suppresses germ-tube growth. Berridge (1984) describes the therapeutic action of lithium in the control of manic depressive illness. Li^{+} is thought to act by inhibiting the enzyme *myo*-inositol 1-phosphatase thus lowering the level of *myo*-inositol which results in a decline in the synthesis of the phosphatidylinositol necessary to maintain the pool of phosphoinositide important in intracellular signalling pathways. Li^{+}, acting in this way in *Py. aphanidermatum* germlings, may explain the suppression of germ-tube growth

The chelation of exogenous Ca^{2+} by BAPTA reduced the growth of *Py. aphanidermatum* germ-tubes. Total Ca^{2+} (Jaffe *et al*, 1975) and cytoplasmic free Ca^{2+} (Brownlee & Wood, 1986) are distributed in a tip-to-base gradient in a variety of tip-growing systems, suggesting that the Ca^{2+} gradient plays a role in tip organisation and growth. Often an ion current enters at the apex of tip-growing cells, and part of this current has been demonstrated to be carried by Ca^{2+} (Weisenseel &

Jaffe, 1976; Saunders, 1986). It has been hypothesised that a Ca^{2+} gradient in tip-growing cells is created by a concentrated influx of Ca^{2+} in the tip region (Kropf & Quatrano, 1987). An increase in external Ca^{2+} concentration may affect the rate of Ca^{2+} influx through Ca^{2+}- channels and thus alter growth rate. Addition of BAPTA will presumably reduce the external Ca^{2+} concentration, which may explain the reduction in germ-tube growth rate shown here for *Py. aphanidermatum* germlings.

The criterion used in this study for scoring a zoospore cyst as germinated is that the emerging germ-tube length is equal to or greater than the cyst diameter (approximately 10µm for *Py. aphanidermatum*). A possible complication in assessment of germination, therefore, is that treatments may reduce germ-tube growth to such an extent that the actual number of cells that have germinated is underestimated. In this study, germ-tubes of cysts treated with BAPTA or Li^+ showed the slowest elongation rate. With 3mM BAPTA treatment, the mean growth rate was 26µm in 3h. Assessment of germination at 1.5h, as carried out with all germination experiments with *Py. aphanidermatum* described in this chapter, would allow the germ-tubes to reach an approximate mean length of 13µm, a cyst would therefore be scored as germinated. More caution would have to be taken in interpreting effects of Li^+ on germination because of its dramatic effect on reducing germ-tube growth. However, careful microscopic examination revealed complete prevention of germination with the lack of any discernable germ-tube.

All the work on Ca^{2+} involvement in germination was done with *Py. aphanidermatum* and *Ph. parasitica*. It supports work previously done to implicate a role of Ca^{2+} in zoospore encystment, adhesion and germination by *Py. aphanidermatum* and *Py. dissotocum* (Donaldson & Deacon, 1992; 1993b), *Ph. palmivora* (Irving & Grant, 1984), *Ph. cinnamomi* (Gubler *et al.*, 1989) and *Ph. parasitica* (von Broembsen & Deacon, 1996). Taken collectively, the results in this chapter suggest a central role of Ca^{2+} in stimulating germination, whereby a Ca^{2+} flux may occur at an early stage during encystment; this may also facilitate zoospore adhesion and trigger germination. *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). *In vitro* experiments demonstrate that treatments sequestering Ca^{2+} or inhibiting Ca^{2+} uptake suppress germination (Tables 4.1, 4.6, 4.10, 4.11; Figures 4.1, 4.2, 4.6, 4.7, 4.8, 4.12). A Ca^{2+} efflux may facilitate adhesion, and a rapid influx of Ca^{2+}, demonstrated for cysts of *Ph. palmivora* (Irving *et al.*, 1984), would ensure that the cyst receives a Ca^{2+} signal leading to differentiation of the spore into a germling capable of host penetration. *In vivo*, as with *in vitro* experiments, non-adhered spores of *Py. aphanidermatum* might be stimulated to germinate by specific amino acids such as L-asparagine or L-glutamate, acting by gating Ca^{2+} channels and/or by an alternative receptor-mediated pathway in which internal Ca^{2+} plays a role. It is possible, then, that spores which dock incorrectly could detect a root due to its amino acid diffusates. These, and possibly other nutrients, might open putative high affinity Ca^{2+} channels facilitating germ-tube tropism towards the root (Jones *et al.*, 1991), leading to host infection.

Throughout this study, difficulties were experienced in obtaining consistent levels of germination of zoospore cysts. Sometimes the germination levels in water controls were as low as 28% (Table 4.10) for *Py. aphanidermatum* whereas at other times the control levels were as high as 81% (Table 4.5). Similar variability has been reported by other workers, such as von Broembsen & Deacon (1996), working with *Ph. parasitica*. Most of this variability is unexplained, but the age of zoospores from which cysts are induced could be a major factor. Table 4.9 shows results of two experiments done in identical conditions and with cysts from the same zoospore population, but done at different times in one day. In the first experiment the zoospores showed a reasonable level of germination in water controls, and they responded to a calcium supplement by increased germination; in the second experiment, with cysts from an older zoospore suspension, the cysts germinated poorly and they were unresponsive to a calcium supplement. Almost all the zoospores used in these two experiments would have been released at the same time, in response to cold-shock to trigger zoospore release from sporangia. It seems, therefore, that the physiological condition of a zoospore population, perhaps including the availability of endogenous nutrient reserves, might influence the germination responses after vortex-encystment.

The responsiveness of zoospore cysts to germination triggers is strongly influenced by the time at which the triggers are applied. Cysts lose their germination responsiveness within 5-15 minutes after the induction of encystment (Cerenius & Soderhall, 1985; Persson & Soderhall, 1986; Donaldson & Deacon, 1992; von Broembsen & Deacon, 1996) and at the same time the level of germination in water controls has been found to be insensitive to the application of EGTA (Donaldson & Deacon, 1992). In work with *A. astaci*, Persson & Soderhall (1986) showed that cysts could germinate in response to 50mM Ca^{2+} added up to 15 minutes after vortex-induced encystment but were unresponsive to Ca^{2+} after this time, yet the cysts could still respond to Ca^{2+} added after 45 minutes, by secretion of peptidase. Evidently the cells did not lose all responsiveness to Ca^{2+}, they only lost the ability to respond by germination after 15 minutes. von Broembsen & Deacon (1996) suggested that this could result from an "uncoupling" of one signal response pathway while another (for Ca^{2+}-induced peptidase secretion) remained coupled. They noted that major ultrastructural changes occur in the early stages of encystment: the microtubular arrays near the site of flagellar insertion into the zoospores are disassembled (Holloway & Heath, 1977), and the nucleus changes from a pyriform to a rounded shape and migrates towards the centre of the cell. If microtubules are involved in signal transduction as suggested for germ-tubes of rust fungi (Hoch *et al.*, 1987) then this could explain the loss of germination-sensitivity to Ca^{2+} after the first few minutes following encystment.

Another phenomenon of interest was observed for cysts of *Ph. parasitica* (Table 4.9). It was found that the responsiveness to Ca^{2+} by cysts formed from the younger zoospore population (experiment 1) changed when the cysts were left for different times, and that this was related to a change in behaviour of cysts in the water controls. If the cysts were left in suspension for 3 or 15 minutes after vortex treatment before they were transferred to slides then the controls showed higher germination than if they were transferred immediately. The addition of Ca^{2+} to these cysts transferred at 3 or 15 minutes then caused a significant suppression of germination. Thus, the responses of the cysts to exogenous Ca^{2+} seemed to be inversely related to the level of germinability that the cysts had acquired in the different control treatments. It is premature to offer an explanation of this, but it demonstrates that the effect of exogenous Ca^{2+} might vary according to the degree of calcium-sufficiency of the cysts, perhaps related to their ability to absorb calcium previously released from the cells or to their ability to release Ca^{2+} from intracellular stores.

CHAPTER 5

The use of fluorimetry and fura-2 to measure Ca^{2+} fluxes during zoospore encystment

5.1 Introduction

In the work described in this chapter, fluorimetry of the Ca^{2+}-sensitive dye fura-2 was used to measure changes in the levels of Ca^{2+} surrounding developing zoospores of *Phytophthora parasitica*. Additionally, the possibility of loading cells with fura-2 to measure intracellular free Ca^{2+} levels was investigated. Much of the work described involved adapting and developing a technique, used widely in animal cell research, to study Ca^{2+} fluxes around developing zoospores; this required a critical assessment of dual excitation spectrofluorimetry of the Ca^{2+}-sensitive dye fura-2 as a tool to study Ca^{2+} dynamics.

Calcium ions have been implicated in almost all stages of the homing sequence of zoospores of the Oomycota (Irving *et al.*, 1984; Deacon & Donaldson, 1993), in development of other fungal cells (Read *et al.*, 1992a, 1992b) in hyphal tip growth (Jackson & Heath, 1993) and in plant cell signalling (Read *et al.*, 1993). Ion fluxes have been implicated in the differentiation stages of fungal zoospores (see section 4.7). Upon receiving an encystment signal, the zoospore undergoes a rapid change in morphology from an ovoid, motile, wall-less spore to a spherical, non-motile, walled cyst. In particular, a Ca^{2+} efflux has been associated with encystment of *Ph. palmivora* zoospores (Irving *et al.*, 1984), and there is also evidence for uptake of radiolabelled Ca^{2+} in the early stages of encystment (Irving *et al.*, 1984). By appropriate techniques, therefore, it should be possible to study the net movement of free Ca^{2+} into and out of encysting zoospores, and to link these changes with zoospore morphogenesis.

One approach to demonstrate the role of Ca^{2+} in linking a stimulus to a response is to detect cytosolic Ca^{2+} dynamics by fluorimetry of Ca^{2+}-sensitive dyes loaded into cells. A large range of fluorescent dyes, highly selective for free Ca^{2+}, are currently available (Haughland, 1992) and there are many approaches for loading cells with these dyes (Thomas & Delaville, 1991; Read *et al.*, 1992). Dyes have been used successfully to measure $[Ca^{2+}]_c$ (cytosolic free calcium) in animal systems (Tsien, 1989) and plant systems (Read *et al.*, 1992, 1993). However, attempts to image $[Ca^{2+}]_c$ in some fungal cells have proved unsuccessful (Schmid &

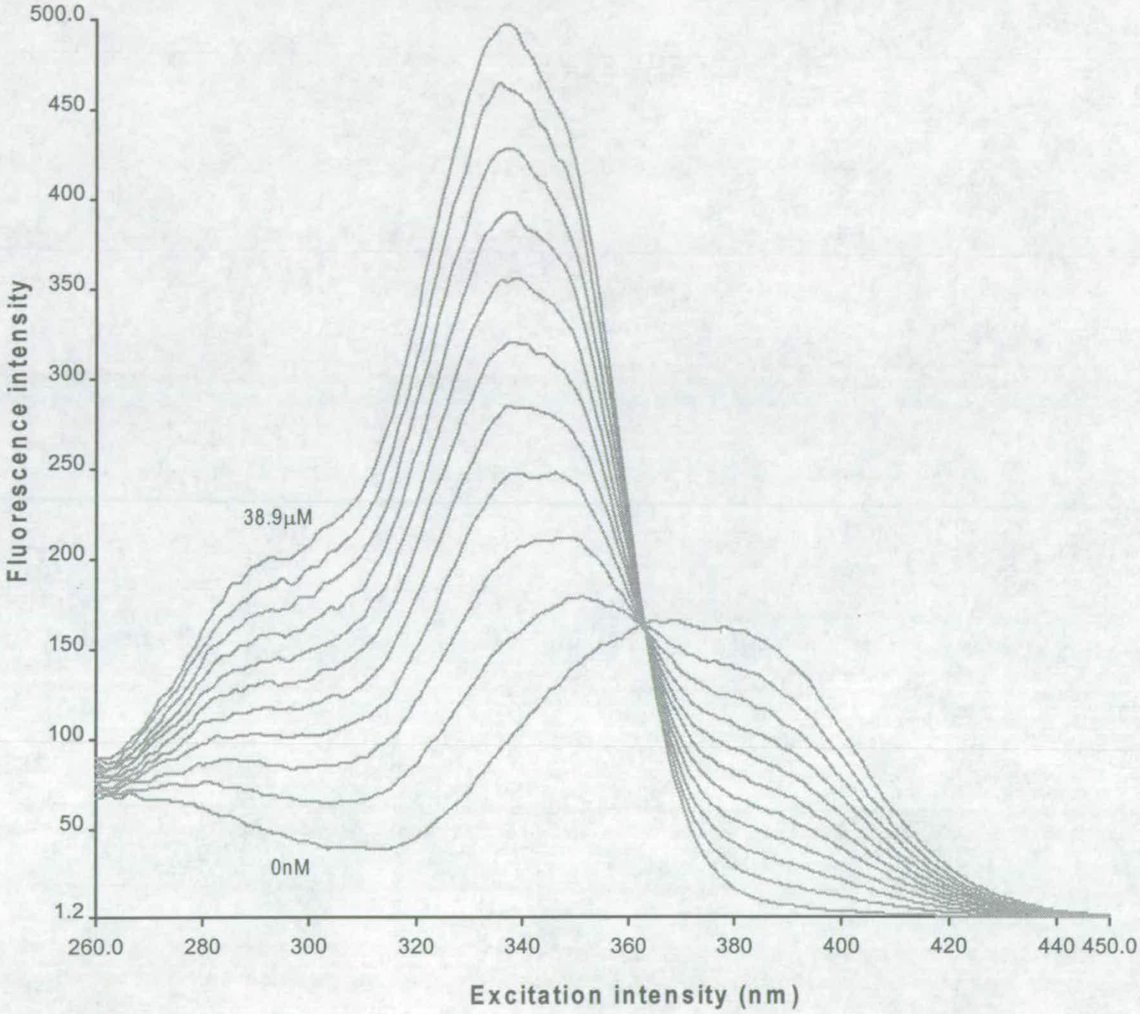
Harold, 1988; Read *et al.*, 1992; Knight *et al.*, 1993). Successful measurements of $[Ca^{2+}]_c$ in fungi have mainly been achieved with protoplasts (Pitt & Kaile, 1990) and yeast cells (Iida *et al.*, 1990). There are several reasons for this variable success, including potential problems associated with dye loading, compartmentalisation of dye within organelles, uneven dye distribution within the cell, dye cytotoxicity, leakage of dye from the cell, cellular autofluorescence contributing to total fluorescence, dye photobleaching and calibration of the dye (Thomas & Delaville, 1991; Read *et al.*, 1992).

5.1.1 Merits of using fura-2 to measure changes in $[Ca^{2+}]$

Fura-2 has become the most popular fluorescent probe with which to monitor dynamic changes in intracellular free Ca^{2+}. Fura-2 exhibits much higher brightness of fluorescence compared with that of its predecessor quin2. The 30-fold greater fluorescence of fura-2 means that intracellular dye concentrations can be lowered, thereby reducing buffering effects of the dye on cytosolic $[Ca^{2+}]$ changes. It has been shown that the fluorescence intensities of 1μM fura-2 and of its Ca^{2+} complex are comparable to 30μM quin2 and its complex under similar conditions (Grynkiewicz *et al.*, 1985). The fluorescence excitation maximum of fura-2 both increases and shifts to a lower wavelength on Ca^{2+}-binding (Figure 5.1) with negligible shift in the emission maximum. This allows fura-2 to be utilised as a dual excitation Ca^{2+}-probe. The spectral shift that occurs upon Ca^{2+} binding allows fura-2 to be used ratiometrically by detecting fluorescence intensity following excitation at 340 and 380nm wavelengths, making the measurement of Ca^{2+} concentration essentially independent of dye concentration (ie. the extent of dye loading), cell thickness, photobleaching and dye leakage.

The acetoxymethyl ester form of fura-2 (fura-2 AM) is widely used to facilitate cell loading. The ester groups mask charges on the fura-2 molecule allowing the dye to passively cross the plasma membrane. Once inside the cell, the ester groups are cleaved by intracellular esterases to release the cell-impermeant fura-2 free-acid molecule.

Figure 5.1 Fluorescence excitation spectra of 1μM fura-2 with increasing [Ca^{2+}] at 20°C, pH 7.20 and 500μM KCl (emission wavelength = 511nm).



The defined free [Ca^{2+}] ranged from 0nM to 39.8μM (as shown) with intermediate values of 16.7, 37.6, 64.5, 100, 150, 225, 351, 602 and 1350nM.

Excitation slits = 3.4nm.

Emission slits = 4.0nm.

5.1.2 Measurements of [Ca^{2+}] in cell populations

The main advantage of cell population measurements is that these are usually easier to perform compared to single-cell studies, calibration is often more straightforward and specialised instrumentation is not required. However, due to the asynchronous nature of most intracellular Ca^{2+} responses, complex patterns may be averaged out and thereby masked in population measurements.

5.1.3 Cell loading techniques

Several techniques have been used to load dyes into a diverse range of cell types, including plant and fungal cells (eg., Read *et al.*, 1992; Knight *et al.*, 1993; Malho *et al.*, 1994). Some of these techniques, such as microinjection, are obviously unsuitable for loading a cell population. Here, the ester loading technique is more applicable. This involves incubating intact cells with an esterified form of the dye and it results in the accumulation of trapped indicator dye in the cytosol unless the dye is subsequently sequestered into organelles. Once extracellular dye has been removed, fluorescence signals from the cytosolic dye can be recorded and used to calculate [Ca^{2+}] after calibration.

5.2 Experimental procedures

5.2.1 Attempts to load cells with fura-2

An attempt was made to load motile *P. parasitica* zoospores (5×10^5 zoospores ml^{-1}) with the Ca^{2+}-sensitive fluorescent dye fura-2 obtained from either Molecular Probes Inc. or Calbiochem-Novabiochem (U.K.) Inc. The cells were incubated with the esterified form of the dye, washed twice with buffer (by centrifugation at 200g for 4 minutes) to remove extracellular dye and finally resuspended in buffer (10mM Tris-Cl, pH 7.20). Control cells treated with water were also washed twice by centrifugation. An aliquot of the zoospore suspension was then transferred to microcentrifuge tubes containing a formaldehyde/glutaraldehyde (2/1.5% final concentration) solution to fix the cells.

Microscopic examination (X40 objective) was then used to ascertain the percentage of spores that retain the motile shape, to test whether incubation in the dye had caused encystment.

Use of the minimum concentration of AM ester to obtain an adequate signal (typically as low as 0.1μM and rarely above 5μM) is strongly recommended to reduce artefacts that result from incomplete enzymatic hydrolysis of the AM esters (Molecular Probes Inc., product information sheet). So, a range of concentrations was tested (Table 5.1).

5.2.1.1 Results

To determine whether motile zoospores had been loaded with the dye, fluorimetry was used to test for the presence of the dye in the cytosol after removing extracellular dye by centrifugation and resuspension in buffer. An excitation scan of the cell population showed a peak of fluorescence at 380nm. This could be due either to the presence of the Ca^{2+}-free form of the dye or to the presence of partially esterified dye in the cells. To test whether the dye had been taken up and was reporting extremely low levels of free Ca^{2+} in the cytosol an ionophore, Br-A23187, was used in the presence of external Ca^{2+} (1-5mM) to elevate cytosolic levels of free Ca^{2+}. Any dye present in the cytosol would then report an increase in $[Ca^{2+}]$ by an increase in the fluorescence intensity with a concomitant shift in the excitation peak to the lower wavelength of 340nm.

Table 5.1. Percentage* of *Phytophthora parasitica* zoospores remaining motile after incubation with either sterile distilled water or with a range of fura-2 AM concentrations for 30 or 60 minutes. The spores were then washed twice by centrifugation and resupended in buffer (10mM Tris-Cl, pH 7.20) before fixation# to assess the level of encystment.

	Percentage of zoospores retaining motile shape	
Fura-2 final concentration	Incubation time 30 minutes	Incubation time 60 minutes
0μM (water control)	84 ± 3.4	94 ± 2.0
2.5μM	83 ± 2.3	87 ± 4.3
5μM	90 ± 2.4	91 ± 5.8
10μM	92 ± 1.7	97 ± 1.4
20μM	87 ± 2.2	90 ± 2.6
30μM	89 ± 2.8	97 ± 1.4

#Spores were fixed using a formaldehyde/glutaraldehyde mixture (2%/1.5% final concentration) and assessed by microscopic examination (x40 objective).

*Data are means ± s.e. of 4 replicate experiments, based on counts of 100 spores in each replicate.

When fluorimetry was used after incubation of cells with 2.5μM fura-2 for 30 minutes, the peak of the fluorescence spectrum was at 380nm and did not change in the presence of ionophore plus Ca^{2+}. Increasing the incubation time to 1 hour also did not cause the fluorescence peak to shift, and the same result was found even when the dye concentration was increased to 30μM.

To test further for dye loading, cells that had been incubated for 1 hour with 30μM fura-2 were lysed with Triton X-100 or by homogenisation for 30 seconds using an Ultra-turrax T25 homogeniser (Janke & Kunkel GmbH & Co. KG, IKA-Labortechnic, Staufen). If loading has been successful, lysis should release any dye from the cytosol into the suspending buffer. Addition of Ca^{2+} at this point should then cause an increase in fluorescence intensity of the dye and a consequent spectral shift, whereas addition of EGTA should have the opposite effect when measured by fluorimetry. Tests using each lysis method were replicated three times but a change in the excitation spectrum did not occur in any of the tests. Longer loading times (2 hours) caused unacceptable levels of encystment, with less than 50% of the spores remaining motile (data not shown).

5.2.2 Effects of physical and chemical treatments on fura-2

Due to the nature of the experiments to be described in this section, whereby fura-2 free-acid was used to report $[Ca^{2+}]$ in the zoospore bathing medium, it was essential that any compounds used in tests on zoospore development had no effect on the properties of fura-2 and its ability accurately to report Ca^{2+} levels. Table 5.2 summarises some known effects of some physical and chemical treatments on the spectral properties of fura-2.

Table 5.2. Known effects that some treatments have on the properties of fura-2 (Grynkiewicz *et al.*, 1985).

Chemical/physical treatment		Effect on fura-2*
pH	increase	K_d decreases
	decrease	K_d increases
Temperature	increase	K_d decreases
	decrease	K_d increases
$[Mn^{2+}]$	increase	Quenching of the dye.

*K_d = dissociation constant

5.2.2.1 Results

It was found that Gd^{3+} at 1μM caused an elevation in fura-2 fluorescence (appendix 1) and when Ca^{2+} was subsequently added, the fluorescence increased but there was a perturbing effect on the isosbestic point at 360nm excitation wavelength. Sr^{2+} (100μM) also altered the spectral properties of fura-2 (not shown). Similarly, addition of 67mM K^+ caused a change in the spectral properties of fura-2 (appendix 2), probably due to the effects of a change in ionic strength on the K_d of fura-2 for Ca^{2+}. It was also found that caffeine (1.25mM) led to an unacceptable alteration in the excitation spectrum of fura-2 (appendix 3) due to the optical properties of the caffeine molecule itself (D. Dryden, personal communication). Verapamil (10μM), La^{3+} (1μM) and TMB-8 (5μM) had no appreciable perturbing effect (appendices 4-6).

Temperature, ionic strength and pH are known to influence the dissociation constant (K_d) of fura-2 binding to Ca^{2+} (Grynkiewicz *et al.*, 1985), and therefore alter the properties of the probe at fixed $[Ca^{2+}]$. Based on this, the environment of the medium surrounding the zoospore population, to which the probe was added, had to be carefully designed and controlled. The medium was made up of 10mM Tris Cl in UHQ water (resistivity 18.88MΩ.cm), pH 7.20, the assumption being that any acidification or alkalinisation which might occur during zoospore development would be buffered at pH 7.20, with negligible effect on the K_d of fura-2 for Ca^{2+}. It was found that after each experiment no change in the pH of the zoospore suspension had occurred. The temperature was maintained at 20°C by a thermostatically controlled cuvette compartment, so that any temperature change during an experiment would be minimal and would have negligible effect on the K_d.

Griffith *et al.* (1988) found that the supernatent from sporangia of *Ph. palmivora*, brushed into glass distilled water, resulted in the transfer of contaminant ions into the medium into which the zoospores were ultimately released. Sodium and K^+ were the most abundant contaminating ion species which contributed to an ionic strength of approximately 500-600μM. As long as the $[Ca^{2+}]$ was buffered at 200nM, zoospore populations remained fully motile for more than 1h, with very little spontaneous encystment and no lysis occurring during this time.

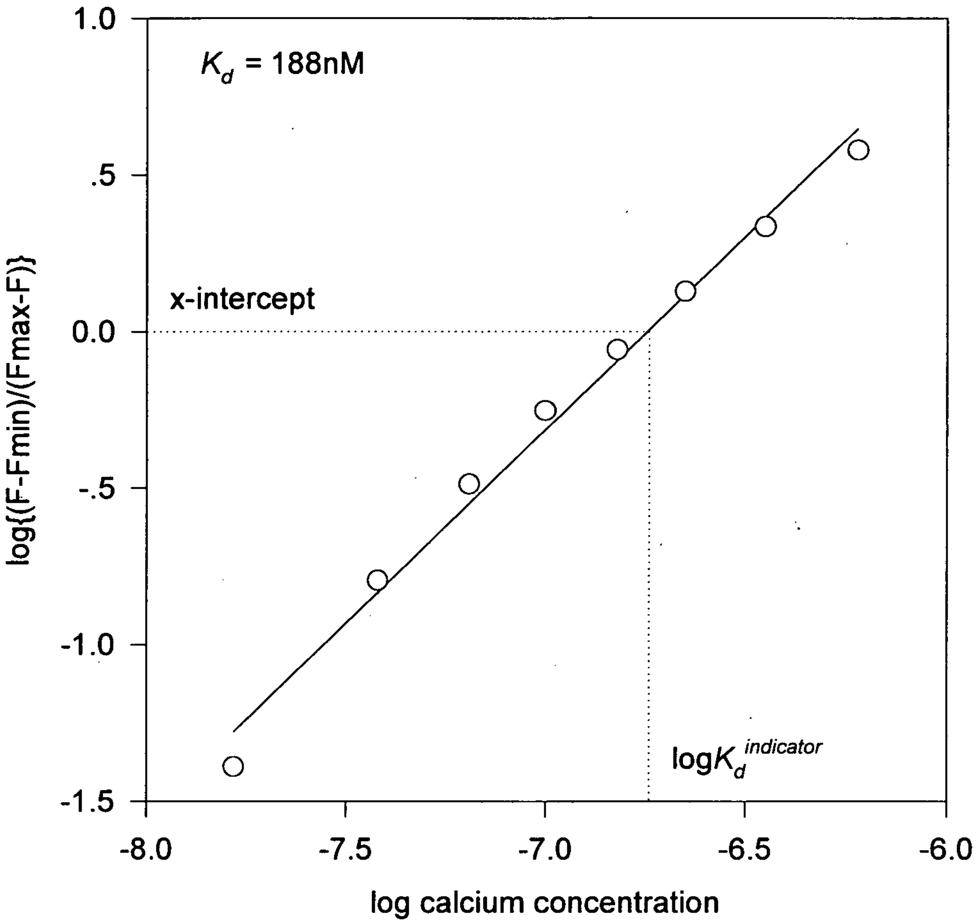
No comparable data exist for *Ph. parasitica*, but the method for production of zoospores as described in sections 2.2 and 2.9.1 was designed to keep the level of contaminant ions to a minimum. It was therefore assumed that the medium into which zoospores were ultimately released had an ionic strength of around 500μM and that this did not change to any significant extent during zoospore development. The dissociation constant of fura-2 for Ca^{2+} was therefore determined in a medium which resembled, as closely as possible, that in which fura-2 was employed during fluorimetry experiments (see section 5.2.3.1).

5.2.3 Calibration of the fura-2 response to Ca^{2+}

5.2.3.1 *In vitro* calibration of fura-2 to obtain $K_d^{fura\text{-}2}$ for Ca^{2+}

As described in detail in section 2.9.5, Calcium Calibration kits can be used to determine accurately the Ca^{2+} dissociation constant of fura-2 under known environmental conditions. It is important to determine the K_d of fura-2 under conditions which mimic, as closely as possible, the environment in which the dye is to be used. For example, cellular cytoplasm has a certain viscosity, pH and ionic

Figure 5.2 Calibration curve of fura-2. As a double log plot, the Ca^{2+} response of the indicator is linear, with the intercept being equal to the apparent $\log K_d^{indicator}$ (dotted lines).



strength (with contribution from other ions in addition to Ca^{2+}). All of these factors must be considered in determination of the dissociation constant of the dye. The calibration buffer kits obtained from Molecular Probes Inc. had an ionic strength of 100mM KCl; this ionic strength would cause motile *Ph. parasitica* zoospores to lyse. It was therefore necessary to re-calibrate fura-2 to obtain a dissociation constant for the conditions in the buffer used for motile zoospores. A concentrated form of the Calcium Calibration Buffer Kit was obtained from Molecular Probes Inc. The solutions in the kit were diluted in ion-free Tris-Cl (10mM) to obtain an ionic strength of 500μM KCl; pH 7.20 (ionic strength as found by Griffith *et al.*, 1988). Following the method described in detail in the protocol provided with the kits, these diluted solutions were used to determine the dissociation constant of fura-2 for Ca^{2+} under the following environmental conditions: 20°C, pH 7.20 and 500μM KCl. Successive excitation scans of fura-2 at an accurately defined free [Ca^{2+}] gave rise to spectra as shown in Figure 5.1. The peak fluorescence intensities at different known free [Ca^{2+}], as obtained from Figure 5.1, are shown in Table 5.3. A double log plot from these data (Figure 5.2) was now used to determine the K_d of fura-2 for Ca^{2+} under

Table 5.3 Data for plot of fura-2 (1μM) calcium response at 20°C, pH 7.20 and 500μM KCl.

[Ca^{2+}] (nM)	Peak fluorescence Intensity (F)	log [Ca^{2+}]	$\log \frac{(F-Fmin)}{(Fmax-F)}$
0	165.7		Fmin
16.7	178.7	-7.78	-1.389
37.6	211.6	-7.42	-0.794
64.5	247.2	-7.19	-0.487
100	284.5	-7.00	-0.253
150	320.6	-6.82	-0.057
225	355.8	-6.65	0.129
351	392.3	-6.45	0.335
602	427.9	-6.22	0.579
1350	464.0	-5.87	0.955
39800	497.1	-4.40	Fmax

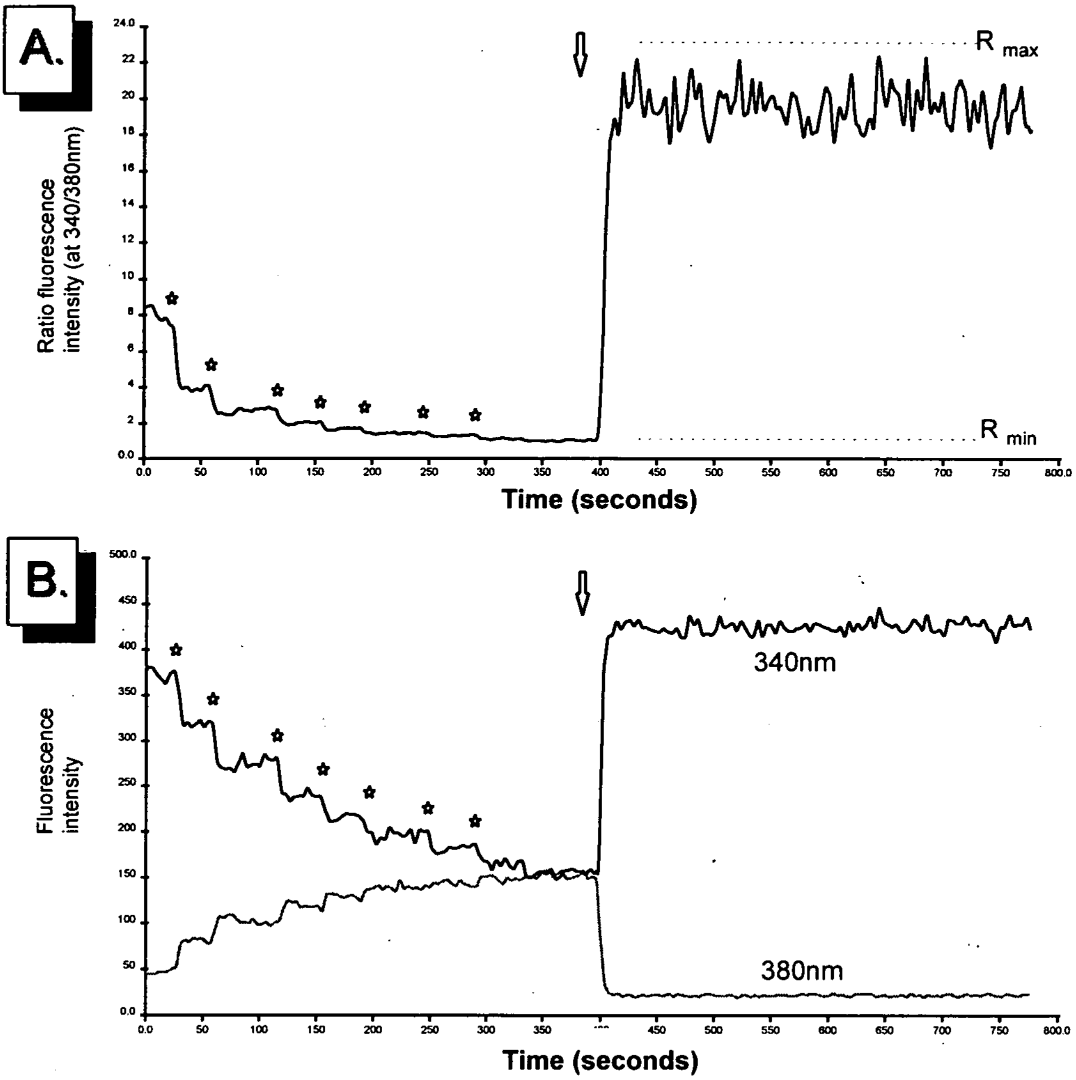
these known environmental conditions in which the probe was to be employed in fluorimetry experiments. The K_d of fura-2 for Ca^{2+} was found to be 188nM at 20°C, pH 7.20 and 500μM Kcl.

5.2.3.2 *In situ* calibration of fluorescence ratio data to obtain [Ca^{2+}]

Fura-2 binds Ca^{2+}, resulting in a shift in excitation wavelength as seen in Figure 5.1. Any spectrum will contain contributions from both bound and unbound probe, so the ratio of the intensities at the characteristic maxima for completely bound and unbound forms will be indicative of the concentration of Ca^{2+} present. Fura-2 responds to changes in [Ca^{2+}] with a change in this ratio (ie. the intensities at 340/380nm), thus allowing measurements to be made irrespective of cell path length, dye concentration, instrument-specific factors, etc. Cellular autofluorescence, however, must be taken into account.

The data were collected as the ratio fluorescence intensity at 340nm and 380nm excitation wavelength. The ratio data were calibrated at the end of each experiment in order to obtain the concentration of Ca^{2+}. Such an *in situ* calibration is shown in Figure 5.3. The ratio when fura-2 was saturated with Ca^{2+} (R_{max} value) and the ratio when fura-2 was in the unbound form only (R_{min} value) were both determined from an *in situ* calibration run at the end of each experiment, which was collected separately from the data run. During this calibration run the ratio was displayed in real time while the R_{max} and R_{min} were determined. R_{min} was first obtained by adding aliquots of 500mM EGTA in 10mM Tris-Cl, pH 7.20, until the minimal ratio was achieved. R_{max} was then obtained by adding aliquots of 1M Ca^{2+} in 10mM Tris-Cl, pH 7.20, until the maximal ratio was achieved. Autofluorescence was then measured on a population of cells of the same density but in the absence of fura-2; ten intensity measurements were made on the cell suspension at each of the two wavelengths. These ten autofluorescence intensity values were then subtracted from the analysis data at the relevant wavelengths during the calculation of [Ca^{2+}]. A computer program was employed to calculate [Ca^{2+}] from the ratio and calibration data using the following equation (Grynkiewicz *et al.*, 1985).

Figure 5.3. Representative *in situ* calibration of the fura-2 response to Ca^{2+} in determination of R_{min} and R_{max}, as performed at the end of each fluorimetry experiment. "A" shows the ratio of the fluorescence intensities at the excitation wavelengths of 340 and 380nm; the actual intensities at these wavelengths are shown in "B".



✿ 500mM Tris-EGTA (pH 7.20) addition.

⇩ 1mM Ca^{2+} addition.

$$[Ca^{2+}] = K_d \times \frac{(R - Rmin)}{(Rmax - R)} \times \frac{Sf2}{Sb2} \quad \text{at any time t}$$

Where: $[Ca^{2+}]$ is the calcium concentration at time t,
K_d is the dissociation constant of fura-2 as calculated under known environmental conditions (Section 5.2.3.1),
R is an experimental ratio value at time t,
R_{min} is the ratio value of unbound fura-2,
R_{max} is the ratio value of completely bound fura-2 in saturating Ca^{2+},
Sf2/Sb2 is the ratio of the intensities at the denominator wavelength for free and bound fura-2. This corrects for any wavelength biasing due to instrumental artefacts.

5.3 Experimental procedure

5.3.1 Development of a method using cell-impermeant fura-2 free acid to detect external Ca^{2+} in an encysting zoospore population

The failure of all attempts to load zoospores with fura-2 and to detect intracellular levels of free Ca^{2+}, described above, led to an alternative approach. It was decided to test whether fluorimetry and Ca^{2+}-sensitive fluorescent dyes could be used to report fluxes of Ca^{2+} in and out of encysting *Ph. parasitica* zoospores.

Dense suspensions of motile *Ph. parasitica* zoospores ($5x10^5$ zoospores ml^{-1} of 10mM Tris buffer, pH 7.20) were produced as described in sections 2.2 and 2.9.1. After addition of fura-2 (1µM final concentration) the background free Ca^{2+} concentration in these suspensions was between 250nM and 1µM, as detected by fluorimetry. Aliquots of 2.5ml zoospore suspension were transferred to 3ml glass cuvettes containing a magnetic stirrer bar and fura-2 (1mM stock) was added to give a final concentration of 1µM. The suspension was left for 5-10 minutes to allow the cuvette contents to attain a temperature of 20°C. An excitation scan was then performed to check that contaminating Ca^{2+} ions had not saturated the fura-2. This is a necessary step because it was found that fura-2 is saturated at a free $[Ca^{2+}]$ of

1.35μM (Figure 5.1) and any further Ca^{2+} addition is not detected by fura-2. Also, for free Ca^{2+} concentrations above 1μM, fura-2 is at its least sensitive. All experiments in which contaminating Ca^{2+} in the zoospore suspension was not in the range 250nM-1μM were discarded. A free [Ca^{2+}] below 200nM caused zoospores to lyse.

Time-drive experiments were performed in which fluorescence intensity was detected by dual excitation at 340nm and 380nm wavelengths. The ratio of fluorescence intensity at the two wavelengths was recorded for motile zoospores surrounded by 1μM fura-2 (Figure 5.1). After 2-3 minutes the experiment was paused, the zoospore suspension was rapidly pipetted into an acid washed, glass HPLC tube and vortexed for 70 seconds to induce encystment. The newly forming cyst population was rapidly returned to the cuvette and the experiment continued for up to 25-30 minutes to detect any changes in the Ca^{2+} levels in the buffer surrounding the developing zoospores. The entire process of vortex-induced encystment of the zoospore suspension and its subsequent transfer back into the cuvette took no longer than 80s. No significant difference in zoospore number was found at the end of the experiment, as tested using a haemocytometer, thereby excluding the possibility that changes in medium [Ca^{2+}] had occurred due to cell lysis.

When the experiment was complete, an *in situ* calibration of fura-2 was performed as described in detail in section 5.2.3.2. Any changes in the ratio of fluorescence levels at the two wavelengths could thereby be converted into values for [Ca^{2+}] in the bathing medium.

5.3.2 Results

5.3.2.1 Detection of external [Ca^{2+}] around an encysting zoospore population

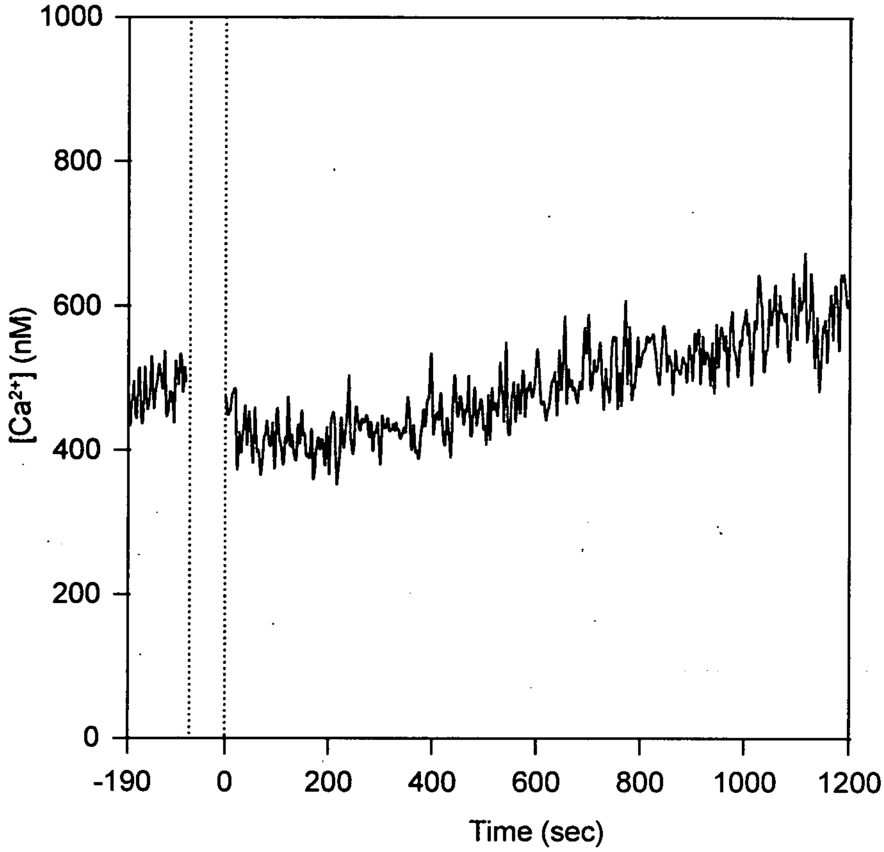
Data from 3 replicate experiments indicated that no net change in the level of Ca^{2+} in the medium surrounding motile zoospores occurred during the 2-3 minute period prior to induction of encystment (Figures 5.4-5.6). After this initial 2-3 minutes the zoospore suspension was vortexed for 70s to induce encystment, then the suspension was immediately transferred back into the cuvette and the experiment was continued for a further 20-30 minutes.

Within the first 3 minutes after the induction of encystment a highly significant reduction in the [Ca^{2+}] of the buffer surrounding the encysting zoospores occurred in each of the 3 replicate experiments (Figures 5.4-5.6, Table 5.4). After this initial 3 minutes, the [Ca^{2+}] in the medium slowly increased, reaching the initial level at typically 10-15 minutes after the induction of encystment (Figures 5.4-5.6). This elevation in [Ca^{2+}] in the medium continued until the experiments were terminated at 20 or 30 minutes after induction of encystment (eg. Figure 5.5). Figures 5.4-5.6 show that this pattern was highly reproducable in replicate tests. In all cases the final [Ca^{2+}] of the bathing medium was significantly higher than the initial concentration before or after vortex treatment of the spores (Table 5.4).

To test if fluorimetry had any deleterious effect on zoospores, a 100μl aliquot of the initial motile zoospore suspension (with added fura-2 but no other treatment) was encysted by vortexing for 70s. A 40μl aliquot was then transferred to a glass slide and incubated with an equal volume of distilled water for 1.5h, after which time the level of cyst germination was assessed by microscopic examination. Another 40μl aliquot was incubated for 30 minutes in a microcentrifuge tube before addition of 40μl of 2M KOH solution to test for the degree of encystment, assessed by counting the number of cysts with an alkali-resistant wall, using a haemocytometer. KOH has previously been used to distinguish between encysted (walled) and non-encysted (wall-less) zoospores because it lyses any non-walled cells (Tokunaga & Bartnicki-Garcia, 1971). Furthermore, at the end of each fluorimetry experiment a 40μl aliquot of the zoospore suspension was either transferred to a glass slide containing an equal volume of water (and 1mM Ca^{2+} final concentration in some experiments) to assess the level of cyst germination after a further 1.5h or an aliquot of cyst suspension was treated with KOH or water to determine the degree of encystment.

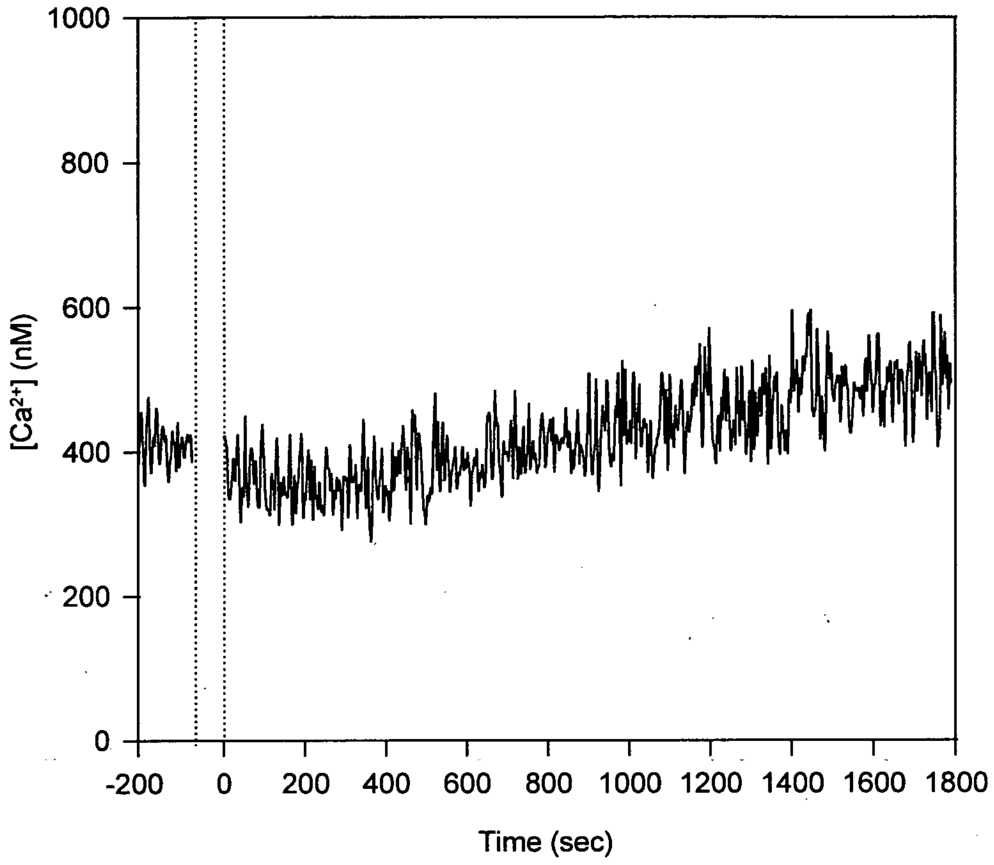
For each fluorimetry experiment, described below, a motile spore suspension was used at a cell concentration of $5x10^5$ zoospores ml^{-1}. Two minutes prior to fluorimetry experiments an aliquot of zoospore suspension was vortex

Figure 5.4 Change with time in the [Ca^{2+}] of the medium surrounding encysting *Phytophthora parasitica* zoospores, as detected by fluorimetry using fura-2 (1μM). Time scan from control replicate 1.



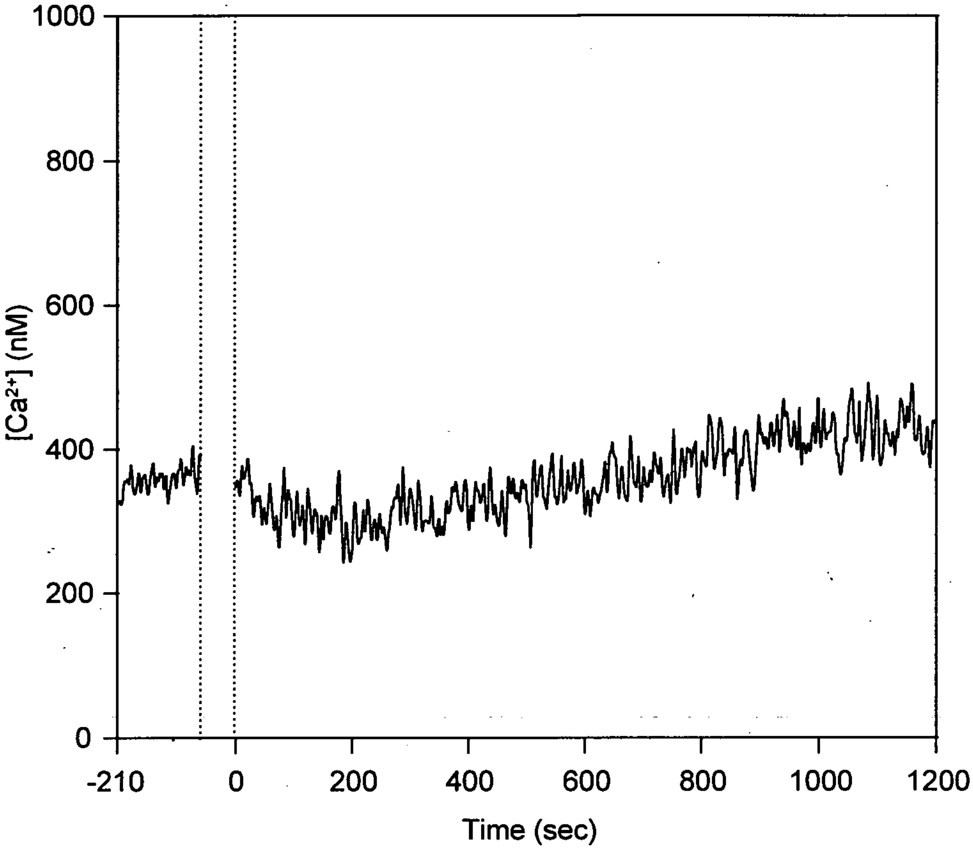
The period between the dotted lines indicates the time when the motile zoospore suspension was removed from the cuvette, encysted by vortex agitation for 70s and transferred back to the cuvette. The experiment was then continued.

Figure 5.5 Change with time in the [Ca^{2+}] of the medium surrounding encysting *Phytophthora parasitica* zoospores, as detected by fluorimetry using fura-2 (1 μM). Time scan from control replicate 2.



The period between the dotted lines indicates the time when the motile zoospore suspension was removed from the cuvette, encysted by vortex agitation for 70s and transferred back to the cuvette. The experiment was then continued.

Figure 5.6 Change with time in the [Ca^{2+}] of the medium surrounding encysting *Phytophthora parasitica* zoospores, as detected by fluorimetry using fura-2 (1μM). Time scan from control replicate 3.



The period between the dotted lines indicates the time when the motile zoospore suspension was removed from the cuvette, encysted by vortex agitation for 70s and transferred back to the cuvette. The experiment was then continued.

encysted and subsequently treated after 30 minutes with 1M KOH; it was found that $5.03 \pm 0.03 \times 10^5$ zoospores ml^{-1} (n=3) had successfully acquired an alkali-resistant cell wall. A separate aliquot of vortex encysted spores, incubated on glass slides, had germinated to a level of 26.3 ± 2.2% (n=3) after 1.5h. It was also found that $4.93 \pm 0.03 \times 10^5$ zoospores ml^{-1} (n=3) had acquired an alkali-resistant cell wall at the end of the fluorimetry experiments. Spores transferred from the cuvette at the end of fluorimetry experiments and incubated on glass slides germinated to a level of 24.3 ± 2.2% (n=3) after 1.5h. These findings indicated that fluorimetry experiments had not prevented zoospore development into cysts and had no deleterious effects on the germinability of cysts.

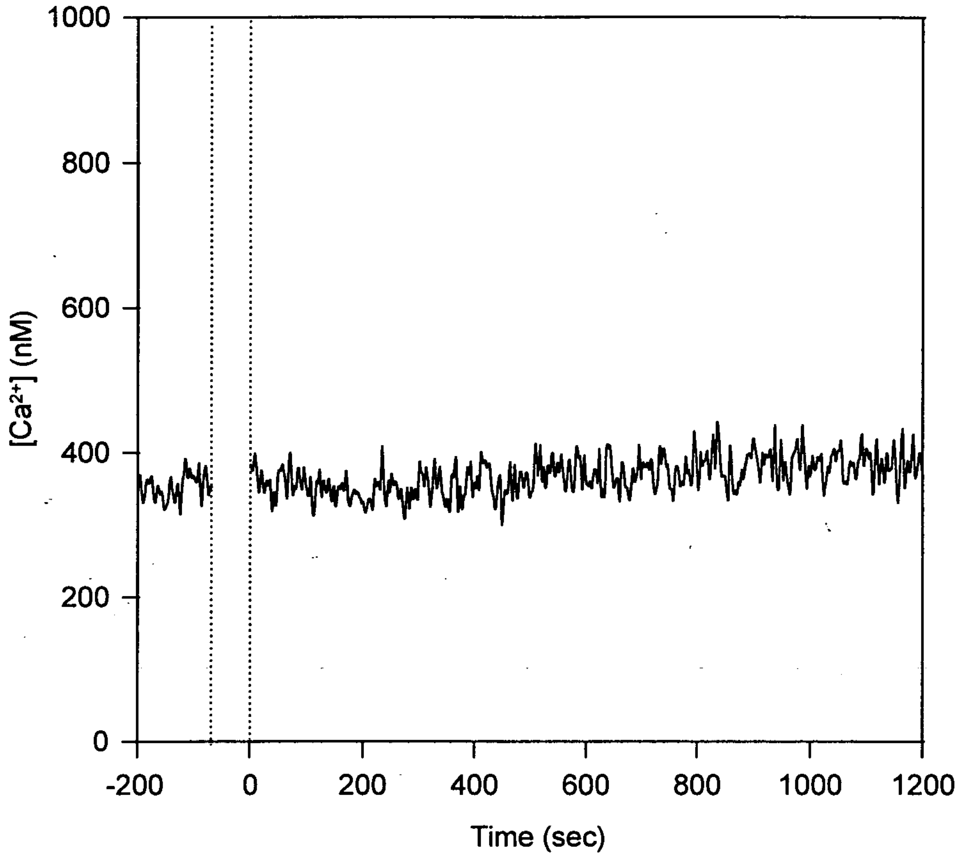
5.3.2.2 The effects of verapamil and lanthanum

A motile zoospore suspension containing 1μM fura-2 was treated with either verapamil (10μM final concentration) or La^{3+} (1μM final concentration) 1 minute prior to the start of fluorimetry measurements. Then a scan was performed and, after 2-3 min, the zoospores were encysted and the experiment was continued as described in section 5.3.2.1. No significant reduction in cell number was observed at the end of the experiment: $4.98 \pm 0.02 \times 10^5$ zoospores ml^{-1} (n=2) compared with an original value of $5.00 \pm 0.02 \times 10^5$ ml^{-1}, assessed with a haemocytometer.

The concentration of Ca^{2+} in the medium surrounding motile spores (5×10^5 zoospores ml^{-1}) suspended in the presence of either verapamil (10μM) or La^{3+} (1μM) was stable, with no obvious increase or decrease during the initial 2-3 minute period before vortex treatment (Figures 5.7, 5.8 and 5.9, 5.10 respectively). After the zoospore suspension was vortexed for 70 seconds, transferred back to the cuvette and the experiment resumed; the level of Ca^{2+} in the medium around La^{3+}-treated spores did not decrease, and during further incubation the $[Ca^{2+}]$ of the medium rose slightly and progressively so that by the end of the experiments this level was significantly higher than it was initially (Table 5.4). Similarly, the Ca^{2+} level surrounding zoospores treated with verapamil did not decrease after vortex-induced encystment. There was a significant overall elevation in the Ca^{2+} level at the end of one experiment that was statistically analysed (Table 5.4) although this was small

Figure 5.7 Change with time in the [Ca^{2+}] of the medium surrounding encysting *Phytophthora parasitica* zoospores in the presence of verapamil (10μM), as detected by fluorimetry using fura-2 (1μM).

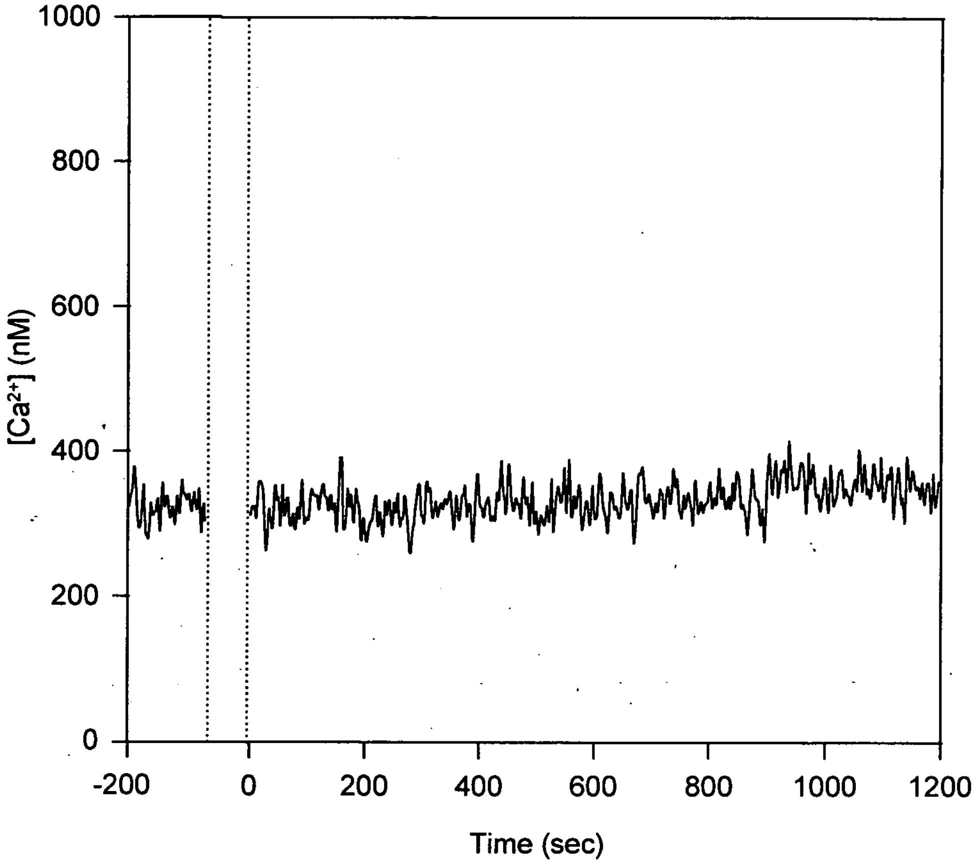
Time scan from replicate 1.



The period between the dotted lines indicates the time when the motile zoospore suspension was removed from the cuvette, encysted by vortex agitation for 70s and transferred back to the cuvette. The experiment was then continued.

Figure 5.8 Change with time in the [Ca^{2+}] of the medium surrounding encysting *Phytophthora parasitica* zoospores in the presence of verapamil (10μM), as detected by fluorimetry using fura-2 (1μM).

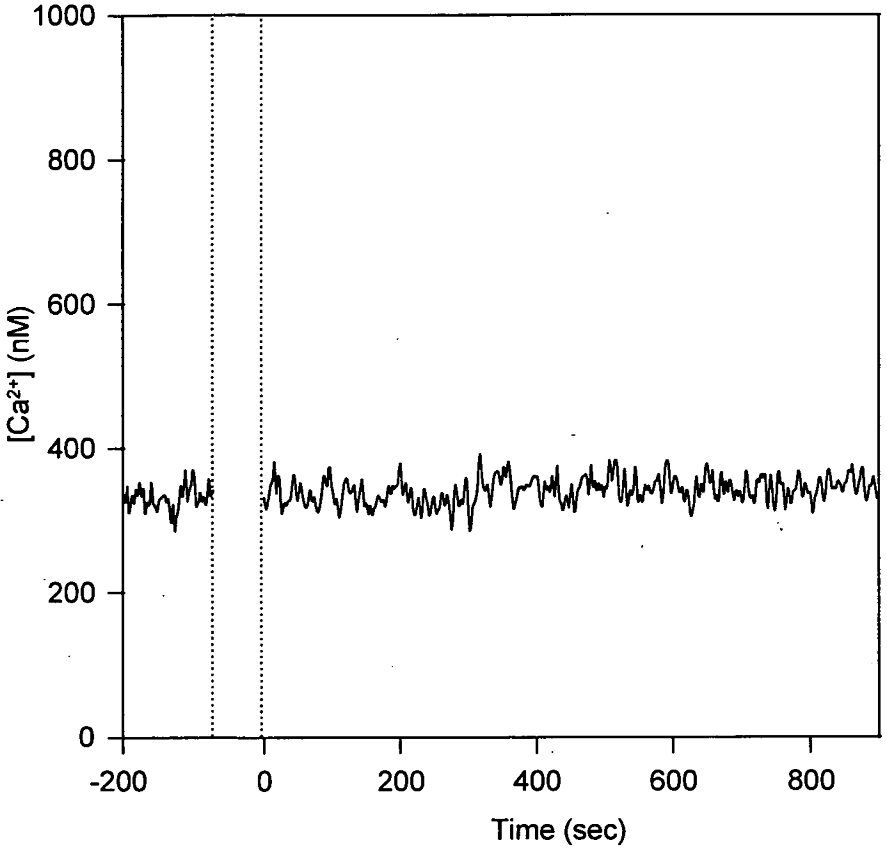
Time scan from replicate 2.



The time period between the dotted lines indicates the time when the motile zoospore suspension was removed from the cuvette, encysted by vortex agitation for 70s and transferred back to the cuvette. The experiment was then continued.

Figure 5.9 Change with time in the [Ca^{2+}] of the medium surrounding encysting *Phytophthora parasitica* zoospores in the presence of La^{3+} (1μM), as detected by fluorimetry using fura-2 (1μM).

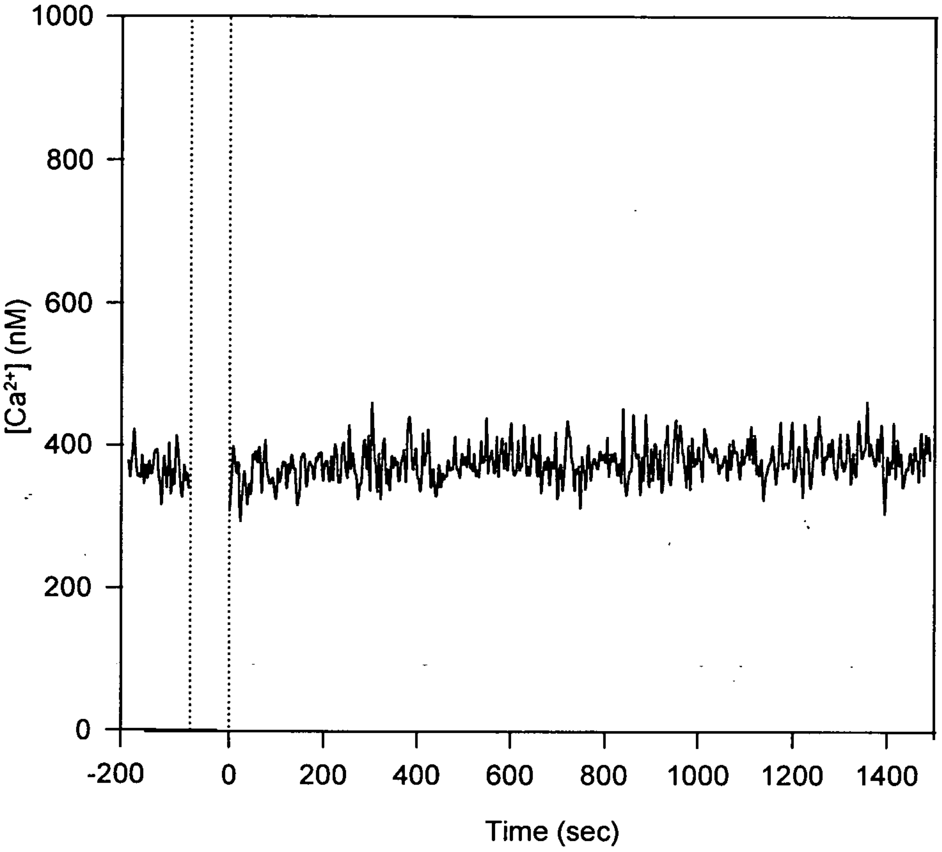
Time scan from replicate 1.



The period between the dotted lines indicates the time when the motile zoospore suspension was removed from the cuvette, encysted by vortex agitation for 70s and transferred back to the cuvette. The experiment was then continued.

Figure 5.10 Change with time in the [Ca^{2+}] of the medium surrounding encysting *Phytophthora parasitica* zoospores in the presence of La^{3+} (1μM), as detected by fluorimetry using fura-2 (1μM).

Time scan from replicate 2.



The period between the dotted lines indicates the time when the motile zoospore suspension was removed from the cuvette, encysted by vortex agitation for 70s and transferred back to the cuvette. The experiment was then continued.

compared with the changes seen in the control experiments.

Prior to fluorimetry experiments and before addition of verapamil and La^{3+}, 4.95 ± 0.05×10^5 (n=2) and 5.0 ± 0.0×10^5 (n=2) zoospores ml^{-1}, respectively, were found to have successfully developed an alkali-resistant cyst wall 30 minutes after vortex encystment. A similar test at the end of each fluorimetry experiment, showed that only 0.45 ± 0.05×10^5 zoospores ml^{-1} (n=2) of verapamil treated spores had successfully developed an alkali-resistant cell wall, and only 0.25 ± 0.05×10^5 zoospores ml^{-1} (n=2) of La^{3+}-treated spores had developed an alkali-resistant cell wall. At the end of fluorimetry experiments, verapamil treated spores transferred to slides containing an equal volume of distilled water had lysed after 1.5h; incubation with an equal volume of 2mM Ca^{2+} in microcentrifuge tubes at the time when cells were added to slides resulted in 3.20 ± 0.05 zoospores ml^{-1} (n=2) having developed an alkali-resistant cell wall after 1.5h; 21.0 ± 1.0% (based on counts of 100 spores in 2 replicate experiments) of the observed cells had germinated. Extensive lysis also occurred in spore populations treated with La^{3+} and incubated in water for 1.5h; however, after incubation in Ca^{2+} (1mM) for 1.5h, 2.50 ± 0.05 zoospores ml^{-1} (n=2) had developed an alkali-resistant cell wall, and 14.0 ± 1.0% (based on counts of 100 spores in 2 replicate experiments) of these observed cells had germinated after 1.5h.

?

$\times 10^5$?

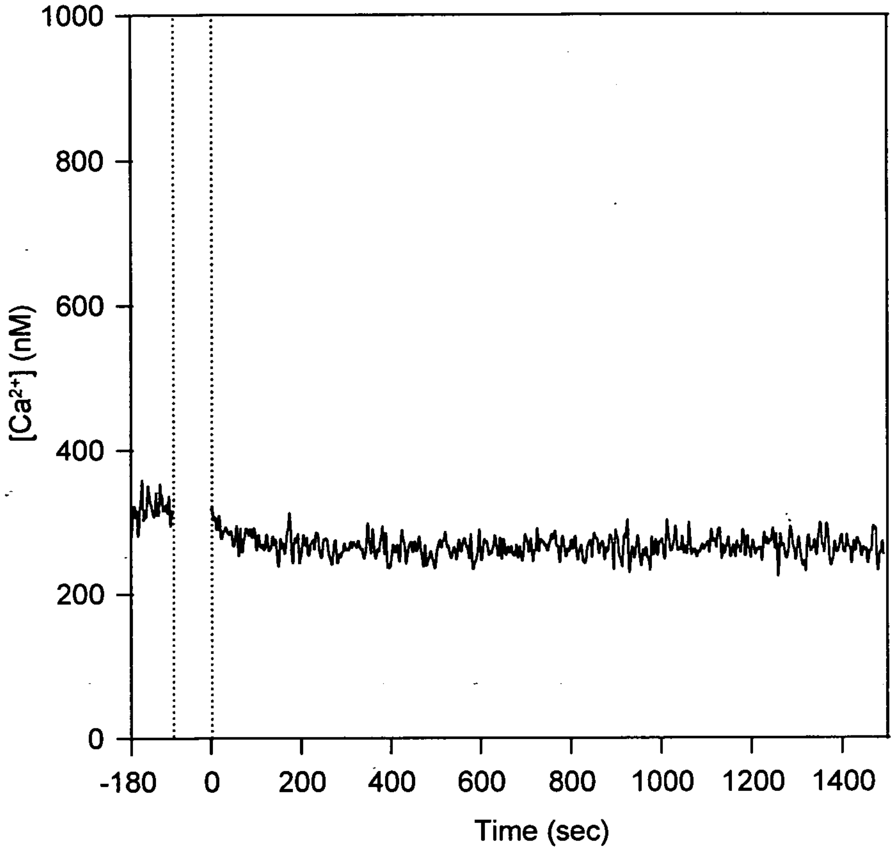
5.3.2.3 The effects of TMB-8

A motile zoospore suspension containing 1μM fura-2 was treated with TMB-8 (5μM final concentration) and subjected to the same procedure as in section 5.3.2.1. No significant reduction in cell number was observed at the end of the experiment: 5.00 ± 0.02×10^5 zoospores ml^{-1} (n=2) compared with an original value of 5.02 ± 0.02×10^5 zoospores ml^{-1} (n=2) , assessed with a haemocytometer.

No apparent change in [Ca^{2+}] of the medium occurred during the initial 2-3 minute period of the experiment (Figures 5.11, 5.12). However, after vortexing the zoospore suspension for 70 seconds and resuming the experiment, a highly

Figure 5.11 Change with time in the [Ca^{2+}] of the medium surrounding encysting *Phytophthora parasitica* zoospores in the presence of TMB-8 (5μM), as detected by fluorimetry using fura-2 (1μM).

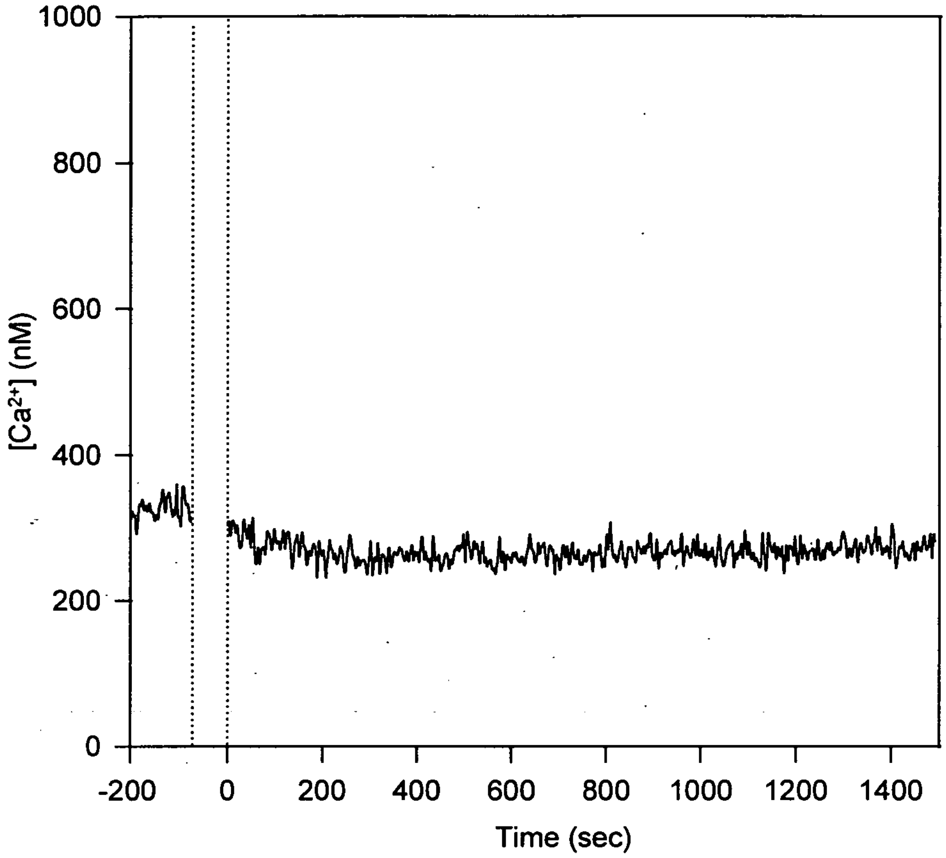
Time scan from replicate 1.



The period between the dotted lines indicates the time when the motile zoospore suspension was removed from the cuvette, encysted by vortex agitation for 70s and transferred back to the cuvette. The experiment was then continued.

Figure 5.12 Change with time in the [Ca^{2+}] of the medium surrounding encysting *Phytophthora parasitica* zoospores in the presence of TMB-8 (5μM), as detected by fluorimetry using fura-2 (1μM).

Time scan from replicate 2.



The period between the dotted lines indicates the time when the motile zoospore suspension was removed from the cuvette, encysted by vortex agitation for 70s and transferred back to the cuvette. The experiment was then continued.

Table 5.4 Calcium concentrations (nM) detected by fluorimetry of the dye fura-2 in the medium surrounding zoospores or encysting zoospores of *Ph. parasitica*. Fluorimetry of cell suspensions was assessed before vortex-encystment and at representative times after vortex treatment[*].

Treatment	[Ca^{2+}] (nM) before vortex treatment (-89 to -17 s)	[Ca^{2+}] (nM) at different times after vortex-induced encystment					
		Time 1 (0-72 s)	Time 2 (603-675 s)[+]	Time 3 (1125-1197 s)[+]	Time 4 (1718-1790 s)	5%LSD	0.1%LSD
Control Expt. 1 (Figure 5.4)	**482.3**	**427.77**	**489.83**	**580.73**		**15.5**	**26.38**
Control Expt. 2 (Figure 5.5)	**417.08**	**365.43**	**388.25**	**486.14**	**524.43**	**21.66**	**36.87**
Control Expt. 3 (Figure 5.6)	**343.02**	**329.89**	**336.67**	**391.67**		**3.78**	**6.44**
Verapamil (10μM; Figure 5.7)	**350.58**	**363.46**	**366.66**	**398.8** (1419-1491 s)		**9.69**	**16.5**
Lanthanum (1μM; Figure 5.9)	**330.19**	**336.13**	**339.69** (401-473 s)	**350.53** (826-898 s)		**6.49**	**11.05**
TMB-8 (5μM) Expt. 1 (Figure 5.11)	**328.45**	**287.47**	**264.32**	**266.14**		**6.23**	**10.61**
TMB-8 (5μM) Expt. 2 (Figure 5.12)	**321.54**	**284.83**	**261.98**	**265.9**		**6.38**	**10.85**

[*] Blocks of 39 data points corresponding to 72 s intervals in the traces in Figures 5.4-5.7, 5.9, 5.11-5.12) were chosen arbitrarily for analysis of [Ca^{2+}] of the bathing medium of the spores; the data were subjected to 1-way ANOVA. In all cases the data blocks represented the periods (1) -89 to -17 s before vortex treatment; (2) 0-72 s immediately after vortex treatment; (3) the final 72 s before an experiment was terminated; (4) one or two intermediate periods, usually about half-way through the post-vortex incubation

[+] Except where shown in parenthesis in the table.

significant decrease in the extracellular [Ca^{2+}] occurred within the first 3 minutes (Table 5.4) and this decrease continued up to 10 min, then there was with no further decrease or increase observable after this time (up to 25 minutes).

Prior to fluorimetry experiments on zoospore populations ($5x10^5$ zoospores ml^{-1}), and before treatment with TMB-8, an aliquot of motile zoospores was vortex agitated and left for 30 minutes. It was found that $4.95 \pm 0.05x10^5$ zoospores ml^{-1} (n=2) had acquired an alkali-resistant cyst wall. After treatment with TMB-8 and fluorimetry, only 2.1 ± 0.1 $x10^5$ zoospores ml^{-1} (n=2) had successfully developed an alkali-resistant cell wall, although $5.00 \pm 0.02x10^5$ zoospores ml^{-1} (n=2) were counted before alkali-treatment. At the end of the experiments, zoospores incubated with distilled water on glass slides for 1.5h germinated to a level of 7.0 ± 2.0% (n=2) whereas addition of Ca^{2+} (1mM) elevated the germination level to 27.0 ± 1.0% (n=2).

5.4 Discussion

The experiments here have involved developing and exploiting a method for assessing fluorimetry of the Ca^{2+}-sensitive dye fura-2 as a potentially precise means of quantification of Ca^{2+} around populations of motile zoospores during their development into cysts. The experiments have investigated transients in [Ca^{2+}] in the medium surrounding *Ph. parasitica* zoospore populations induced to encyst synchronously in response to vortex agitation. It has been shown that changes in medium [Ca^{2+}] occur during the development of motile zoospores into cysts and that treatment of zoospores with Ca^{2+}-channel blocking agents and an intracellular Ca^{2+} antagonist can lead to an alteration in these transients. In addition, these Ca^{2+} modulators were found to inhibit the formation of an alkali-resistant cyst wall and suppress subsequent germination. A relationship between Ca^{2+} transients and zoospore development was thereby demonstrated.

Critical evaluation of the role of Ca^{2+} during *Ph. parasitica* zoospore development required its quantitative measurement in real time. Fura-2 has become the most popular fluorescent probe with which to monitor dynamic changes in intracellular free [Ca^{2+}] during cell development. With the synthesis of fura-2 by Tsien and colleagues (Grynkiewicz *et al.*, 1985), measurement of [Ca^{2+}] has become relatively easy to perform. The major advantage of fura-2 over other Ca^{2+} titration methods is its high affinity for Ca^{2+} and rather low affinity for H^+ (especially in the physiological range), Mg^{2+} and heavy metals. Fura-2 also undergoes large absorbance changes upon binding to Ca^{2+} and negligible binding to membranes compared to its predecessor quin-2. Fura-2 shows much stronger fluorescence than quin-2 and undergoes a greater spectral shift upon binding to Ca^{2+}. This allows fura-2 to be used ratiometrically, making measurement of [Ca^{2+}] essentially independent of such factors as the extent of dye loading, cell thickness, photobleaching and dye leakage.

To facilitate cell loading, Molecular Probes Inc. and Calbiochem offer AM esters of fura-2. Attempts to demonstrate that changes in intracellular [Ca^{2+}] accompany encystment were hampered because evidence presented here suggests that fura-2 AM is not taken up by motile *Ph. parasitica* zoospores, or at least fura-2 is not present in the cytosol in a responsive state.

Calcium Calibration Buffer Kits from Molecular Probes Inc. allow accurate determination of the Ca^{2+} dissociation constant of fura-2 under known environmental conditions using particular instrumentation. The method produces a clearly defined isosbestic point for ratiometric indicators such as fura-2, an effect observed during its use in this chapter (Figure 5.1).

Cell population experiments were chosen due to the availability of a dual excitation spectrofluorimeter although it is probable that the kinetics of Ca^{2+} responses in single cells (Jaffe, 1993) may be averaged out in population measurements. However, employing this technique, it was found that changes in the [Ca^{2+}] of the medium surrounding encysting *Ph. parasitica* zoospores could be accurately measured and lie within the dynamic range detectable by fura-2, with no apparent deleterious effect on the zoospores. *In situ* calibration was relatively straightforward compared with calibration of dyes in an intracellular location

(Thomas & Delaville, 1991). However, *in vitro* re-calibration had to be performed to obtain the K_d of fura-2 for Ca^{2+} under the environmental conditions in which it was to be employed. Furthermore, certain tests could not be carried out for comparison with agitation-induced encystment, such as the effects of K^+ or Sr^{2+} -induced encystment, because the required concentration of these ions would alter the dissociation constant of fura-2 for Ca^{2+} and lead to inaccuracies in determination of $[Ca^{2+}]$.

Initially, to investigate whether zoospore encystment involved changes in cytosolic free Ca^{2+}, denoted $[Ca^{2+}]_c$, attempts were made to load a motile zoospore population with an esterified form of fura-2 (fura-2 AM). Incubation with 30μM fura-2 AM for up to 60 minutes (Table 5.1) did not cause premature encystment of the motile zoospore population whereas longer loading times did so. After attempts to remove extracellular dye, fluorimetry was used to determine if the dye had been taken up by the cells. An excitation scan was performed and the spectrum obtained indicated that the dye was present in the cell population sample. However, the dye fluorescence was non-responsive to experimentally-induced increases in $[Ca^{2+}]_c$ by addition of an ionophore in the presence of excess Ca^{2+}. Reducing $[Ca^{2+}]_c$ by addition of the Ca^{2+} chelator EGTA to the external medium (Read *et al.*, 1992a) also had no effect on dye fluorescence. Lysing cells with detergent to release the dye, and subsequent adjustment of the medium $[Ca^{2+}]$ with Tris-EGTA and Ca^{2+} (Thomas & Delaville, 1991) had no effect on the fluorescence spectrum of the dye, indicating that the spectrum obtained was from extracellular fura-2 AM which had not been removed by washing. That the extracellular dye was unresponsive to changes in $[Ca^{2+}]$ suggested that extracellular hydrolysis of the esterified dye had not occurred. Similarly, *Ph. cinnamomi* zoospores failed to load fluorescent dye when incubated with quin-2 AM (Cork, 1986); however, *Ph. parasitica* zoospores take up fluorescein diacetate (data not shown), so the cells do contain cytosolic esterases. So, it seems that the ability of cells to load a particular dye is related to the type of dye used (Knight *et al.*, 1993).

It is known that rapid, synchronous zoospore encystment is initiated in *Phytophthora* spp. and *Pythium* spp. by mechanical agitation (Tokanunga &

Bartnicki Garcia, 1971; Irving *et al.*, 1984; Donaldson & Deacon, 1992). Synchronous encystment can also be induced in *Phytophthora* by addition of Ca^{2+}, Sr^{2+} or cyclic nucleotides (Irving *et al.*, 1984).

By using fura-2 to detect changes in $[Ca^{2+}]$ in a zoospore bathing medium, a consistent decrease in medium $[Ca^{2+}]$ was found after synchronous encystment of a motile *Ph. parasitica* population was induced by vortex treatment. The $[Ca^{2+}]$ of the medium surrounding the spores decreased within the first 3 minutes after the stimulus to encyst. After this time, the $[Ca^{2+}]$ of the medium gradually increased to above that measured around the initially untreated zoospores. This provides evidence that the stimulus to encyst induces a net inflow of Ca^{2+} from the external medium. If so, then the resulting transitory elevation in cytosolic $[Ca^{2+}]$ could act as a primary intracellular signal in activating the events involved in encystment or post-encystment. A subsequent Ca^{2+} efflux then occurred, possibly as a result of activation of Ca^{2+}-ATPase to lower the cytosolic $[Ca^{2+}]$ to the resting level, thereby avoiding Ca^{2+} cytotoxicity (Carafoli, 1987; Jackson & Heath, 1993; Gadd, 1995). For technical reasons it was not possible to monitor fluorescence during the application of an encystment stimulus because treatments known to cause synchronous encystment, such as K^+, would have an effect on the fura-2 molecule, leading to an inaccurate estimation of $[Ca^{2+}]$. Increases in medium $[Ca^{2+}]$ have also been detected adjacent to large aggregates of encysted *Ph. palmivora* zoospores using Ca^{2+}-sensitive microelectrodes (Reid *et al.* 1995). The morphological changes which accompany encystment, such as exocytosis and cytoskeletal rearrangements, are thought to be Ca^{2+}-dependent (Hemmes & Pinto Da Silva, 1980; Jaffee, 1983; Sobue *et al.*, 1983).

Calcium channel blocking drugs are commonly used to demonstrate inhibition of Ca^{2+}-entry-mediated stimulation of development in a wide variety of fungi, including the encystment of *Ph. palmivora* (Iser *et al.*, 1989) and germination of *Pythium* zoospores (Donaldson & Deacon, 1992). Verapamil hydrochloride is an organic Ca^{2+}-channel blocker in mammalian cells (Godfraind *et al.*, 1986). This drug slowed the rate at which zoospores encysted and delayed the entry of $^{45}Ca^{2+}$ into pectin-stimulated *Ph. palmivora* zoospores (Iser *et al.*, 1986). Both verapamil and

La^{3+} (an inorganic blocker of Ca^{2+}-channels) inhibited germination of encysted *Pythium* zoospores (Donaldson & Deacon, 1992).

In the work reported here, when verapamil and La^{3+} were tested for their effect during encystment of *Ph. parasitica*, the initial decrease in medium [Ca^{2+}] around control (untreated zoospores) did not occur, suggesting that influx of Ca^{2+} into spores, during the early stages after the stimulus to encyst, occurs via Ca^{2+}-entry channels. In fact, both verapamil and La^{3+} caused a significantly early increase in the medium [Ca^{2+}] after vortex-induction of encystment, and there was a slower, progressive increase in the medium [Ca^{2+}] during further incubation of the cells. Blockage of Ca^{2+} influx by La^{3+} and verapamil resulted in a dramatic decrease in the number of immobilised zoospores which had developed an alkali-resistant cyst wall, suggesting that a Ca^{2+} influx, after the stimulus to encyst, is involved in the events leading to successful development of a cyst wall.

A similar mechanism, whereby contact with a surface facilitates an influx of cations leading to spore 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. Furthermore, differentiation could be inhibited by Gd^{3+}, a blocker of mechanosensitive ion channels, which can be partly overcome in the presence of Ca^{2+} (Zhou *et al*, 1991).

In experiments where zoospores were incubated with TMB-8, an early decrease in medium [Ca^{2+}] occurred following vortex treatment but this was not followed by an increase as observed in the control experiments. Instead, there was a continuing slow decrease in the medium [Ca^{2+}] following the initial sharp decrease, although this decrease was not sustained during the whole incubation time. TMB-8 is an inhibitor of intracellular Ca^{2+} release (Europe-Finner & Newell, 1984) but may also affect various other signalling systems, including inhibition of the protein kinase C system (Simpson *et al.*, 1984) which is present in fungi (Favre & Turian, 1987). This hypothesis was not tested, but while Ca^{2+} influx occurs at an early stage in encystment it is possible that intracellular Ca^{2+} also contributes to the signal transduction chain either by being released from intracellular stores directly by the primary developmental signal or through Ca^{2+}-induced Ca^{2+} release (Endo, 1977). Consistent with this, blockage of Ca^{2+} entry with channel blocking drugs also prevented a subsequent Ca^{2+} release into the medium, suggesting that elevation of cytosolic $[Ca^{2+}]$ by influx can influence Ca^{2+} release from intracellular stores thereby amplifying the signal. Such amplification could result in Ca^{2+} cytotoxicity if the Ca^{2+} level was not tightly regulated by Ca^{2+} efflux transporter systems such as the plasma membrane H^+/Ca^{2+}-ATPase proposed to be present in fungal cells (Miller *et al.*, 1990; Grzemski *et al.*, 1994).

Recently, Reid *et al.* (1995) found that TMB-8 (10-50μM) suppressed encystment of *Ph. palmivora* zoospores. In the present study, and in contrast to the dramatic reduction in the number of spores which had successfully developed an alkali-resistant cyst wall when treated with Ca^{2+}-channels blockers, TMB-8 (5μM) treatment only reduced the number of spores which formed a wall to approximately 40%. It is possible, therefore, that Ca^{2+} influx is sufficient to trigger the events leading to cyst wall formation in *Ph. parasitica* as long as a threshold concentration is attained.

Germination levels in the control experiment were low: only one quarter of the zoospores had germinated on glass slides 1.5h after the fluorimetry experiment had been completed. This low germination could have resulted from low Ca^{2+} levels in the medium due to the nature of the experiment - a balance had to be struck between maintaining the $[Ca^{2+}]$ such that zoospores would differentiate into cysts, but that fura-2 would not become saturated with Ca^{2+} and therefore be unresponsive to further increases in Ca^{2+}. Of the 50% of zoospores which encysted in the presence of TMB-8, 7% went on to germinate after incubation with distilled water for 1.5h on slides. Treatment with 1mM Ca^{2+} elevated germination to a level comparable with that of the control experiment. Therefore, it seems that the trigger for germination of *Ph. parasitica* spores involves an influx of Ca^{2+} and a contribution from release from intracellular stores if the external Ca^{2+} concentration is not high enough to offset this requirement.

In an encysting *Ph. palmivora* zoospore suspension it is suggested that Ca^{2+} is rapidly reabsorbed by cells after it has been released (Irving *et al.*, 1984; Iser *et al.*, 1989). In view of this, Donaldson & Deacon (1992) suggested that an influx of Ca^{2+} triggers germination of *Pythium* cysts, that EGTA suppresses germination by complexing with Ca^{2+} released from the spores or present in 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. Donaldson & Deacon (1992) also showed that zoospores transferred rapidly to glass slides after induction of encystment germinated at a higher percentage than did cells kept in suspension. This was suggested to be because the zoospores adhere to a glass surface and at least some released Ca^{2+} is trapped in the adhesive and thus available for reuptake.

The hypothesis, in short, is that Ca^{2+} fluxes across the cell membrane play a central role during encystment and germination, but Mg^{2+} and Sr^{2+} can act as surrogate cations and that 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 (Iser *et al.*, 1989; Donaldson & Deacon, 1992). However, the results presented in this chapter suggest that an influx of Ca^{2+} precedes an efflux when *Ph. parasitica* zoospores are stimulated to encyst synchronously by vortex agitation. Furthermore, pharmacological agents which alter these fluxes also perturb the successful transformation of the motile zoospore into a cyst.

Irving *et al.* (1984) reported that an efflux of Ca^{2+} occurred when *Ph. palmivora* zoospores were induced to encyst by addition of pectin, Sr^{2+}, cyclic nucleotides and by mechanical agitation, and that this was followed by resorption of Ca^{2+}. However, Irving *et al.* (1984) do not provide data for encystment induced by agitation. For experiments using this encystment method, these workers reported that a reduction in cell Ca^{2+} occurred but a corresponding rise in supernatent Ca^{2+} was not detected, and they suggested that a rise in supernatent Ca^{2+} may have occurred before sampling began, but was missed due to the nature of these particular experiments. Unfortunately, the experiments in this chapter were limited to vortex-induced encystment and it was not possible to monitor [Ca^{2+}] during the 70 sec vortexing period. However, if there had been an initial major efflux of Ca^{2+} during vortex treatment then it would need to have been more than compensated by an uptake of Ca^{2+}, all within 70-80 sec; otherwise the fluorimetry traces would not have recorded a major **decrease** in medium [Ca^{2+}] immediately after vortex treatment in the controls. In short, the results obtained by fluorimetry are at variance with those of Irving *et al.* (1984) who, at least for Sr^{2+} or pectin induced encystment of *Ph. palmivora*, recorded an initial increase of radiolabelled $^{45}Ca^{2+}$ equivalent to about 750 nmoles in the supernatent around zoospores at a cell concentration of $2.3x10^6$ ml^{-1}. Thereafter, there was a small influx equivalent to about 200 nmoles of Ca^{2+} over a 10-15 minute period. In the present study, fluorimetry indicated that vortex encysted zoospores of *Ph. parasitica* ($5x10^5$ zoospores ml^{-1}) absorbed about 100nM Ca^{2+} from the bathing medium during vortex treatment and then slowly and progressively released Ca^{2+} into the medium, so that the medium [Ca^{2+}] after 10 to 12 minutes was similar to that around the zoospores before encystment, and the medium [Ca^{2+}] then increased further to a level significantly above the initial level. It is difficult to reconcile these findings with those of Irving *et al.* (1984), especially as these workers provided no data for vortex-induced encystment.

Iser *et al.* (1986) reported an inward flux of Na^+ ions into *Ph. palmivora* zoospores 3-5 min after pectin-induced encystment. Influxes or effluxes of ions such as Na^+ would presumably alter the ionic strength of the medium surrounding encysting zoospore populations, and this could potentially have an effect of the dissociation constant of fura-2 for Ca^{2+}, thereby giving an inaccurate estimate of [Ca^{2+}]. To determine whether changes in ionic strength alter the K_d of fura-2 for Ca^{2+}, a calculation was performed which employed the Debye-Huckel limiting law of ionic solutions. It was found that a change in ionic strength during encystment, of up to 100µM due to monovalent cation species, would have no significant effect on the K_d of fura-2 for Ca^{2+} (Appendix 7). Iser *et al.* (1986) reported an uptake of approximately 100 pmoles of Na^+ per 10^6 cells for *Ph. palmivora*. In this study, $5x10^5$ zoospores ml^{-1} were used so it is likely that any exchange of ions other than Ca^{2+} would have negligible effect on the K_d of fura-2 for Ca^{2+} during fluorimetry experiments.

Some contradictions concerning a role of Ca^{2+} exist within the available data on zoospore encystment. For example, while external Ca^{2+} at 5mM alone, or at 1mM in the presence of a Ca^{2+}-ionophore (A23187), initiates encystment and germination in *Ph. palmivora* (Irving *et al.*, 1984), and Sr^{2+} substitutes for Ca^{2+}, the addition of A23187 in the presence of only 100µM Ca^{2+} did not initiate encystment even though A23187 did initiate Ca^{2+} uptake (Irving *et al.*, 1984). Furthermore, encystment induced by pectin and mechanical agitation occurred when zoospores had been released into and maintained in a Ca^{2+}-free medium, by EGTA addition (Irving *et al.*, 1984). In separate experiments on *Ph. palmivora* it was found that an external $[Ca^{2+}]$ of >100nM was necessary for encystment and Ca^{2+} alone at a concentration of ≥2µM could induce encystment (Griffith *et al.*, 1988). Also, cAMP applied externally induced both encystment and Ca^{2+} efflux in *Ph. palmivora* (Irving *et al.*, 1984) and pectin-induced encystment was followed rapidly (20s) by a marked increase in phophatidic acid (PA) in *Ph. palmivora* zoospores, with small elevations in cAMP and inositol phosphates during the same period (Zhang *et al.*, 1992). Externally supplied PA also induced encystment but only if Ca^{2+} was present in the bathing medium (Zhang *et al.*, 1992).

Irving *et al.*, (1984) predict that increases in cytosolic $[Ca^{2+}]$ result from an intracellular source rather than an external one. Evidence presented here suggests than an elevation in cytosolic $[Ca^{2+}]$ by Ca^{2+} influx could be accompanied by release from intracellular stores, as blocked by TMB-8. The specific intracellular locations of Ca^{2+} stores and the source of Ca^{2+} release from zoospores are still not known. It has been implied that some of the Ca^{2+} released might originate from the peripheral vesicles (Irving *et al.*, 1984), but a contribution from intracellular stores such as the vacuole or endoplasmic reticulum cannot be ruled out. Specific Ca^{2+}-binding sites are suggested to exist on the cytoplasmic side of the plasma membrane and also in the intermembrane spaces of mitochondria and the endoplasmic reticulum (Hemmes & Pinto Da Silva, 1980). These workers suggest that this may reflect the energy dependent nature of Ca^{2+}-mediated secretory events and/or represent a stage in the mobilisation of intracellular Ca^{2+}.

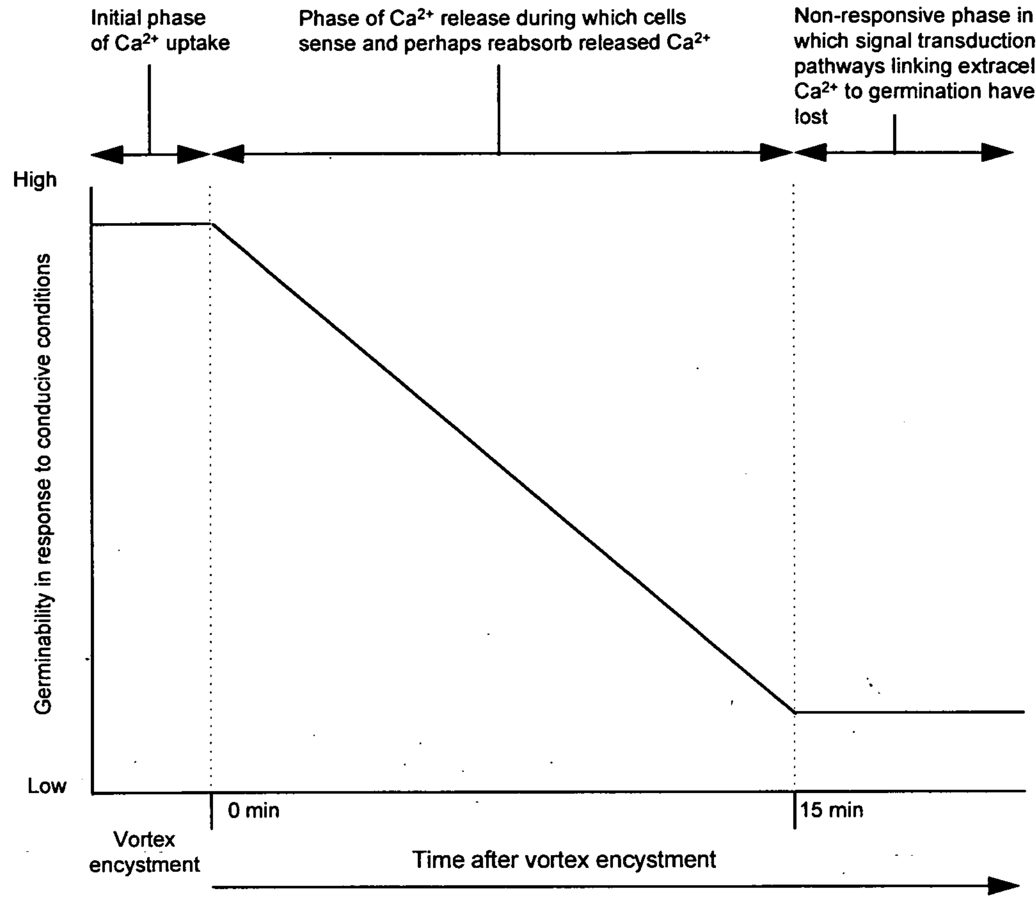
Sing & Bartnicki-Garcia (1972a, b) reported a rapid loss in adhesiveness during encystment of *Ph. palmivora*, for which the adhesive phase lasts only 30-60 seconds. Similarly, adhesiveness was lost after 5 min for *Ph. cinnamomi* (Gubler *et al.*, 1989). Furthermore, Donaldson & Deacon (1992) showed a relationship between adhesion and germination of *Py. aphanidermatum* cysts. These workers found that transfer of cysts to glass slides immediately (t_0) after encystment resulted in good adhesion and germination, whereas a delay of 10 min (t_{10}) resulted in poor adhesion and germination. Addition of Ca^{2+} to t_{10} cysts resulted in good adhesion and germination, whereas treatment with EGTA caused poor adhesion and germination of t_0 cysts (Donaldson & Deacon, 1992). Using the same isolate of *Ph. parasitica* as used in the work presented here, von Broembsen & Deacon (1996) found that cysts maintained in suspension germinated poorly, but cysts that were transferred to slides within the first few minutes of encystment germinated well. Furthermore, the germinability of *Ph. parasitica* cysts declined with time when cysts were maintained in suspension, and if zoospores were maintained in suspension for 9 min before being transferred to slides the ability of the cysts to germinate was at a low level. Results in this chapter have shown that the $[Ca^{2+}]$ of the medium surrounding encysting zoospores, induced by vortex agitation, consistently decreased after application of the encystment stimulus, but after approximately 10 min the $[Ca^{2+}]$ was at a similar level to that around the initial motile zoospore population. It is possible, therefore, that during the first few minutes after vortex treatment major uptake of Ca^{2+} occurs, as demonstrated by fluorimetry when cells were maintained in suspension, but that this uptake of Ca^{2+} is not directly involved in germination. Thereafter, Ca^{2+} is released slowly from the cells, and germination is at least partly dependent on the cells being able to sense this Ca^{2+} externally and perhaps to reabsorb it. The fluorimetry traces in Figures 5.4-5.6 indicate that the medium $[Ca^{2+}]$ is restored to the level that originally surrounded the motile zoospores, at about 9 to 11 minutes after vortex treatment. Several studies have shown that zoospore cysts maintained in suspension decline in their ability to germinate in response to treatments if the treatment is delayed beyond about 10 minutes. For example, von Broembsen & Deacon (1996) found that a delay of 9 minutes in transfer of cysts of *Ph. parasitica* to a glass surface led to a significant reduction in germinability; Cerenius & Soderhall (1984) found that cysts of

Aphanomyces astaci lost most of their ability to germinate in response to Ca^{2+} if treatment was delayed for 15 minutes; Donaldson & Deacon (1992) found that cysts of *Py. aphanidermatum* lost much of their ability to germinate if they were not transferred from suspension to glass slides within the first 4-5 minutes after vortex treatment. All these findings suggest a possible revised working hypothesis of the role of Ca^{2+} in zoospore cyst germination (Figure 5.13)

It is proposed that there is an initial net uptake of Ca^{2+} when cells are induced to encyst by vortex treatment but that this uptake need not necessarily lead to germination. It may, instead, regulate the changes leading to development of a cyst wall. During encystment, vesicles fuse with the plasma membrane to release cyst coat material and protein/glycoprotein adhesive. Over the subsequent 10-15 minutes Ca^{2+} is released from the cells. This Ca^{2+} may interact with the released glycoprotein to confer upon the cell its adhesive properties. In suspension, this Ca^{2+} may diffuse away from the cell and become diluted. However, zoospores can absorb Ca^{2+} during encystment, and adhesion to a surface within the first 10 min may reduce the rate at which Ca^{2+} diffuses away from the cell so that Ca^{2+} may be reabsorbed at a high enough concentration to trigger germination. Within 10-15 min, cells lose their ability to germinate in response to applied Ca^{2+} treatments, presumably because the signal transduction pathways linking Ca^{2+} uptake to germination have been lost.

Taken collectively, the results in this chapter suggest a central role of Ca^{2+} fluxes in the development of motile zoospores into cysts. Ca^{2+} efflux may facilitate adhesion (Gubler *et al.*, 1989; Donaldson & Deacon, 1992) by interaction with the released protein/glycoprotein, and early adhesion may prevent dispersal of the released Ca^{2+} which could influence subsequent germ-tube outgrowth. The importance of a role of Ca^{2+} in zoospore function is clear from many studies, although evidence also exists for the involvement of other putative second messenger molecules such as PA and cyclic nucleotides. A diverse range of

Figure 5.13 Revised model of the role of exogenous (or released and reabsorbed) Ca^{2+} in triggering germination of zoospore cysts.



signalling systems exist which may act co-operatively or synergistically and interaction between different signal transduction systems should not be overlooked (Gadd, 1995). Indeed, different encystment stimuli such as physical agitation or receptor-mediated encystment may lead to differentiation by involving alternative signalling pathways; encystment and germination may also involve slightly different signalling systems. In *Zoophthora radicans*, appressorium formation is strongly dependent on external Ca^{2+} whereas germ-tube initiation requires little or none (Magalhaes *et al.*, 1991). Although convincing evidence exists that both encystment and germination of *Pythium* and *Phytophthora* zoospores is strongly dependent on Ca^{2+} (Donaldson & Deacon, 1992; Grant *et al.*, 1986; Griffith *et al.*, 1988) the possible involvement of other signal transduction pathways must also be recognised.

CHAPTER 6

Confocal microscopy

6.1 Introduction

Laser scanning confocal microscopy (LSCM) has been used to image Ca^{2+} within many types of cell (Read *et al.*, 1992a, b; Knight *et al.*, 1993; Read *et al*, 1993; Malho *et al.*, 1995; Malho & Trewavas, 1996; Taylor *et al.*, 1996). To demonstrate that signal-response coupling is mediated by Ca^{2+} would require its localisation and measurement within living cells (Read *et al.*, 1992a). The possibility of doing this is becoming greater due to the development and availability of a range of fluorescent dyes which exhibit high selectivity for intracellular Ca^{2+}. Confocal microscopy produces high resolution optical sections free of out-of-focus blur and such images of cells loaded with Ca^{2+}-sensitive fluorescent dyes are readily obtained (Fricker & White, 1992).

Many Ca^{2+} dyes are now obtainable in an esterified form (eg., fura-2 - see section 5.1.1). So, cells can be loaded with dyes as membrane-permeant esters thus avoiding the need to load cells by methods such as microinjection which could disrupt the fragile cell membrane. Esterified dyes are uncharged and therefore hydrophobic and so can readily cross the cell membrane. Once inside the cell, cytosolic esterases cleave off the ester groups, releasing the free acid form of the dye which cannot cross the cell membrane, essentially resulting in accumulation of trapped dye within the cell.

Because Ca^{2+}-sensing dyes must bind Ca^{2+} within the cell to report intracellular Ca^{2+} concentration, this can cause problems by altering the level of intracellular free Ca^{2+}. The extent of this problem is directly related to the level of dye loading. Sequestration of dye within organelles, the presence of partially esterified dye, dye photobleaching and autofluorescence of certain cells (Knight *et al.*, 1993; Read *et al.*, 1992a) are some other problems associated with using Ca^{2+} dyes to image intracellular free Ca^{2+}.

For confocal microscopy, laser irradiation is used and only a limited number of laser lines are available, restricting the number of dyes which can be utilised with this system. Single wavelength imaging with LSCM has so far given satisfactory results (Malhó *et al.*, 1994) for detection of gradients and changes in cytosolic free Ca^{2+} concentration, but the only suitable dyes for this are Calcium Green-1 and Fluo-3.

The work described in this chapter involved attempts to load these dyes into fungal zoospores so that intracellular calcium levels could be assessed by LSCM. It was hoped that, by doing so, changes in intracellular calcium could be monitored during the differentiation of zoospores into cysts and then during the germination of cysts.

6.2 Experimental procedure

6.2.1 Laser scanning confocal microscopy

Confocal fluorescence imaging was performed using an MRC-600 system (Bio-Rad, Hemel Hempstead, U.K.) interfaced with a 25mW argon laser and mounted on a Nikon Diaphot TMD inverted microscope. Image capture and analysis were carried out using a Research Machines Nimbus 486sx personal computer running a COMOS (version 6.03) software package supplied by Bio-Rad. The 488nm laser line combined with a ND2 (3% transmission) neutral density filter was used to excite CG-1 and fluo-3. Fluorescence images were obtained using a filter block containing a 510nm dichroic mirror and 488/10nm filters. Either a dry 40x objective (Nikon Plan Apo; 0.95 NA) or a dry 60x objective (Nikon Plan Apo; 0.95 NA) were used for imaging.

Bright field images were collected via a fibre optic cable which delivered transmitted laser light directly to the second photomultiplier tube.

The optimal settings required for confocal fluorescence imaging of the Ca^{2+}-dyes were then set up as shown in Table 6.1. Black levels were set to give a background signal pixel intensity average of 10, determined using the histogram command. For each captured fluorescence image the background signal was

Table 6.1 Conditions used for confocal imaging of calcium dyes loaded into zoospores.

Confocal aperture	50% opened
Electronic signal amplification:	
Channel 1 (488nm excitation)	low signal on; gain 5.0
Scan speed	0.75 sec/image (128 lines)
Objective	x40 or x60 plan apo, 0.95 numerical aperture
Electronic zoom	x5

removed by a "dark image" subtraction. Images were displayed with a pseudocolour look-up table (LUT).

6.2.2 Dye loading of fungal zoospores

Zoospores of *Py. aphanidermatum* and *Ph. parasitica* were produced by the methods described in sections 2.1 and 2.2, respectively.

The motile zoospores were incubated with various concentrations of the AM ester form of either Calcium Green-1 or Fluo-3, then examined by LSCM as described in section 6.2.1. The dye concentrations used were based on recommendations in the product information sheet obtained from Molecular Probes Inc. and were chosen to give a minimum concentration of dye within the cells (0.1μM-5μM) for an adequate fluorescence signal, while reducing the possibility of artefacts which can result from incomplete hydrolysis of the AM ester (Roe *et al.*, 1990) .

When motile *Py. aphanidermatum* zoospores were incubated in 5μM CG-1 AM at 20°C for 1.5h in the dark, the fluorescence from the cells, obtained during LSCM, was indistinguishable from background fluorescence, suggesting that insufficient free dye had accumulated within the cells (Table 6.2). Increasing the concentration of CG-1 AM to 20μM gave no improvement. In experiments where motile *Ph. parasitica* zoospores where incubated in the dark with 20-25μM Fluo-3 AM for up to 2h at 20°C, the fluorescence signal obtained by LSCM was not sufficiently greater than background fluorescence. Further increasing the concentrations of Fluo-3 AM and CG-1 AM to 80μM resulted in encystment of *Ph. parasitica* zoospores especially after 2h of incubation; incubation of either motile or pre-encysted spores of *Ph. parasitica* with 80μM CG-1 AM or 80μM Fluo-3 AM for 2h did not result in appreciable dye loading. However, incubation of motile zoospores of *Py. aphanidermatum* in 80μM CG-1 AM or Fluo-3 AM for 1h did result in detectable fluorescence above that of the background. Some encystment was observed under these conditions.

Table 6.2 The ability of *Pythium aphanidermatum* and *Phytophthora parasitica* zoospores to load Ca^{2+}-sensitive dyes as detected by fluorescence during LSCM.

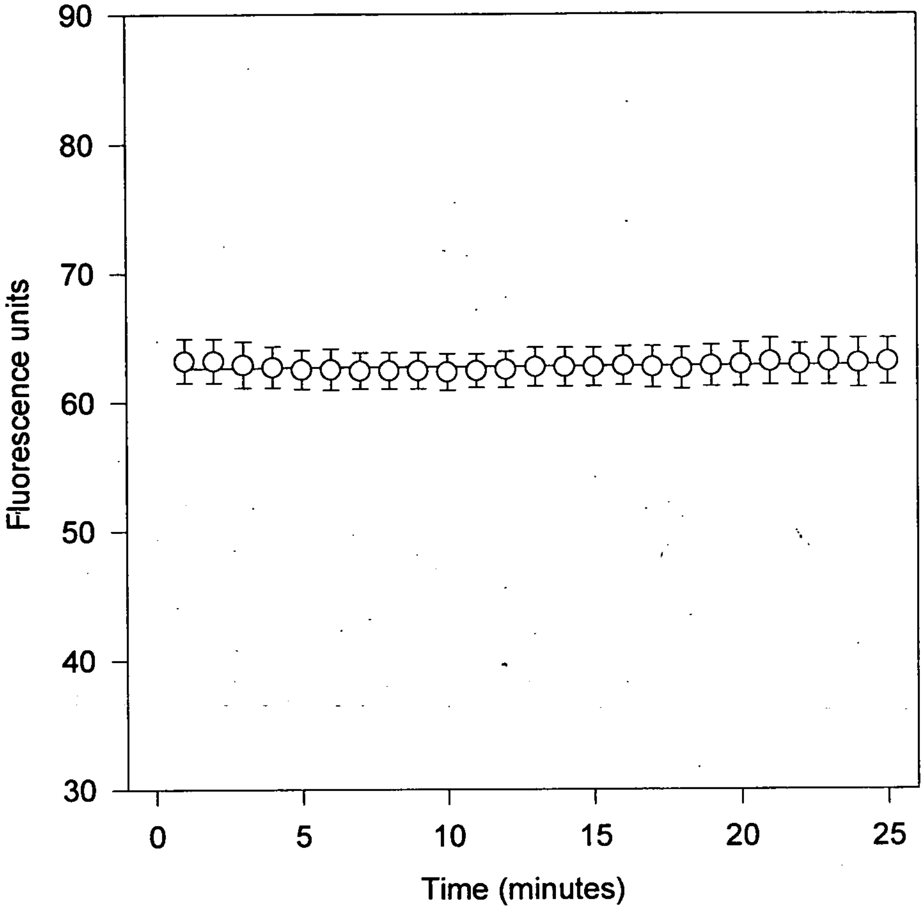
		Fluorescence detected			
Dye	Incubation	*Phytophthora*	*parasitica*	*Pythium*	*aphanidermatum*
concentration	time	Fluo-3 AM	CG-1 AM	Fluo-3 AM	CG-1 AM
5μM	1.5h	-	-	No	-
20μM	1.0h	-	No	-	-
	1.5h	-	-	No	-
	2.0h	-	No	-	-
25μM	1.0h	No	-	-	-
80μM	1.0h	No	No	Yes	Yes
	2.0h	No	No	-	-

6.2.3 Assessment of dye photobleaching

When fluorescent probes are exposed to excitation light they can produce potentially harmful reactive metabolic intermediates and undergo irreversible photobleaching leading to loss of responsiveness of the probe (Becker & Fay, 1987; Roe *et al.*, 1990; Oparka & Read, 1994). Calcium-sensitive dyes are no exception to this, although some dyes are more susceptible than others; for example, fura-2 is much less susceptible to photobleaching than is quin-2. The rate of photobleaching and problems of photodamage can, however, be reduced by attenuating the excitation intensity and duration of exposure (Roe *et al.*, 1990). CG-1 is also susceptible to photobleaching so it was necessary to determine whether photobleaching would be a potential problem during LSCM of CG-1 in single cell studies on *Py. aphanidermatum*. Therefore, all LSCM experiments were carried out in a semi-dark laboratory environment, and the minimum level of excitation light, required to produce a sufficiently high fluorescence intensity for analysis, was used (Table 6.1). By setting up the imaging parameters on a nearby cell and then moving to the zoospore to be studied, the duration of exposure of the spores was greatly reduced.

To test for the rate of photobleaching, dye-loaded zoospores were encysted by vortex agitation for 70s and imaged at 1 minute intervals for up to 25 minutes using the fastest scan speed (0.75 sec/frame at 128 lines/frame). The fluorescence intensity was then measured from the acquired images of 9 replicate experiments (Figure 6.1). Under these imaging conditions, it was found that photobleaching was not a significant problem - there was no decrease in fluorescence intensity up to 25 minutes after initiation of the experiment.

Figure 6.1 Assessment of Calcium Green-1 dye photobleaching with time. Loaded *Pythium aphanidermatum* zoospores were encysted and successive laser scans were performed at one minute intervals using the parameters outlined in Table 6.1. Data are means ± s.e. for 9 replicate experiments



6.2.4 Assessment of background fluorescence

Calcium imaging often involves the detection of low levels of dye fluorescence which can be masked by background fluorescence and stray light (Read *et al.*, 1992a). It is important, therefore, that the dye fluorescence to background signal (signal-to-noise) ratio is maximised. Most of the background signal results from autofluorescence from the biological specimen, but contributions from sources such as glass slides, coverslips, immersion oil and liquid nutrient media can also be significant (Oparka & Read, 1994; Knight *et al.*, 1993). To investigate whether these other sources of light would present problems during LSCM of single *Py. aphanidermatum* zoospores in 10mM Tris-Cl (pH 7.20), unloaded cysts were examined under the same conditions used to image CG-1 and fluo-3 emission. Using the parameters used for LSCM imaging (Table 6.1), no light other than from the probe was detected. A probable explanatory factor is that LSCM produces images of optical sections free from out-of-focus light, much of which can originate from background fluorescence and stray light.

6.2.5 Assessment of zoospore health after loading and during imaging

Confocal imaging can cause undue stress to cells, resulting mainly from a combination of laser irradiation and dye loading (Callaham & Hepler, 1991; Read *et al.*, 1992a; Knight *et al.*, 1993). Dye cytotoxicity can be a problem with fluorescent dyes and will depend on the concentration of the probe and the duration of its application. It is important, therefore, to assess the health of the cells after dye-loading and during imaging.

To investigate whether incubation in the dye ester had any deleterious effects on *Py. aphanidermatum* zoospores, the following three approaches were used.

1. Microscopic examination of loaded cells.
2. Quantification of germination after incubation in the dye ester.
3. Observation of encystment and germination under imaging conditions.

After incubation of *Py. aphanidermatum* zoospores in 80μM CG-1 AM for 1 hour it was observed that only a very small proportion of the motile population had encysted. The majority of zoospores remained motile and displayed the helical motility pattern seen under normal laboratory conditions. Even the encysted zoospores had not lysed.

After incubation in the dye an aliquot of the zoospore suspension was then encysted by vortex agitation for 70s, transferred to a slide and incubated for 1.5h. At the same time, an unloaded population of zoospores in 10mM Tris-Cl (pH 7.20) was vortex encysted and incubated on a slide for 1.5h. Germination was assessed by microscopic examination of 3 replicate experiments, based on counts of 100 spores for each replicate. It was found that dye-loaded zoospores germinated to a level of 63.2 ± 6.7% (n=3) which did not differ significantly from that of the control (65.5 ± 8.2, n=3).

During LSCM imaging using the parameters outlined in Table 6.1, the ability of dye loaded zoospores to encyst (Figure 6.2) and subsequently germinate was also taken as an indication of cell viability.

6.2.6 Assessment of intracellular location of dye

Calcium-sensitive dyes must remain within the cytosol in a responsive state if they are to be useful for measuring cytoplasmic Ca^{2+} levels accurately. Sequestration of dye within organelles can reduce its cytoplasmic concentration to levels which prevent precise intracellular [Ca^{2+}] measurement. Total fluorescence from a cell will contain contributions of fluorescence from each of the compartments. Furthermore, the concentration of free Ca^{2+} within organelles is typically much greater than in the cytoplasm and so dyes partitioned within organelles are mostly Ca^{2+}-saturated and show maximal fluorescence (Bush & Jones, 1990).

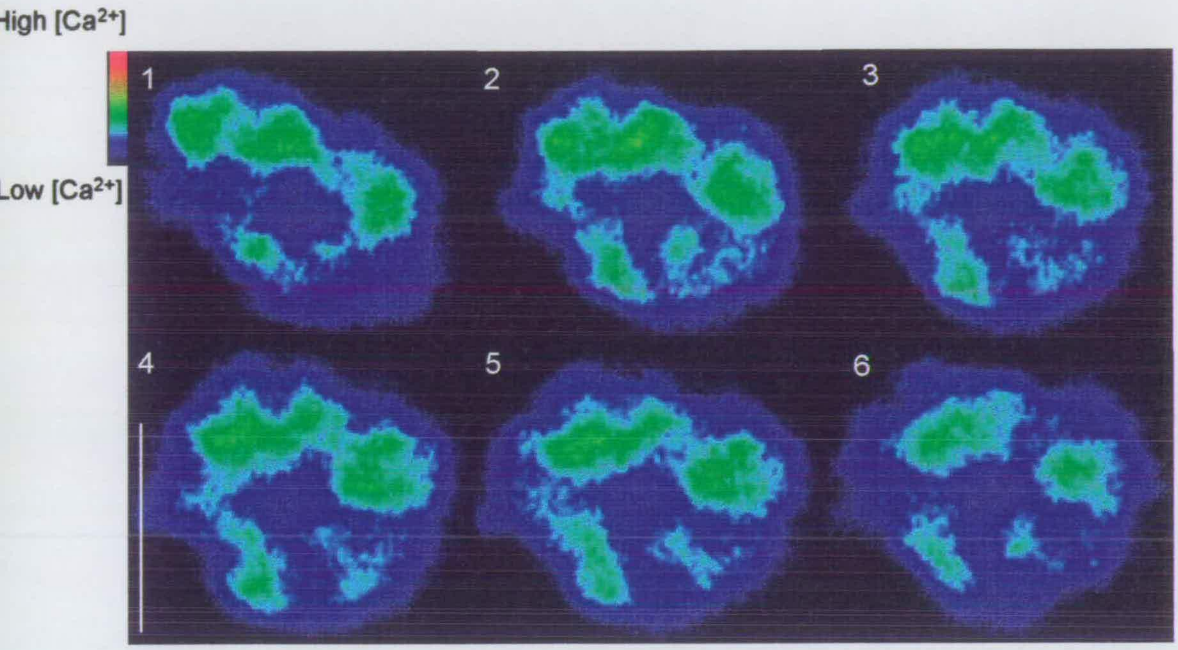


Figure 6.2 Successive confocal images (1-6) of a *Pythium aphanidermatum* zoospore loaded with Calcium Green-1 and immobilised with 4% D-mannitol. Images were collected at 1 minute intervals. The motile zoospore encysted during the experiment to form a rounded cyst by 5 minutes. Bar = 10 μm.

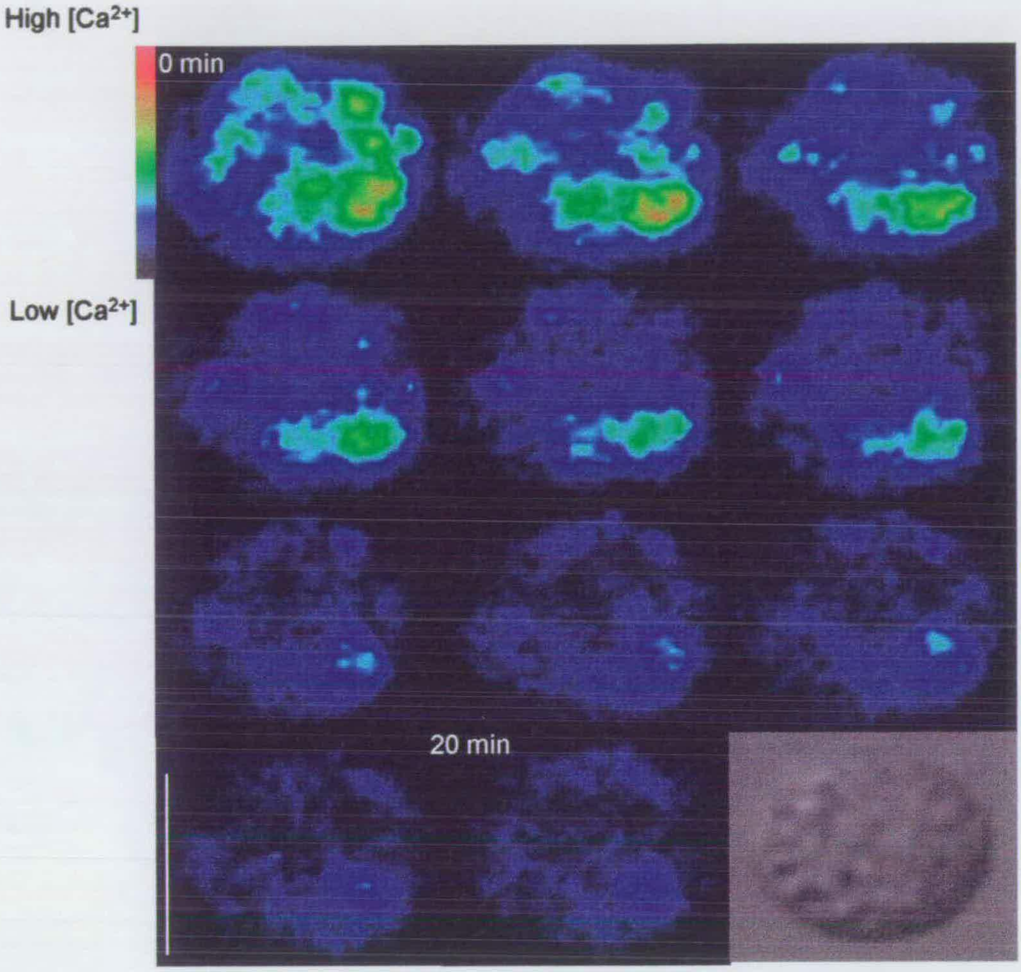


Figure 6.3 Successive images of a *Pythium aphanidermatum* cyst loaded with Calcium Green-1. Immediately after the initial image was obtained (0 min), Br-A23187 was added to a final concentration of 10 μM in the presence of EGTA (1mM) and successive images were obtained at 2 min intervals up to 20 min. A bright field image was obtained after a further 1 min. Bar = 10 μm

Calcium-dyes have been reported to be sequestered into plant and fungal cell organelles (Bush & Jones, 1990; Kiss *et al.*, 1991; Knight *et al.*, 1993), in particular when the ester-loading method is employed. Dye sequestration within organelles is often easy to visualise as punctate fluorescence using LSCM. The organelles in which dye becomes compartmentalised vary between cell types and loading procedures, but are thought to include the fungal vacuolar network (Knight *et al.*, 1993).

Experimental manipulation of cytosolic [Ca^{2+}] can give an indication as to the presence of Ca^{2+}-dye within the cytosol. An immediate response of the dye by a change in fluorescence often indicates the location of measurable dye within the cytosol; a slower response, or none at all, can indicate an organellar location (Thomas & Delaville, 1991).

Dye-loaded zoospores were examined under LSCM for any punctate fluorescence indicative of sequestration of dye into cytoplasmic organelles. In almost all cases where esterified dye was introduced into motile zoospores, punctate fluorescence was observed (Figures 6.2 and 6.3). However, in most cases a region within the zoospore lacked fluorescence and, therefore, presumably dye (Figure 6.4). This region often corresponded to the position of the water expulsion vacuole and/or nucleus which could be seen by conventional light microscopy during the LSCM experiments.

Ca^{2+} (1-5mM) or EGTA (500μM-1mM), alone or in the presence of 10μM Br-A23187 or ionomycin, was added to dye loaded zoospores. Any change in cytosolic [Ca^{2+}] mediated by any of these treatments will alter the fluorescence of any dye located in the cytosol.

Figure 6.3 gives a representative example of a cell observed over 20 min. There was a slow decrease in fluorescence of intracellular CG-1 over the 20 minutes when a *Py. aphanidermatum* zoospore cyst, in the presence of 10μM Br-A23187 and 1mM EGTA, was imaged by LSCM. Furthermore, addition of up to 5mM Ca^{2+} to pre-encysted *Py. aphanidermatum* zoospores did not result in an

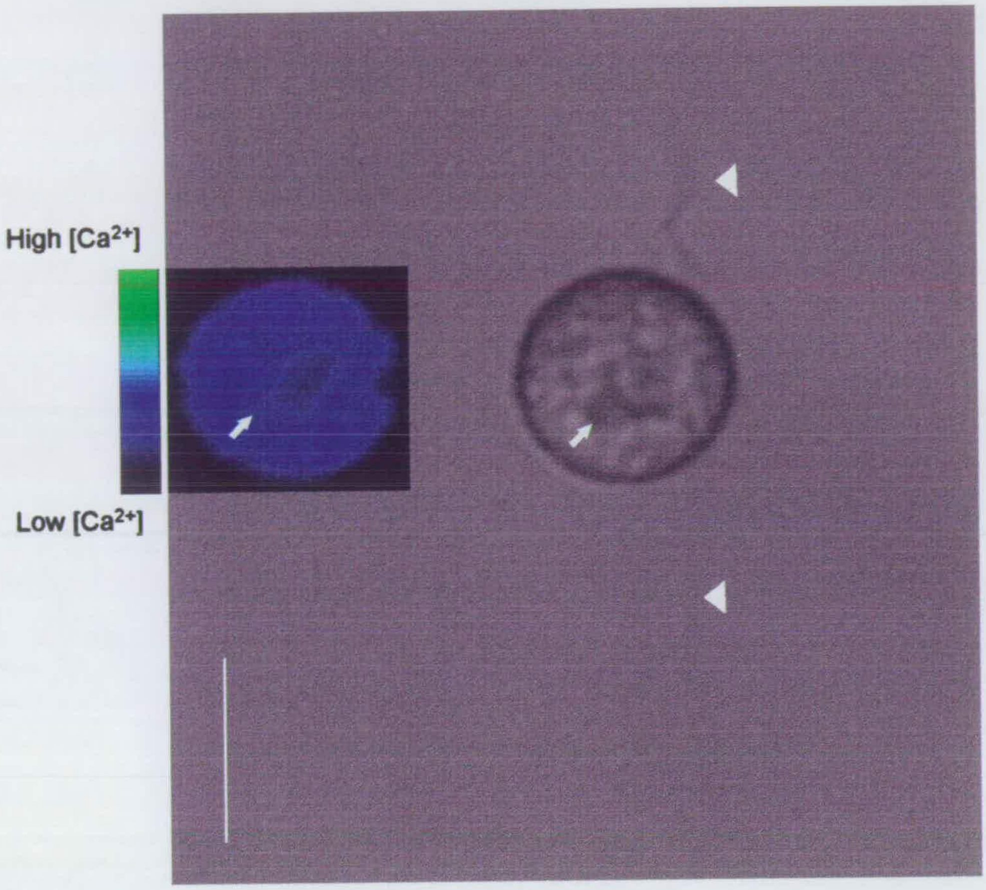


Figure 6.4 Confocal images of a newly formed *Pythium aphanidermatum* cyst loaded with Calcium Green-1. The arrow indicates a region within the zoospore which lacks fluorescence, and corresponds to the position of the water expulsion vacuole and/or the nucleus seen in the bright field image. The attached flagella (arrowheads) are also visible in the bright field image. Bar = 10 μm.

increase in intracellular dye fluorescence when added alone or in the presence of Br-A23187 (10μM) or ionomycin (10μM).

In order to image cytosolic free Ca^{2+} within a motile zoospore and during its development into an encysted zoospore it is essential that the cell remain stationary. By adding D-mannitol to a final of 4% it was possible to keep the zoospores in a stationary position while they underwent their transition from motile cells to germinating cysts. Mannitol solutions at 3.5%, 4.0% and 4.5% have an osmolality of 200mOsm, 230mOsm and 260mOsm respectively. At these final concentrations, mannitol caused immediate immobilisation of *Py. aphanidermatum* zoospores; encystment occurred within 5 min and after 1.5h the cysts had germinated to levels of 96.0 ± 1.4%, 96.2 ± 1.1% and 94.0 ± 1.7% respectively (mean ± s.e.m for 5 replicate experiments). Sodium chloride at final concentrations of 0.6% and 0.8% has an osmolality of 192mOsm and 256mOsm respectively. NaCl at 0.6% caused zoospores to rapidly encyst and they germinated to a level of 34.2 ± 9.0% after 1.5h (n=5) whereas 0.8% NaCl caused many of the zoospores to lyse. KCl at 1% final concentration has an osmolality of 249mOsm and also caused lysis. Addition of D-mannitol (4% final concentration) to a suspension of *Py. aphanidermatum* zoospores resulted in 94.8 ± 1.2% (mean ± s.e.m for 5 replicate experiments) of the spores being immobilised immediately after mannitol addition. If an equal volume of water was added 30s after mannitol addition, 32.8 ± 4.4% (n=5) of the zoospores resumed swimming. If water was added 3 min after mannitol addition only 9.3 ± 4.4% (n=5) of the zoospores could be released to swim again. Figures 6.2 shows a motile zoospore of *Py. aphanidermatum*, loaded with CG-1, and suspended in 4% D-mannitol, encysting during an LSCM experiment. Loading zoospores with dye and then subsequent addition of D-mannitol did not appear to have any deleterious effect on cell viability; in fact zoospores started to encyst within 5 minutes of mannitol addition and 34 of the 49 zoospores imaged under these conditions germinated during LSCM experiments. That most cells imaged using LSCM successfully encysted and germinated indicated that the combination of dye-loading and laser irradiation had not damaged the developing spore. The zoospores rounded up in a manner observed with encysting spores that are not subjected to such treatment.

6.3 Discussion

The results presented in this chapter have demonstrated that fluorescent dyes can be loaded into living, motile fungal zoospores of *Py. aphanidermatum*, and that the introduced dyes can be imaged by LSCM during encystment and germination. Incubation of motile zoospores in the esterified form of the dye did not cause unacceptable levels of encystment nor did it inhibit subsequent germination. Furthermore, imaging using LSCM did not prevent *Py. aphanidermatum* zoospores from encysting and germinating. However, in other respects the findings were discouraging, so further attempts to measure cytosolic [Ca^{2+}] by dye-loading were abandoned.

Intracellular dye fluorescence could be detected in zoospores of *Py. aphanidermatum* loaded with CG-1 and fluo-3. In contrast, no such signal was obtained from *Ph. parasitica* zoospores incubated with these dyes. Longer incubation times and higher dye-ester concentrations resulted in unacceptable levels of encystment of *Py. aphanidermatum* and *Ph. parasitica* zoospores, with extensive cell-lysis also occurring (data not shown).

Most attempts to experimentally alter cytosolic [Ca^{2+}] of dye-loaded cells did not result in a rapid change of dye fluorescence, this often being indicative of the sequestration of dye within organelles. Compartmentation of Ca^{2+}-dyes within organelles has also been a significant problem in some studies with plant (Bush & Jones, 1990; Kiss *et al.*, 1991) and animal (Roe *et al.*, 1990) cells and to a greater extent in filamentous fungi (Read *et al.*, 1992a, b; Knight *et al.*, 1993). In the few instances where EGTA in the presence of an ionophore caused a decrease in dye fluorescence within the zoospore, there was only a gradual change over a period of 20 minutes. Knight *et al.* (1993) reported that Ca^{2+} in the presence of Br-A23187 caused an elevation in dye fluorescence within 1 minute but that this only occurred in hyphae that were presumed dead.

By masking the acidic Ca^{2+}-binding sites of fluorescent dye molecules by hydrophobic ester groups, the molecule is rendered Ca^{2+}-insensitive but able to permeate the plasma membrane (Grynkiewicz *et al.*, 1985). Accumulation of probe in the cytoplasm relies upon cleavage of ester linkages by cytoplasmic esterases, rendering the free dye Ca^{2+}-sensitive and highly charged, thereby unable to cross cellular membranes and thus theoretically trapped within the cytosol.

The inability of a cell to accumulate trapped dye which is in a Ca^{2+}-sensitive form could result from a lack of necessary esterase activity in the cytosol or by extracellular hydrolysis of the dyes (Bush & Jones, 1990). Sequestration of dyes may occur if cells contain low levels of esterase activity such that incomplete hydrolysis of the ester linkages occurs in the cytosol; the dye could then cross the membranes of intracellular organelles which also contain esterase activity (Roe *et al.*, 1990).

Dye molecules differ in the extent to which they are esterified in the commercially available AM form; the fluorescent Ca^{2+} dyes typically having up to 5 ester groups attached whereas the pH-sensitive dye BCECF may have 1-3 in the commercially available mixture. This may explain why cells reportedly take up BCECF more readily than Ca^{2+}-dyes and its compartmentation occurs more slowly (Knight *et al.*, 1993). It seems, therefore, that problems of dye loading and sequestration depend upon the type of cell and nature of the dye used (Knight *et al.*, 1993). Indeed, the ester loading method has been used to successfully load fluo-3 and CG-1 into germ-tubes of *Uromyces vignae* (Read *et al.*, 1992a; Knight *et al.*, 1993) and CG-1 into hyphae of *Basidiobolus ranarum* (Knight *et al.*, 1993) whereas this method proved unsuccessful for loading hyphae of *Neurospora crassa* (Schmid & Harold, 1988; Knight *et al.*, 1993). Similarly, Cork (1986) reported a failure to ester-load quin-2 into zoospores of *Ph. cinnamomi* zoospores. More recently, Grzenski *et al.* (1994) have reported successful ester-loading of BCECF into *Ph. palmivora* zoospores. In work not presented in this chapter, it was possible successfully to load the pH-dye SNARF-1 into motile *Py. aphanidermatum* zoospores but no further work was done with this dye. Although CG-1 and fluo-3 could also be loaded into the zoospores of *Py. aphanidermatum*, evidence presented in this chapter suggests that the Ca^{2+}-dyes had become sequestered into organelles, but not in the region of the cells containing the WEV and nucleus.

In short, the present study has shown that some fungal zoospores can be loaded with fluorescent dyes and can be imaged, in an apparently unperturbed state, by LSCM during their transition from a motile to an encysted spore. By carefully controlling the operational conditions of the confocal microscope, further problems of dye photobleaching and photodamage of the zoospores were not evident. However, when loaded as the AM-esters the dyes accumulated within organelles, indicating that at present this approach is not useful for measurement of cytoplasmic Ca^{2+} in developing zoospores. The future development of new dyes with different chemical properties, and which do not sequester into organelles, may enable cytoplasmic $[Ca^{2+}]$ to be measured in developing zoospores using LSCM.

Chapter 7

Concluding discussion

The work in this thesis has involved the study of factors influencing the behaviour and infection-related processes of zoospores of several plant-pathogenic fungi in the Oomycota. The work has revealed novel features of zoospore motile behaviour, and has shown that calcium-chelating compounds can alter the motile behaviour of zoospores of *Pythium* spp., *Phytophthora* spp. and *Aphanomyces euteiches* and, in particular, their responses to attractant amino acids. In addition, zoospores were prevented from germinating by treatments which lower extracellular [Ca^{2+}], interfere with Ca^{2+} uptake mechanisms or antagonise intracellular Ca^{2+}-mediated events. Dose response experiments have identified the levels of these compounds required to achieve these effects for *Py. aphanidermatum* and *Ph. parasitica*. Furthermore, application of these compounds at a precise time during the developmental stages was found to be important, otherwise they had poor effects. Development of a technique, used widely in animal cell research, which employed fluorimetry of a Ca^{2+}-sensitive fluorescent dye to measure Ca^{2+} levels around developing zoospores, revealed that vortex-induced encystment is followed by changes in the levels of Ca^{2+} in the medium surrounding zoospores during and after encystment. Attempts to load Ca^{2+}-sensitive fluorescent dyes into the cytosol of motile fungal zoospores proved problematic, because confocal microscopy revealed that the dyes had become compartmentalised within organelles.

Investigation of the motile characteristics of zoospores of three *Pythium* spp., three *Phytophthora* spp. and *A. euteiches* revealed certain similarities between all these plant-pathogens; zoospores of all these fungi swam in an extended helical fashion, rotating about their long axis as they did so. However, analysis of zoospores swimming in the mid depth of a deep suspension revealed that *Ph. palmivora*, *Ph. parasitica*, *Ph. infestans* and *A. euteiches* made random turns, whereas *Py. aphanidermatum*, *Py. dissotocum* and *Py. catenulatum* did not make frequent random changes of direction unless they made contact with an obstruction, such as the surface of a glass slide. Chelation of exogenous Ca^{2+} using EGTA reduced the frequency with which *Ph. infestans* made turns, whereas EGTA completely prevented random turning behaviour of *Ph. parasitica*, *Ph. palmivora* and *A. euteiches*. EGTA and BAPTA (a more highly specific Ca^{2+}-chelator than

EGTA) also caused zoospores to swim in a "straight" fashion and prevented the avoidance response when zoospores made collisions with each other or with the surface of glass slides. When zoospores in the presence of EGTA were in the straight swimming mode described above, they were unable to respond chemotactically to the attractant L-glutamate (Donaldson & Deacon, 1993a). Similarly, this study has shown that isotropic backgrounds of certain amino acids can alter the normal helical motility pattern of zoospores to one of irregular, jerky swimming with constant changes of direction; addition of EGTA then overrode this response and zoospores exhibited the straight swimming mode. Subsequent addition of Ca^{2+} then reversed this effect and jerky motility resumed. This is the first time that zoospores have been shown to respond sequentially to different treatments that alter their motility characteristics, and it was achieved by careful selection of the concentrations of the motility-modifying treatments. It demonstrates that the underlying control of motility of zoospores of different members of the Oomycota (including members of the Peronosporales and Saprolegniales) is essentially similar.

Furthermore, amino acids altered the swimming attributes in a similar way for all the species studied, although different species responded differently to particular amino acids. The altered form of swimming pattern in response to amino acids resembles that described when zoospores are close to a root or attractive compound(s) (Royle & Hickman, 1964a; Reid *et al.*, 1995). The repeated changes of direction of swimming induced by amino acids may explain the accumulation of zoospores in capillary tubes during chemotaxis assays. However, D-amino acids were found not to be chemoattractants of *Pythium* zoospores (Donaldson & Deacon, 1993b), whereas in this study, some D-amino acids caused jerky motility of the same isolate of *Py. aphanidermatum* as used by Donaldson & Deacon (1993b). Furthermore, *Ph. infestans* is known not to show chemotaxis to any of the tested amino acids (D. E. Grayson, personal communication), but showed irregular, jerky motility in response to L-glutamic acid and L-aspartic acids. These anomalies make it difficult to relate the altered swimming behaviour of zoospores in response to amino acids to the accumulation of zoospores at the mouths of capillaries in chemotaxis assays and near attractant sites on host plants. Nonetheless, trapping within the vicinity of the host may increase the likelihood that zoospores make contact with and infect the host.

It has been assumed that the motile behaviour of zoospores of *Pythium* and *Phytophthora* spp. is broadly similar (Deacon & Donaldson, 1993), and that chemotaxis of zoospores is analagous to the biased random walk of bacteria. However, this study has revealed that zoospores of *Pythium* spp. do not make random turns when swimming in unrestricted conditions, whereas *Phytophthora* spp. and *A. euteiches* do so. It was also found that *Py. aphanidermatum* zoospores accumulate at the bottom of a spore suspension, whereas *Ph. parasitica* and *Ph. infestans* accumulate at the top. These differences are notable because zoospores of all these fungi are able to accumulate at specific sites on host plants. It is possible that they do so by different mechanisms or they may do so by a common mechanism in which attractant compounds override their inherent differences in motility behaviour. Another notable finding was that EGTA suppressed random turning behaviour of *A. euteiches* and the three *Phytophthora* spp. tested, and also negated the altered pattern of zoospore motility of all fungi caused by addition of some amino acids. The most reasonable explanation for this is that exogenous Ca^{2+} is required for maintenance of the normal motility pattern of zoospores, and for their responses to attractive compounds.

Byrt *et al.* (1982) and Grant *et al.* (1985, 1986) working with *Ph. cinnamomi* and *Ph. palmivora* respectively, found that strong encystment responses were obtained with cations - notably Ca^{2+}, Mg^{2+}, Sr^{2+}, Na^{+} and K^{+}. Similarly, Donaldson & Deacon (1993b) found that Ca^{2+}, Mg^{2+} and Sr^{2+} elicited encystment of *Pythium* spp. Furthermore, Griffith *et al.* (1988) found that an external [Ca^{2+}] of >100nM was necessary for encystment, and at a concentration of 2μM, Ca^{2+} alone could initiate encystment. At an early stage during encystment (within the first 1 min of pectin or Sr^{2+} addition) there was a reported efflux of both Ca^{2+} and Na^{+} from zoospores, followed 2-4 min later by a net influx of these ions (Irving *et al.*, 1984; Iser *et al.*, 1989). The Ca^{2+} fluxes in this work were assessed by release of $^{45}Ca^{2+}$ from zoospores formed by mycelia grown in the prescence of $^{45}Ca^{2+}$, or by uptake of exogenously supplied $^{45}Ca^{2+}$. Increasing pH above 7.0 induced encystment, as did the addition of monensin, which catalyses Na^+/H^+ exchange, and A23187, a Ca^{2+} ionophore (Iser *et al.*, 1989). In addition, drugs which inhibit Ca^{2+} uptake, such as verapamil, delayed the entry of Ca^{2+} into cells and slowed the rate at which zoospores encysted (Iser *et al.*, 1989). Subsequent interpretations of zoospore encystment and cyst adhesion have relied heavily on these reported findings (Gubler *et al.*, 1989; Deacon & Donaldson, 1993). In particular it has been suggested that Ca^{2+} released early after the induction of encystment can facilitate adhesion of cysts to surfaces and that the subsequent uptake of Ca^{2+} can facilitate germination.

Work in this thesis has described the development and assessment of fluorimetry of the Ca^{2+}-sensitive dye fura-2 as a precise technique for quantification of Ca^{2+} around populations of zoospores during their development into cysts. It is known that rapid, synchronous zoospore encystment is initiated in *Phytophthora* and *Pythium* spp. by mechanical agitation. By using fura-2 to detect changes in $[Ca^{2+}]$ in a zoospore bathing medium, a consistent decrease in medium $[Ca^{2+}]$ was found after synchronous encystment of a motile *Ph. parasitica* population was induced by vortex treatment. This decrease was detectable immediately after vortex encystment and the lowered $[Ca^{2+}]$ of the bathing medium persisted for several minutes. After this time, the $[Ca^{2+}]$ of the medium gradually increased to above the level around the originally motile zoospores. This is of interest because it seems to be directly contradictory to the findings by Irving *et al.* (1984) and Iser *et al.* (1989) that zoospores release Ca^{2+} and Na^{2+} after encystment induction, and then subsequently take up these ions. In this study, treatment of zoospores with both an organic and an inorganic Ca^{2+} channel blocker before induction of encystment prevented the initial decrease in medium $[Ca^{2+}]$ and instead caused an increase in $[Ca^{2+}]$ of the medium, suggesting that the decrease observed in control experiments, in the absence of Ca^{2+}-modulating drugs, occurred due to uptake of Ca^{2+} into the zoospore via Ca^{2+} channels. Furthermore, treating zoospores with TMB-8, an inhibitor of Ca^{2+} release from intracellular stores, did not prevent the initial decrease in medium $[Ca^{2+}]$ but no subsequent elevation in Ca^{2+} levels of the medium occurred.

Zoospores were encysted by vortex agitation in the fluorimetry experiments. This encystment method required that the zoospore population be removed from the experimental chamber, vortex-encysted for 70s, and transferred back to the chamber before continuing the experiment; the total time taken was 80s. Therefore, a rise in medium [Ca^{2+}] during this period cannot be precluded. However, in order for these findings to be compatible with the magnitude of Ca^{2+} release (up to 30% of total cellular Ca^{2+}) reported by Irving *et al.* (1984), the cells would need to have released this amount of Ca^{2+} and then reabsorbed as much Ca^{2+}, all within 80s, and then the Ca^{2+} flux across the cell membrane would need to have stabilised for at least three minutes because no net change of Ca^{2+} was detected several minutes after vortex-encystment. An alternative possibility is that the early release or uptake of other ions interfered with the fluorometric determination of Ca^{2+} in the medium, but calculations showed that [Na^+] would need to change by millimolar levels in order for this to happen, and this seems implausible. A further possibility is that Ca^{2+} is released early during vortex-encystment but then binds strongly to the protein/glycoprotein cyst coat that is known to be released by exocytosis of peripheral vesicles in the zoospores (Sing & Bartnicki-Garcia, 1975a), so that the released Ca^{2+} is not detectable by the Ca^{2+}-sensitive dye in the bathing medium. The adhesive that binds newly formed zoospore cysts to surfaces is known to interact with Ca^{2+} (Gubler *et al.*, 1989; Donaldson & Deacon, 1992). However, this could not explain the discrepancy between the results of the fluorimetry experiments and those of Irving *et al.* (1984), because these workers separated zoospore cysts (with their attached cyst coats) from the bathing medium prior to assays of Ca^{2+} in the cells and in the medium.

The discrepancies between the findings in this thesis, for an early net uptake of Ca^{2+} by encysting zoospores of *Ph. parasitica*, and those of Irving *et al.* (1984), for an early net efflux of radiolabelled Ca^{2+} by encysting zoospores of *Ph. palmivora*, cannot easily be explained. Irving *et al.* (1984) reported a net Ca^{2+} efflux when zoospores were encysted by each of four treatments: addition of pectin, addition of Sr^{2+}, addition of cAMP, and vortex treatment of zoospores. However, they did not show the results for vortex encystment, and they noted that in this treatment the transient net efflux of Ca^{2+}, assessed by a reduction of $^{45}Ca^{2+}$ from the labelled cells, was not matched by a corresponding increase in $^{45}Ca^{2+}$ in the surrounding medium, due perhaps to technical difficulties.

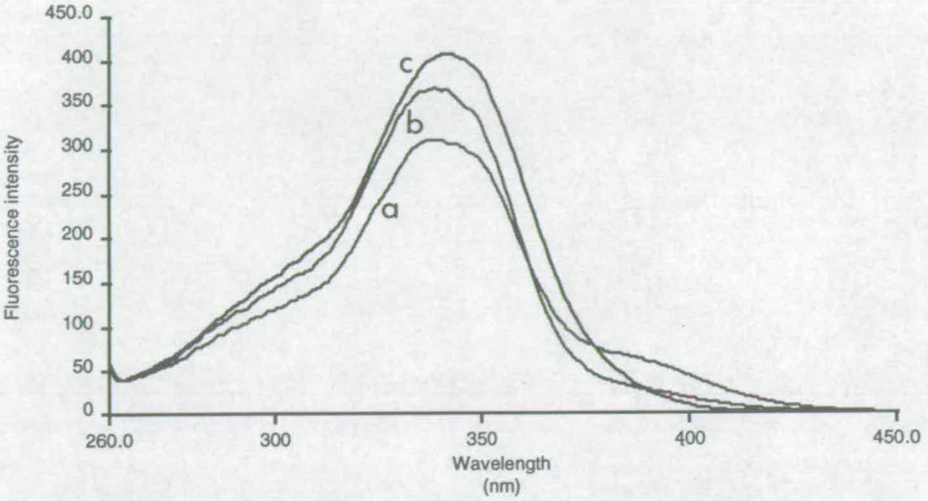
The fluorimetry studies in this thesis yielded consistent results and also clear differences in the net Ca^{2+} flux when zoospore populations were treated with Ca^{2+}-modulating compounds. Of particular interest was the progressive release of Ca^{2+} from encysted cells after an early large Ca^{2+} influx. This finding is compatible with many experimental studies (eg. Donaldson & Deacon, 1993b; von Broembsen & Deacon, 1996) based on the model of Donaldson & Deacon (1992), which predicts that cysts germinate when they adhere to surfaces because Ca^{2+} released from the cells is retained in the adhesive close to the cell and can then be reabsorbed to trigger or facilitate germination. However, a modified hypothesis is proposed here, in which the early large uptake of Ca^{2+} during encystment might be associated with stimulus mediated exocytosis of proteins/glycoproteins from peripheral vesicles, and then the cells require a further stimulus for germination, dependent on the release and subsequent reuptake of Ca^{2+} from the environment. This latter phase of the model is consistent with many experimental studies (Donaldson & Deacon, 1992; Donaldson & Deacon, 1993b; von Broembsen & Deacon, 1996; Deacon & Saxena, 1997).

It was of interest to determine the intracellular role of cytosolic Ca^{2+} during encystment by studies on single cells using confocal microscopy. However, work in this thesis suggests that loading zoospores with the dyes currently available in an esterified form is not a satisfactory method because the dyes become compartmentalised within organelles. Recently, Jackson (1995) successfully microinjected the cytoplasm of *Ph. cinnamomi* sporangia with dextran-conjugated dyes. Presumably, the zoospores released from these sporangia will contain dye in a cytosolic location, and can then be imaged using LSCM during their encystment after addition of mannitol, as found in work in this thesis. Until the role of Ca^{2+} in encystment is studied at the single cell level, the mechanisms by which it acts will remain elusive.

Nonetheless, a rapid influx of Ca^{2+} in response to encystment stimuli, as evidenced by the fluorimetry experiments described in this study, may explain the variability seen in attempts to prevent germination of *Py. aphanidermatum* zoospores using Ca^{2+}-modulating treatments. It is known that in *Phytophthora* and *Pythium* spp., the ability of certain cations to cause encystment does not necessarily mean that they will promote germination. For example, Mg^{2+} elicits encystment but not germination in *Ph. cinnamomi* (Irving & Grant, 1984), whereas Ca^{2+} and Sr^{2+} elicit encystment and germination in *Pythium* and *Phytophthora* zoospores (Byrt *et al.*, 1982; Grant *et al.*, 1985; Donaldson & Deacon, 1993b). So, it seems that although encystment and germination are linked, they are also separable. Interestingly, results obtained here with *Py. aphanidermatum* have shown that germination can be prevented by addition of compounds which reduce the levels of extracellular Ca^{2+} (such as BAPTA), inhibit Ca^{2+} uptake through Ca^{2+} channels (such as La^{3+}), antagonise intracellular Ca^{2+} mediated events, such as release of Ca^{2+} from intracellular stores (TMB-8), or antagonise calmodulin mediated effects (such as TFP). However, it was also apparent that these treatments needed to be applied at precise developmental stages or they had poor effects. In support of this, similar experiments with *Ph. parasitica* revealed that if these compounds were present when the zoospores were induced to encyst, then they had a greater effect in suppressing the ability of zoospores to subsequently germinate. Another notable point was that the concentrations of the compounds needed to obtain these effects could be greatly reduced by adding them before the encystment trigger. The techniques developed in this study should therefore contribute to the elucidation of the roles of both exogenous and intracellular Ca^{2+} levels in differentiation from zoospores of the Oomycota. Ultimately, such studies could lead to the development of new methods for controlling the damage caused by these aggressive plant pathogens. In this respect, one of the most important factors is the requirement of these fungi for exogenous (extracellular) Ca^{2+} in order to exhibit their normal swimming motility and in order for zoospore cysts to germinate. Recent studies have shown that manipulation of the Ca^{2+} level in irrigation water or in mineral nutrient solutions used in commercial cropping environments can markedly affect the release of zoospores from sporangia, the period of motility of zoospores, the ability of zoospore cysts to germinate to form hyphae and the ability of cysts to release a further zoospore (von Broembsen & Deacon, 1997).

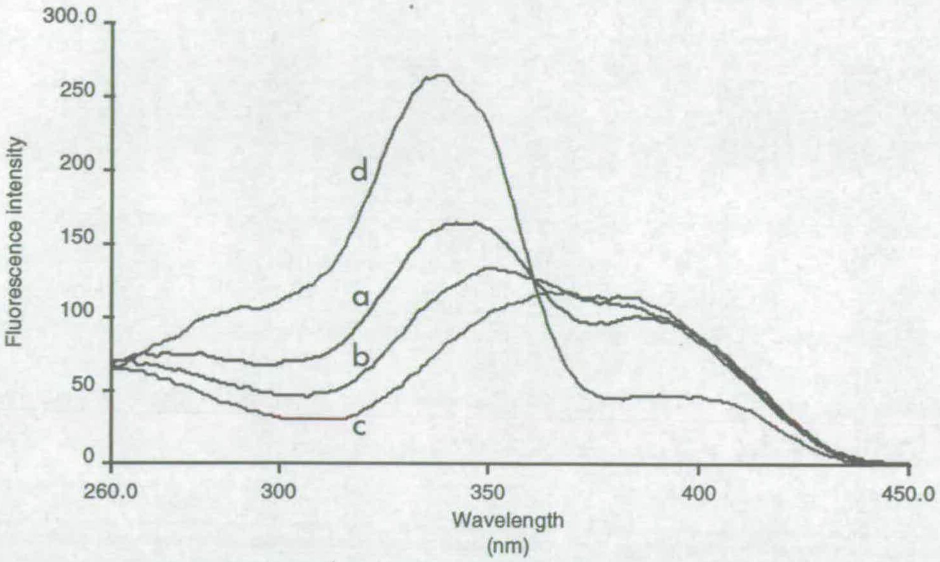
Appendices

Appendix 1 Effect of 1μM Gd^{3+} on fura-2 excitation spectrum.



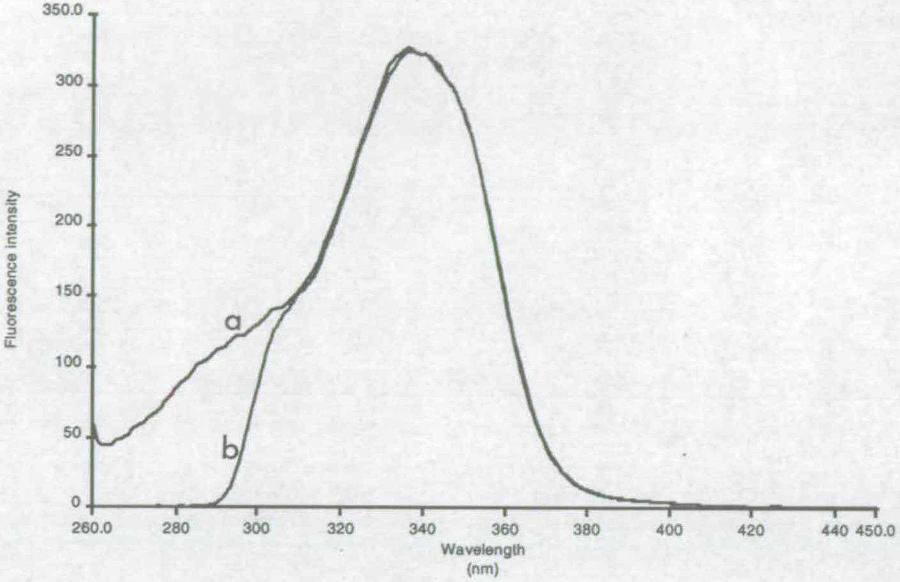
An initial excitation scan (a) was performed, then, Gd^{3+} was added to a final concentration of 1μM. Addition of Gd^{3+} caused an elevation in the fluorescence of fura-2 (b), and when Ca^{2+} was subsequently added to a final concentration of 2mM a further elevation in fura-2 fluorescence occurred (c), but this spectrum did not pass through the isosbestic point at 360nm as for spectra a and b.

Appendix 2 Effect of 67mM K$^+$ on fura-2 excitation spectrum.



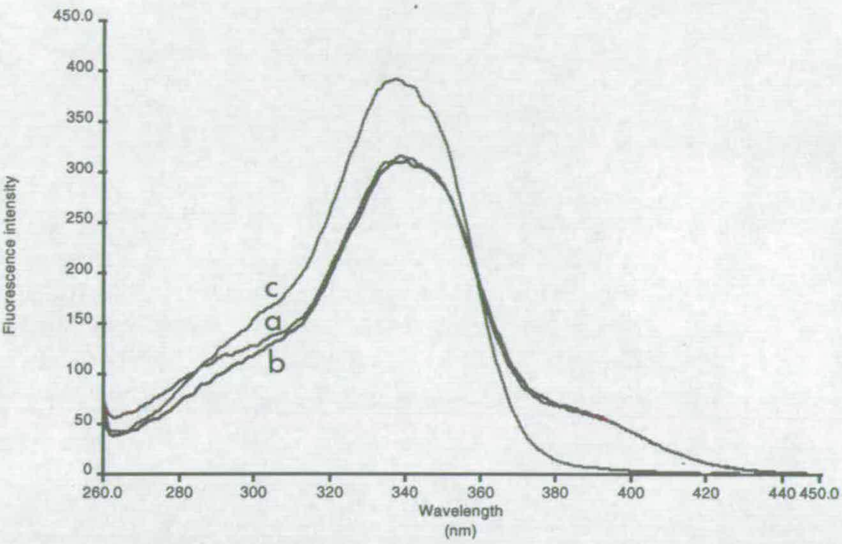
An initial excitation scan (a) was performed, then, K$^+$ was added to a final concentration of 67mM. Addition of K$^+$ caused a decrease in the fluorescence of fura-2 (b). The fluorescence of fura-2 then decreased (c) upon EGTA addition (500µM final concentration) and increased (d) upon addition of Ca^{2+} (1mM final concentration).

Appendix 3 Effect of 1.25mM caffeine on fura-2 excitation spectrum.



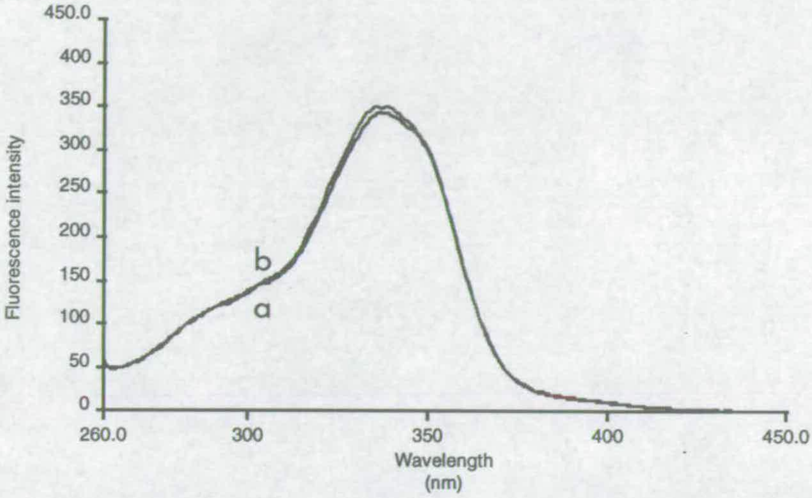
An initial excitation scan was (a) performed, then, caffeine was added to a final concentration of 1.25mM. Addition of caffeine altered the spectral properties of fura-2 (b).

Appendix 4 Effect of 10μM verapamil on fura-2 excitation spectrum.



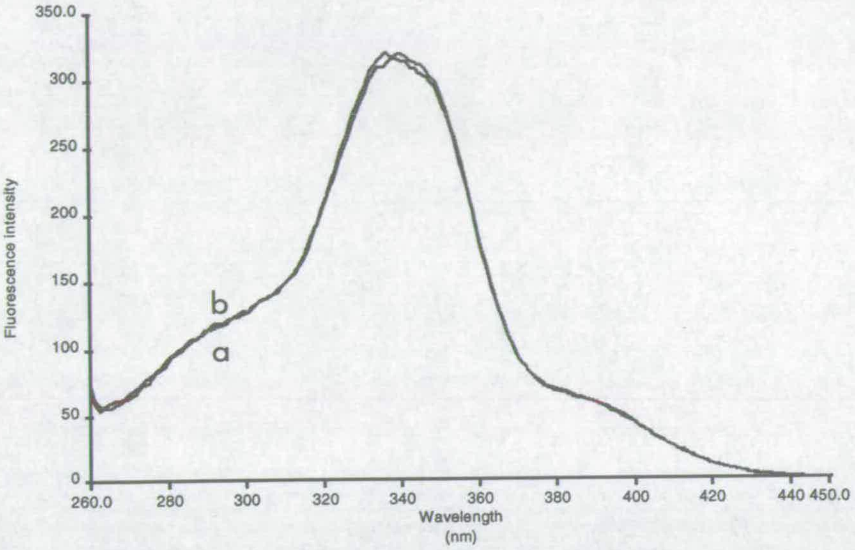
An initial excitation scan (a) was performed, then, verapamil was added to a final concentration of 10μM. Addition of verapamil had no effect on the spectral properties of fura-2 (b), and fura-2 remained responsive to Ca^{2+} (1mM) addition as evidenced by an increase in fluorescence (c).

Appendix 5 Effect of 1μM La^{3+} on fura-2 excitation spectrum.



An initial excitation scan (a) was performed, then, La^{3+} was added to a final concentration of 1μM. Addition of La^{3+} had no effect on the spectral properties of fura-2 (b). Subsequent addition of Ca^{2+} caused an elevation in fura-2 fluorescence (not shown).

Appendix 6 Effect of 5μM TMB-8 on fura-2 excitation spectrum.



An initial excitation scan (a) was performed, then, TMB-8 was added to a final concentration of 5μM. Addition of TMB-8 had no effect on the spectral properties of fura-2 (b). Subsequent addition of Ca^{2+} caused an elevation in fura-2 fluorescence (not shown).

Appendix 7

Consider a chemical equilibrium where D^{4-} denotes fura-2

$$(DCa)^{2-} \leftrightarrow D^{4-} + Ca^{2+}$$

the dissociation constant for this reaction, K_{diss}, is written as an expression of concentration as follows

$$\text{observed } K_{diss} = \frac{[D^{4-}]\,[Ca^{2+}]}{[CaD^{2-}]}$$

and the true K_{diss} as an expression of activities (a) of each species is written as follows

$$\text{true } K_{diss} = \frac{a_{Ca^{2+}}\, a_{D^{4-}}}{a_{(DCa)^{2-}}} \qquad (1)$$

So, for the association reaction

$$D^{4-} + Ca^{2+} \leftrightarrow (DCa)^{2-}$$

the association constant K_{ass} is

$$K_{ass} = \frac{a_{(DCa)^{2-}}}{a_{Ca^{2+}}\, a_{D^{4-}}}$$

Activity, a, is the "effective concentration" of a particular anion or cation and can be related to their concentration, C, by the following equation

$$a = \gamma c$$

where γ is the activity coefficient of the anion or cation. At low concentrations, ie. when c approaches 0M, γ becomes 1.

From equation 1, K_{diss} can be expressed as follows

$$K_{diss} = \frac{a_{Ca^{2+}}\, a_{D^{4-}}}{a_{(DCa)^{2-}}} = \frac{[D^{4-}]\,[Ca^{2+}]}{[CaD^{2-}]} \cdot \frac{\gamma_{Ca^{2+}}\, \gamma_{D^{4-}}}{[CaD^{2-}]} \qquad (2)$$

Debye and Huckel put forward a quantitative theory of electrolyte solution which is based on a model and allows us to calculate the quantity $\gamma_{\pm}$ from the basic properties of a solution. The model assumes that electrolytes are completely dissociated into ions in solution, that the solutions are dilute (that is, the concentration is 0.01M or lower) and that on average each ion is surrounded by ions of opposite charge, forming the ionic atmosphere

Neither the activity coefficient of a cation, γ_{+}, nor of an anion, γ_{-}, can be measured directly, so the final result is expressed in terms of the mean activity coefficient of an electrolyte by the Debye-Huckel limiting law as follows.

$$\log_{10} \gamma_{\pm} = -A \mid Z+ \; Z- \mid (I)0.5$$

where

$$A = \frac{1.8246\text{x}10^{6}}{(\epsilon T)^{1.5}}$$

$$= 0.509 \text{ for water}$$

ϵ = 80.1, the dielectric constant of water

$T = 293°K$

Z_+ and Z_- are the respective charges on specific species (eg., for Ca^{2+}, $Z_+ = 2$)

The quantity I, the ionic strength of the solution, is defined as follows:

$$I = \tfrac{1}{2} \Sigma_i c_i z_i^2$$

z_i is the charge number of an ion i and c_i is its concentration.

For any ion (i)

$$\log_{10}\gamma_i = -A z_i^2 (I)^{0.5}$$

From equation 2:

$$pK_{diss}\,(\text{true}) = pK_{diss}\,(\text{observed}) - \log_{10} \frac{\gamma_{Ca^{2+}}\,\gamma_{D^{4-}}}{[CaD^{2-}]}$$

$$\log_{10}\gamma_{Ca}{}^{2+} = -A\,4\,(I)^{0.5}$$

$$\log_{10}\gamma_{D}{}^{4-} = -A\,16\,(I)^{0.5}$$

$$\log_{10}\gamma_{CaD}{}^{2-} = -A\,4\,(I)^{0.5}$$

$$-\log_{10} \frac{\gamma_{Ca^{2+}}\,\gamma_{D^{4-}}}{[CaD^{2-}]} = \log_{10}\gamma_{Ca}{}^{2+} + \log_{10}\gamma_{D}{}^{4-} - \log_{10}\gamma_{CaD}{}^{2-}$$

$$= -A\,16\,(I)^{0.5}$$

Because KCl is the dominant species in the calibration buffer used to determine the K_{diss} of fura-2 for Ca^{2+}, as described in chapter 5, then $I = [K^+]$

If the buffer surrounding zoospores changed by 100μM due to a flux, either inward or outward, of K^+ or Na^+ as suggested by Iser *et al.* (1986), then the effect on the dissociation constant can be found as follows:

At 500μM KCl

$$-\log_{10} \frac{\gamma_{Ca^{2+}}\gamma_{D^{4-}}}{[CaD^{2-}]} = -\dot{A}\ 16\ \sqrt{5x10^{-4}}$$

= -0.182

At 600μM

$$-\log_{10} \frac{\gamma_{Ca^{2+}}\gamma_{D^{4-}}}{[CaD^{2-}]} = -A\ 16\ \sqrt{6x10^{-4}}$$

= -0.199

Therefore, a change in ionic strength due to K^+ or Na^+ will change the observed pK_{diss} by 0.017.

The observed K_{diss} found by calibration using fluorimetry, as described in chapter 5, was 188nM at 500μM ionic strength.

Therefore, pK_{diss} = -6.73 as found from the intercept from Figure 5.2.

The probable experimental error incurred in determining this pK_{diss} from the intercept from Figure 5.2 is likely to be ± 0.05 based on acceptable levels in physical chemistry and biology. If the intercept has error greater than the calculated difference in observed pK_{diss} when the ionic strength is changed by 100μM, then ionic strength has no significant effect on the K_d of fura-2 for Ca^{2+}.

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