SUPPRESSION OF ZOOSPORE CYST AND SPORANGIAL GERMINATION OF PHYTOPHTHORA INFESTANS BY TREATMENTS THAT MIGHT INTERFERE WITH CALCIUM-MEDIATED FUNCTIONS.

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I would however, like to dedicate this thesis to my Dad and the memory of my mum, without whose constant support and encouragement I would never have made it. Thank you.
### Abbreviations used in this thesis

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AA</td>
<td>Arachidonic acid</td>
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<tr>
<td>ABP</td>
<td>Actin-binding proteins</td>
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<tr>
<td>BAPTA</td>
<td>1,2-bis (2-aminophenoxy) ethane N,N,N', N' tetraacetic acid tetra sodium salt</td>
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<tr>
<td>bp</td>
<td>Base pair</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<tr>
<td>EGTA</td>
<td>Ethylene glycol-bis (β amino ethyl ether) N, N, N', N' tetraacetic acid</td>
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<tr>
<td>Gluc</td>
<td>Glucose</td>
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<tr>
<td>HR</td>
<td>Hypersensitive response</td>
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<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>KDa</td>
<td>Kilo Daltons</td>
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<tr>
<td>l</td>
<td>Litre</td>
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<tr>
<td>L glu</td>
<td>L glutamine</td>
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<tr>
<td>L asn</td>
<td>L asparagine</td>
</tr>
<tr>
<td>MAP's</td>
<td>Microtubule associated proteins</td>
</tr>
<tr>
<td>MCP</td>
<td>Methyl accepting chemotactic proteins</td>
</tr>
<tr>
<td>MEP</td>
<td>Malt extract and Peptone</td>
</tr>
<tr>
<td>ml</td>
<td>Millilitre</td>
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<tr>
<td>MPa</td>
<td>Mega pascals</td>
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<tr>
<td>O$_2^-$</td>
<td>Superoxide</td>
</tr>
<tr>
<td>OH$^-$</td>
<td>Hydroxide ion</td>
</tr>
<tr>
<td>s</td>
<td>Second</td>
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<tr>
<td>TFP</td>
<td>Trifluoperazie hydrochloride</td>
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Abstract

In Laboratory experiments, Ca\(^{2+}\), other divalent cations and various Ca\(^{2+}\) - modulating treatments were tested for effects on germination of zoospore cysts and of sporangia of three isolates of *Phytophthora infestans* and sometimes for an isolate of *P. palmivora*. The aim was to determine whether manipulation of Ca\(^{2+}\) or Ca\(^{2+}\)- mediated events had the potential for control of potato late blight caused by *P. infestans*. This work was based on previous evidence for a central role of Ca\(^{2+}\) in the infection sequence from zoospores of Phytopathogenic *Phytophthora*, *Pythium* and *Aphanomyces* spp.

Zoospore cyst germination of both *P. infestans* and *P. palmivora* was suppressed by early post-encystment addition of chelators EGTA or BAPTA, indicating a requirement for external Ca\(^{2+}\) or other polyvalent cations, by calcium channel blockers (La\(^{3+}\), Gd\(^{3+}\), verapamil) or by amiloride, indicating a requirement for flux of Ca\(^{2+}\) or other ions across the cyst membrane, by calmodulin antagonists (calmidazolium, dibucaine, trifluoperazine) and by intracellular calcium antagonists (caffeine, TMB-8), indicating a role for both calmodulin and for Ca\(^{2+}\) release from intracellular stores. Supplements of Ca\(^{2+}\) or other divalent cations (Ba\(^{2+}\), Mg\(^{2+}\)) also suppressed cyst germination, but sometimes partly or completely overcame the suppression of other treatments if applied early as post-treatments.

Germination of sporangia of *P. infestans* by hyphal outgrowth (direct germination, at 20\(^{\circ}\)C) or zoosporogenesis (indirect germination, at c. 12\(^{\circ}\)C) was suppressed by the same treatments as applied to zoospore cysts. These treatments sometimes caused rapid sporangial death, assessed microscopically by irreversible changes in cytoplasmic organisation. Their suppressive or toxic effects were generally more pronounced when sporangia were incubated to induce indirect rather than direct germination. The suppression caused by Ca\(^{2+}\)-modulating treatments could be rescued only partly by simultaneous or early post-application of divalent cations. Even potentially mild chemical treatments (0.1% pectin or 5mM orthophosphate) caused rapid (20-30 min) death of sporangia (especially when incubated for indirect germination). Simultaneous or early post-treatments with divalaent cations could only partly reverse the suppression. In comparative tests, the suppression of sporangial germination of *P. palmivora* by various treatments could sometimes be reversed by divalent cations or individual nutrients (L-asparagine, L-glutamine) whereas *P. infestans* never responded to these nutrients. In a continuous-flow system the germination of *P. infestans* sporangia was suppressed by Ca\(^{2+}\) concentrations as low as 100 \(\mu\)M, but continuous leaching with distilled water had no effect. All these findings are discussed in relation to the potential for control of *P. infestans* by Ca\(^{2+}\) manipulation leaves.
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CHAPTER I
INTRODUCTION

CHAPTER TWO MATERIALS AND METHODS

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2.2 Induction of indirect germination in Phytophthora infestans

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CHAPTER THREE. The effect of calcium-modulators ions and nutrients on the germination of Phytophthora sporangia
Chapter one

Introduction
1.1 General introduction

*Phytophthora* species have been recognised as prime plant pathogens ever since Anton de Bary first described *Phytophthora infestans* (Mont.) de Bary in 1876; in fact the name *Phytophthora* literally means plant destroyer. *Phytophthora* species have been implicated in foliar diseases (*P. infestans*), fruit rot (*P. palmivora* (Butler) var palmivora), root rot (*P. megasperma* Drechsler), and post harvest diseases (*P. syringe* (Klebahn)), (Coffey, 1991) It is therefore vitally important to understand the method of host location and infection employed so that these stages can be more accurately targeted for potential disease control. The organism of interest is the causative agent of late blight in potatoes (*Solanum tuberosum*), *Phytophthora infestans* (Mont) de Bary. This is one of the economically most important plant pathogens and was responsible for the Irish potato famine of 1845 which led to the death through starvation of over 1 million people (Gregory, 1983). In order to better target the disease, it is essential that all aspects of its life cycle are thoroughly understood.

1.2 The taxonomy of *Phytophthora*

The genus *Phytophthora* is closely related to the genus *Pythium* and so is placed in the family *Pythiaceae* in the class Oomycota. Both *Pythium* and *Phytophthora* species produce aseptate coenocytic hyphae and have the ability under favourable conditions to produce biflagellate zoospores. The Oomycota are one of the five groups of fungi that are capable of producing zoospores (Alexopoulos et al., 1996).

The Oomycota have traditionally been grouped into four main orders: the Lagendiales, Letomitales, Peronosporales and the Saprolegniales. The latter two groups have been used as the basis of two new sub classes the Peronsporomycetidae and the Saprolegniomycetidae (Beakes et al., 1995)
The morphological plasticity and overlap of the Oomycota makes classification difficult by traditional methods. The ultrastructural arrangements of the Oomycota such as the presence of tubular mitochondrial cristae, tripartite mastigonemes and the presence of $\alpha$-1,3 or $\alpha$-1,6 linked glucans in the cell wall are common with the chromophyte algae. By using 16s RNA sequencing (Bhattacharya and Medlin, 1992) and the NAD subunit 5 sequence (Parquin et al., 1995) the chromophyte algae, the diatoms and the Oomycota are seen as a cohesive evolutionary assemblage. On the basis of this evolutionary phylogeny the Oomycota are placed in the recently described Chromista kingdom (Dick, 1995). Cavalier-Smith (1986) characterised this kingdom as being composed of the brown algae and all protists having tripartite tubular hairs (mastigonemes) arranged in two rows along the flagellar shaft or chloroplast reticulum or both. Figure 1.1 shows the now accepted placement of the Oomycota. However although no longer considered as being a member of the true fungi *Phytophthora* shows the physiology, morphology and life cycle consistent with many fungi.

![Diagram of phylogenetic placement of the Oomycota](image)

Figure 1.1 The phylogenetic placement of the Oomycota, based on Judleson (1997)
1.3 Growth requirements of *Phytophthora* spp.

Growth of *Phytophthora* is typically fungal, exhibiting tip growth and a filamentous growth pattern. The hyphae of *Phytophthora* are unpigmented, coenocytic and aseptate. *Phytophthora* species absorb simple nutrients directly across the cell wall and membrane system but more complex polymers need to be degraded extracellularly by secreted enzymes before they can be taken up (Erwin and Ribierio, 1996).

*Phytophthora infestans* occupies the nutritional middle ground between the nutritionally less demanding Oomycota such as the saprophytic *Pythium* and *Saprolegnia* spp. and the obligate biotrophic downy mildews. The basic requirements for growth are an organic carbon source, O, H, P, S, K, Ca, Mg, Fe, N and several trace elements and thiamine (Hohl, 1991). Despite having the enzymes required *Phytophthora infestans*, is unable to utilise host polysaccharides such as starch and cellulose, and grows best when supplied with hexose sugars (Hohl, 1993). *P. infestans* has a preference for L-amino acids and appears to have lost the supposedly primitive trait of being able to assimilate oxidised nitrogen (Hohl, 1975), although Fothergill and Child (1964) reported that highly oxidised forms of both phosphorus and sulphur were readily utilised. *Phytophthora* species have an incomplete sterol synthesis pathway and display a requirement for the presence of β hydroxy-sterol which are essential for membrane integrity (Hohl, 1991) and sporangia production (Lancake, 1971). *Phytophthora infestans* shows a preference for ferrous iron compared to ferric iron and reducing agents such as ascorbic acid also promote growth by acting as a pH buffer (Cuppert and Lilly, 1973).
1.4. The fungal mode of growth

Apical or tip growth is common to the fungi, pollen tubes and root hairs in plants. It involves a controlled expansion of cell wall and plasmalemma to produce a polarised apical pattern of surface expansion and an elongated tubular hyphal shape (Heath, 1995; Bartnicki-Garcia, 1990).

This polarised pattern of cell growth can only occur when the expansion of the cell surface is focused at one location (Gow, 1994). Hyphal extension is therefore an extreme example of polarised cell growth, since cell extension is restricted to a narrow zone defined by a tapering hyphal apex. The vast majority of growth occurs within 1µM from the tip (Gooday, 1971).

Robertson (1958, 1959) was one of the first workers to suggest a feasible explanation for the process of tip growth. In his model he envisaged a system were a plastic deformable tip is produced and extends apically, while wall rigidification occurs behind the extending tip. This model was refined by Wessels et al. (1983) as the steady state growth theory. In its simplest form this assumes a continuous secretion at the apex of an expansible mixture of wall polymers that is continually removed backwards towards the base of the extension zone where a rigid complex arises by interactions between and among the constituent polymer chains. As the new wall material is added it becomes progressively more cross-linked and so able to resist turgor.

In order for tip growth to occur all the precursors for wall growth must be deposited in one place only. In fungi the wall biosynthesis precursors are transported in microvesicles. Fusion of these microvesicles with the surface provides new membrane for expansion and enzymes for wall synthesis (Gooday, 1994). The vesicles must be prevented from fusing with the lateral wall prior to arriving at the tip. At the
hyphal apex large numbers of microvesicles form a body called the Spitzenkorper (apical body) (Bracker et al., 1976).

The Spitzenkorper is confined to the higher fungi and is a single spheroid complex lacking discrete boundaries and is located in the apex of growing fungi. It is essential for growth and is thought to be the likely source of vesicles for exocytosis and cell surface expansion (Lopez-Franco and Bracker, 1996). The Spitzenkorper is very sensitive to changes in the environment of a hyphal apex and applied stresses induce extreme changes. Prosser (1994) and Harold (1997) proposed that the Spitzenkorper corresponds to a vesicle supply centre (VSC) and therefore functions as a holding station for secretory vesicles. The VSC is an essential component of the mathematical model for hyphal growth derived by Bartnicki-Garcia (1990). In this model the VSC releases vesicles equally in all directions, but as the VSC advances linearly the number of vesicles reaching the cell surface become increasingly disproportionate giving rise to the polarised apical growth pattern of surface expansion and the formation of the typical elongated tubular hyphal shape.

1.4.1 What drives the expansion of the hyphal tip?

The traditional view of tip growth is that it is a process fueled by turgor (Robertson 1958, 1969). However recent evidence seems to indicate that this is not always the case. In Saprolegnia ferax (gruithuisen) Thuret, Kaminskyj et al. (1992) only found a correlation between increased turgor and increased hyphal growth when the rate of hyphal growth exceeded 12µM min⁻¹, below this increasing turgor had no noticeable effect. In Achyla bisexualis Money and Harold (1992) found that at lower turgor pressure, the rate of hyphal growth was maintained by biogenesis of a softer wall at the apex; the production of this fluid cell wall enables growth to occur at turgor pressures as low as 0.01Mpa (Money and Harold 1993). It appears that there
is an interplay between turgor pressure and wall biogenesis which ultimately has to be controlled by the cytoplasm and the cytoskeleton (Harold, 1997).

In fungi the cytoskeleton is composed of microtubules and actin filaments with a number of actin-binding proteins (ABPs) and microtubule associated proteins (MAPs) (Heath, 1994). The cytoskeleton is a skeletal system permeating the cytoplasm that contains stress-bearing elements that can both order and shape cellular components and provide a base against which force can be applied (Gooday, 1994).

There is growing evidence for the role of the cytoskeleton in tip growth. Jackson and Heath (1990) and Heath (1996) showed that in Saprolegnia ferax actin at the tip is in the form of an actin cap that is seemingly involved in stabilising the plastic hyphal apex and localising vesicle exocytosis. F-actin at the tip is believed to play a role in the synthesis and organisation of the cytoplasm to support tip growth (Jackson and Heath, 1993). In Heath’s membrane skeletal model (Heath, 1995) F-actin associates with other proteins and the plasmalemma in order to better resist turgor. Further evidence comes from the work of That et al. (1980) in which actin cytoskeleton inhibitors stopped the growth of Neurospora crassa (Shear and Dodge), and the work of Temperli et al. (1991) in which microtubule inhibitors stopped the growth of P.infestans. As fungi contain calcium-binding cytoskeletal proteins such as calmodulin, ionic regulation, via Ca²⁺, of the cytoskeleton and ultimately of apical growth would be expected (Heath, 1994).

Garrill et al. (1992) found a polarised concentration of stretch activated-calcium channels and calcium-activated potassium channels within hyphal tips of S. ferax. This asymmetric distribution may lead to a biased entry of calcium into the cell, perhaps explaining the calcium gradient reported by Garrill et al. (1993) in S.ferax. This apical influx of calcium appears to be common to all tip-growing organisms, having recently been demonstrated in pollen tubes (Kuhtreiber and Jaffe, 1990) and root hairs (Schieffelbein et al., 1992). In order to ensure the maintenance of this
calcium gradient the vacuole appears to function together with the reticulum apex, to which it is connected, as an infinitely expandable calcium sink (Jackson and Heath, 1993; Allaway et al., 1997). The calcium gradient appears to enhance hyphal extension (Jackson and Heath, 1989) and to ensure normal tapered hyphal growth and a low level of branching (Robson et al., 1991). However Levina et al. (1995) found that the presence of stretch-activated calcium channels within hyphal tips of *N. crassa* were not involved in the establishment of a calcium gradient and they were not essential for growth.

In the higher fungi the Spitzenkorper is essential for the occurrence of tip growth but in the lower fungi such as the Oomycota Harold (1997) proposed a different model. In this model secretory vesicles carrying calcium channel proteins are transported to the apex where they undergo exocytosis and insertion into the membrane. This leads to a localised influx of calcium promoting the fusion and polymerisation of actin and localised cell wall deposition. As the hypha grows these channels are then swept backwards. Therefore it is the proposed distribution of these open stretch-activated calcium channels that dictates the pattern of exocytosis over the apical surface and the localised insertion of the new cell wall.

1.5 Disease symptoms and spread

1.5.1 Symptoms

The first symptoms that become apparent during infection of potato by *P. infestans* occur on the leaves, where purple-black or brown-black lesions appear at the leaf margins. These can appear at anytime during the development of the plant and spread to the petiole and the stem (Hooker, 1981). The tubers become affected later in the season with the early symptoms being confined to brown or purple patches on the skin which in damp soil progresses rapidly through the tuber and is accompanied by secondary infection by bacteria and other fungi. If the tuber is in dry soil, infection
is confined to the surface of the tuber; however if the tubers are incorrectly stored in moist conditions after harvest the infection can progress rapidly throughout the tuber (Erwin and Riberio, 1996).

The infection of the tuber is essential to ensure the survival of the pathogen from season to season: *P. infestans* overwinters in infected tubers either left in the soil or in cull piles and one viable infected tuber in 400 acres was sufficient to function as a primary focus for new infections in the following growing season. The infected tubers give rise to shoots with living mycelium in the cortex from which sporangiophores emerge and produce sporangia. These sporangia are then able to disseminate and establish new foci of infection. If an epidemic is to start then favourable environmental conditions are vital. Figure 1.2 shows the life cycle of *P. infestans*, based on Judelson (1997).

![Life cycle of P. infestans](image)

*Figure 1.2* The life cycle of *P. infestans* showing the distinct sexual and asexual stages.
1.5.2 The influence of prevailing conditions on the development of an epidemic

Foliar *Phytophthora* diseases such as *P. infestans* can increase from almost undetectable levels to very high levels within days (MacKenzie *et al.*, 1983). This increase in inoculum is caused by the rapid production of sporangia and zoospores from infected tissue under suitable conditions. Potato blight can therefore be considered to be a multicyclic disease and is able to rapidly cause epidemics when the prevailing conditions are suitable. Therefore it is essential to understand the conditions that drive an epidemic.

**Temperature**

Temperature influences all the stages of disease development, its effect being a summation of the effects on the different steps in the life cycle of *P. infestans* (Muller and Haig, 1953).

Schober and Ullrich (1985) reported that potato leaves could not be infected by directly germinating sporangia (germination via germ tubes) so in order for foliar infection to occur zoosporogenesis must take place. Crosier (1934) reported that the optimum temperature for indirect germination (zoosporogenesis) was 12-13°C. Zoospores of *P. infestans* can remain motile for 15 min at 24°C which is close to the optimum temperature for sporangia formation of 21°C. However Grainger (1979) reported that *P.infestans* was inactive in potato crops in Scotland at temperatures greater than 23°C, perhaps due to the fact that at temperatures above 20°C zoospores readily encyst to avoid the adverse conditions - most likely the absence of free water.
Water

The presence of free water is essential, as neither sporangia nor zoospores can infect dry foliage. As temperature increase, so the length of leaf wetness needs to be extended to ensure successful infection (Rotem et al., 1971; Harthill et. al., 1990). Periods of leaf wetness followed by short periods of leaf dryness substantially reduce the number of lesions produced. This is explained by the observations of Choen et al. (1974) who reported that when sporangia of *P. infestans* were exposed to brief periods of wetness followed by a short period of drying then irreversible damage to the sporangia occurred. If there is no initial period of wetness then a much longer period of dryness is required to cause the same effect. However Stewart et al. (1993) showed that potato leaves are more susceptible to blight in conditions of low soil moisture. This is most probably due to a reduction in the number of potentially antagonistic organisms on the tuber surface and a general increase in disease susceptibility due to water stress.

Humidity

High humidity has a major effect on the development of an epidemic of foliage late blight because two key stages in the life cycle - the production and germination of sporangia - are dependent upon high humidity. Unless the air is saturated the gradient of humidity decreases with distance from the leaf surface. Harrison and Lowe (1989) reported that anything that lowered the humidity such as increased airflow had a detrimental affect on disease progression. High humidity increases the length of survival of sporangia, and a sudden drop in humidity causes the sporangia to become detached from the sporangiophores by hygroscopic movements (Rotem and Cohen, 1974).
Air currents

Air currents and wind are essential for the long distance spread of the disease. The sporangia of *P. infestans* are hydrophilic and are therefore readily incorporated into raindrops: it is this splash dispersal mechanism that is responsible for the short distance dispersal of the pathogen through the crop canopy (MacKenzie *et al.*, 1983). For the rapid long distance spread as seen in Europe in 1845, high wind speeds are essential.

For successful spread of the pathogen the prevailing environmental conditions must be such as to ensure that the sporangia are viable. Rogoshin and Filippov (1983) reported an almost antagonistic role of the wind in the spread of the disease, as high wind speeds although moving the sporangia great distances, also reduce the relative humidity, thereby reducing the viability of the sporangia.

The length of the photoperiod

Dowley (1981) reported that the rate of lesion development increased on tomato as the length of the photoperiod was reduced. This is perhaps due to the reduced temperature associated with a decrease in solar radiation although Harrison *et al.* (1994) reported that the expression of non-race-specific resistance of potato leaves could be modified by temperature and light intensity.

Summary

As we can see from the information concerning the effect of climatic conditions on the development of an epidemic, there is not one variable that, when changed, does not effect one of the other variables. Therefore it must be realised that
the development of an epidemic is due to a complex interaction of the prevailing weather conditions, as some conditions favour development of sporangia but not their spread and vice-versa.

1.5.3 The role of the oospore

The vast majority of work looking at the effect of environment on disease development has focused on the sporangia. However, with the increasing frequency of naturally occurring oospores (Drenth et al., 1994) recent research has been directed at the effect of the environment on the oospores.

Oospores are formed at temperatures between 8 and 23°C as long as free water is present (Cohen et al., 1997) and are viable after being exposed to temperatures ranging from -20°C to +45°C (Fay and Fry, 1997). Oospores are viable under field conditions for at least eight months, and preliminary indications show that they may be able to survive more than one growing season (Pittis and Shattock, 1994). Furthermore the oospores exhibit asynchronous germination, and together with the potential to produce sporangia, provide a constant supply of infectious material enabling the fungus to survive adverse conditions and rapidly establish new foci of infection once conditions become more favourable (Drenth et al., 1994).

More research is needed on the effect of environmental conditions on oospore survival and the increasingly important role of the oospore in disease establishment so that these parameters can be included in new potato blight forecasting systems.

1.6 Sporangial germination

Although sporangia are the wind-dispersed propagules of *P. infestans* it is actually the zoospore that is the infective agent. Mature sporangia have a uniform
granular appearance, with a basal septum separating the sporangial cytoplasm from the connection to the sporangiophore. The sporangial apex is capped by a thick discoidal plug. Sporangia are generally about 50 μm by 15μm, with regularly spaced nuclei surrounded by vesicles. Sporangia of *Phytophthora* can germinate either directly via germ tubes or indirectly via zoospores. The germination pathway that the sporangium takes is mainly dependent upon the temperature of incubation (Crosier, 1934).

1.6.1 Direct germination

In *P. palmivora*, flagella are pre-formed in the sporangium without regard to the mode of germination, although they have no role in direct germination and are rapidly broken down upon the commencement of direct germination (Hemmes and Hohl, 1969). The presence of these pre-formed flagella suggests that indirect germination is the default mode of germination and direct germination only occurs under specific conditions. In order to obtain high levels of direct germination incubation at the appropriate temperature is often not sufficient and specific nutrients may need to be added (Hemmes and Hohl, 1972). The direct germination of *Phytophthora* is influenced by temperature, oxygen, pH and nutrients (Harvet, 1954; Aragaki *et al.*, 1967).

In direct germination the germ tube normally emerges from the tip of the sporangium (Figure 1.3) but in certain circumstances a swelling, termed the wall swelling, is produced from which several hyphae may emerge (Clerk, 1972) (Figure 1.3). Occasionally during the course of direct germination zoospores may be formed which encyst in the sporangia and germinate through the sporangial wall (Figure 1.3). A new sporangial wall is then laid down within the old wall and this is termed the germination wall (Hemmes and Hohl, 1969).
In indirect germination each nucleus is incorporated into a zoospore but in direct germination only a few nuclei enter the germ tubes whilst the remaining nuclei remain in the sporangia (Clerk, 1972).

Figure 1.3 Direct germination of *P. palmivora* showing the three characteristic forms. Photomicrographs taken by the author. Scale bar = 10µM
1.6.2 Indirect germination (zoosporogenesis)

The process of zoosporogenesis has to be induced by a temperature change (cold shock). During the first 20 minutes after the induction of zoosporogenesis large clusters of vesicles become apparent surrounding each nucleus. Within 75 minutes post-induction the sporangia in *P. cinnamomi* has become fully cellularised with the cytoplasm being divided into regular hexagons each containing a nucleus, a functional water expulsion vacuole and flagella (Hyde *et al.*, 1991). In *P. cinnamomi* cleavage and flagellum differentiation are concurrent events (Beakes, 1991). The zoospores are discharged in a membranous vesicle that soon breaks away (Figure 1.4). Once the papillar plug is displaced zoospore discharge is dependent upon osmotically generated pressure gradients (Gisi, 1983).

The zoospores of *Phytophthora* species can be considered to be naked motile protoplasts covered in a plasmamembrane. They are heterokont biflagellates with the point of flagellar insertion lying within the ventral groove next to the water expulsion vacuole which functions to maintain a constant osmotic environment (Carlile, 1983). The anterior flagellum is a tinsel-type flagellum and is covered in tripartite tubular hairs (mastigonemes), although Dick (1997) considers this terminology obsolete and refers to it as a straminipilous flagellum. The posterior flagellum is longer and smooth. The flagella extend up to 40 µm beyond the zoospore soma which is generally between 9 and 13 µm long (Lange and Olsen, 1983). Motile zoospores are responsible for the location and infection of the host (Figure 1.5).
Figure 1.4 Zoospore release from a sporangium of *P. palmivora*. Photomicrographs were taken by the author. Scale bar = 10μM
The zoospore, once released from the sporangium, must undergo a pre-determined series of events in order to locate and infect the host. From zoospore release to host penetration can take as little as 30 minutes (Deacon, 1988).

The responsive motile zoospore is converted into a walled cyst that adheres to the host before developing into a germling with a hyphal apex. Germlings can show tropic responses to external stimuli thereby maximising recognition and subsequent colonisation of the host (figure 1.6).

Occasionally a zoospore will encyst prematurely and these cysts may release a secondary zoospore to begin the process anew (Duniway, 1983).
1.7 Zoospore motility

The movement of zoospores is dependent upon the presence of free water, not only to enable the zoospore to swim, but also for the formation and discharge of the zoospore (Lange and Olsen, 1983). Without natural water flow, zoospores of Phytophthora megasperma can swim distances of up to 6cm towards the surface of a suspension (Pfender et al., 1977) and this is confirmed by the work of Newhook et al. (1981) who reported that although zoospores of P. cinnamomi (Rands) var. cinnamomi, had the ability to swim up to 6m, based on their swimming speed and duration of motility they only actually dispersed a distance of 6cm when released into a pool of water from a point source.

Phytophthora zoospores swim in straight lines with frequent random turns; as they swim they rotate about their axis producing a helical path (Carlile, 1983). They have an angular displacement of between 100 and 150μm s⁻¹ for P. cinnamomi and 77± 7.3 μm s⁻¹ for P. infestans (Allen and Newhook, 1974; Warburton, 1997). The large difference in the reported rates of swimming for P. cinnamomi is probably due to small differences in temperature and metabolic rate. The energy for this is supplied by endogenous lipid reserve, and (1-3) β-D glucan mycolaminarin (Bimpong, 1975; Carlile, 1986).
Swimming thrust is supplied by the beating of the flagella, with waves being propagated from the flagellar base towards the tip, creating a fluid flow that is parallel to the flagellar axis. Due to the presence of the mastigonemes the anterior flagellum pulls the cell in the direction of wave propagation (Holwill, 1982). The anterior flagellum is reported to contribute about 90% of the thrust and the posterior flagellum about 10%. The posterior flagellum may periodically stop beating for 50–100ms during which time it acts as a rudder, causing swift but smooth changes in direction. The tubular mastigonemes are essential for smooth swimming as the addition of monoclonal antibodies specific for them disrupts the zoospore motility (Cahill et al., 1996).

Young et al. (1979) reported that zoospores were freely motile in ideal soils composed of glass beads as long as the pore sizes were greater than the diameter of the zoospore soma. Newhook et al. (1981) further showed that zoospores of P. cinnamomi were able to swim without encystment through “soils” composed of sand. This suggests that zoospores produced in soil would be able to swim freely through the soil to reach suitable host material.

1.7.1 The role of calcium in motility

Several studies have shown that the addition of ion treatments can influence the swimming patterns of motile zoospores (Allen and Harvey, 1974; Thomas and Butler, 1989; Ersek et al., 1991). Thomas and Butler demonstrated that the addition of Ca²⁺ caused Achyla to swim in straight lines, and Ersek et al. were able to immobilise zoospores of P. infestans by the addition of Li⁺, although this inhibition could be overcome by the addition of Ca²⁺. Phytophthora zoospores show a requirement for micromolar levels of Ca²⁺ and Mg²⁺ for the maintenance of long term motility (Carlile, 1983).
Calcium has been implicated in flagellar activity of trout sperm (Boiteno and Omoto, 1992) and it appears to regulate the activity and function of the axeome by controlling the levels of protein phosphorylation and consequently cell motility (Bloodgood, 1991). The calcium-binding protein calmodulin acts as a signal transducer in calcium-regulated motility, and together with another calcium-binding protein, centrin, and has been found to be concentrated at the base of the anterior flagella in *P. cinnamomi* (Gubler et al., 1990). Several studies have shown that calcium has a central role in motility in other organisms such as protozoa (Ekert, 1972), algae (Harz and Hagman, 1991) and marine invertebrates (Tamm, 1989).

Donaldson and Deacon (1993a) found that the addition of calcium modulating pharmacological agents altered the normal helical swimming pattern of *Pythium aphanidermatum* zoospores, with different modulators producing a different characteristic response. Calcium chelators such as EGTA produced periods of prolonged straight swimming. The Ca$^{2+}$ channel blocker verapamil causes the zoospore to swim in circles, and the Ca$^{2+}$ ionophore A23187 and the Na$^{+}$/Ca$^{2+}$ membrane flux inhibitor amiloride caused jerky swimming. It was only calmodulin antagonists such as dibucaine that caused a reduction in swimming speed, and these treatments also induced a posterior drift, of the zoospore.

Warburton (1997) applied several of these modulator treatments to a range of *Phytophthora* spp. and obtained broadly similar results. This together with the work of Reid et al. (1995) in which calcium modulator treatments were found to block the autoaggregation of *Phytophthora* zoospores provides firm evidence for the central role of calcium in the motility of *Phytophthora* zoospores. The regulation of motility in these Oomycota is probably broadly similar to that in *Paramecium* in which variations in intracellular Ca$^{2+}$ concentrations regulated the beating of the cilia (Eckert, 1972).
1.8 Kinesis and taxis

The external environment may influence the motile behaviour of cells by kinesis or taxis so that they either accumulate at, or move away, from a particular site (Carlile, 1980a, 1983; Deacon and Donaldson, 1993).

Kinesis

Kinesis is a unidirectional response to specific environmental stimuli (Dunn, 1990). Klinokenesis is the regulation of the turning frequency to produce a biased random walk. If the organism is moving in the correct direction (towards an attractant or away from a repellent) the frequency of random turning is reduced and the organism swims in a straight line. If the cell is moving in the wrong direction the frequency of random turns is increased. Orthokenesis is a change in the swimming speed produced by an environmental stimulus. If the cell is close to an attractant source the speed of swimming is reduced so that the cell spends longer in the vicinity of the attractant, for example in the case of phytopathogenic fungi on plant roots (Carlile, 1983).

Taxis

Taxis which more correctly is referred to as topotaxis is a directionally orientated responses so that if the cell moves towards a stimulus it is said to be a positive response and if it moves away it is said to be a negative response. Fungal zoospores respond to a wide range of environmental stimuli, details of which are listed below.
Adelphotaxis

Adelphotaxis is the mutual attraction between zoospores which may lead to autoaggregation. This phenomenon is most often associated with very high zoospore concentrations and has often been considered to be a laboratory artefact; however pattern swimming has been reported for *P. drechsleri*, *P. capsici* and *P. palmivora* (Ko and Chan, 1974; Ko and Chase, 1973).

Thomas and Patterson (1990) proposed a two stage model for autoaggregation in *Achlya bisexualis* in which a few zoospores respond to an initial signal (attractant) which is reinforced by an autoaggregation signal released by the gathering zoospores. In all cases there is an obvious need for an attractant. Calcium is released by zoospores during the process of encystment (Irving et al., 1984) and has also been shown to influence zoospore motility (Ersek et al., 1991; Deacon and Donaldson 1993) it is therefore a likely candidate for the attractant in autoaggregation, although this has not been proven.

Reid et al. (1995) investigated autoaggregation in *P. palmivora*. Autoaggregation was only found to occur at zoospore densities of greater than $10^5$ zoospores per ml and once an autoaggregation clump started to form it developed exponentially, creating water currents which could draw in more zoospores. Zoospores show taxis towards these clumps at distances of up to 300µm and zoospores which encyst away from the clump show germtube tropism towards it. Calcium plays a central role in this autoaggregation as the addition of calcium modulator treatments blocks the process and the zoospores also showed attraction towards crystals of CaCO$_3$ (Reid et al., 1995).

Using calcium-sensitive microelectrodes, an increase in calcium concentration was measured in the vicinity of the clumps(Reid et al., 1995). This increase in concentration could be due to the large calcium release associated with encystment and could serve as the secondary signal described by Thomas and Peterson (1989).
However Reid et al., showed that the process of autoaggregation was species-specific such that clumps of *P. palmivora* zoospores did not attract zoospores of *Py. aphanidermatum*, and vice-versa. Therefore the role of Ca\(^{2+}\) in autoaggregation seems unclear. The role of the process *in vivo* is unclear due to the very high zoospore concentrations required; however if some zoospores locate a suitable infection site then the release of an autoaggregation factor would increase the number of zoospores in the vicinity and increase the likelihood of successful infection.

*Electrotaxis*

Electrotaxis is the movement of a cell in response to an electrical field. The effect of an applied electrical field on zoospore motility was studied by Troutman and Wills (1964), Ho and Hickman (1967), Katsura and Miyata (1971) and Khew and Zentmyer (1974).

The observation that zoospores of *Phytophthora* are influenced by electrical fields is probably due to their surface properties, as zoospores of *Phytophthora* have been shown to carry a net negative charge (Khew and Zentmyer, 1974). Electrotaxis is significant because it may improve the ability of the zoospore to perceive and locate host roots.

Miller *et al.* (1988) detected ion currents associated with the roots of *Nicotiana tabacum* and concluded that they were due to the spatial heterogeneities in the electrogenic ion transport system. A positive electric current enters at the zone of cell elongation and exits at the mature tissue. Inward directed ion currents are also found at wound sites and where lateral roots emerge (Morris *et al.*, 1992). These sites of maximal current intensity are also the sites at which zoospores accumulate most frequently. The phenomenon of electrotaxis was often considered to be an artefact, but Morris *et al.*, (1992) demonstrated taxis to electrodes at physiologically relevant field strengths.
Although zoospores carry an overall net negative charge, different species of *Phytophthora* distribute this charge in contrasting fashion which leads to differences in their alignment when ethanol-immobilised zoospores are placed in an electrical field. In *P. palmivora* the anterior flagellum carries a net negative charge and therefore the zoospore orientates towards the anode whereas in *Py. aphanidermatum* the anterior flagellum carries a net positive charge and therefore the zoospore orientates towards the cathode (Morris and Gow, 1993).

In plants the current is carried mainly in the form of protons, so it would be expected that zoospores would be able to respond to a proton gradient (pH gradient). Morris *et al.* (1995) showed that zoospores of *P. palmivora* were attracted to capillary tubes containing acidic buffers and repelled by those containing buffers of alkaline pH. However in the rhizosphere root-generated pH gradients had little if any role in taxis to roots. A possible attractant is Ca$^{2+}$ which has a central role in zoospore motility and has been found to be associated with plant roots (Miller *et al.*, 1988).

In *P. palmivora* the mechanism of electrotaxis seems to involve orientation of the zoospore (topotaxis) according to its natural electrical dipole and the voltage dependent stimulation of turning frequency (klinokinesis). A change in the charge at the cell surface will, provided the internal charge is unaltered, cause a change in the transmembrane potential altering the flagellar activity and thereby causing turning. Electrotaxis alone seems unlikely to be sufficient to bring the zoospore into direct contact with the root and probably functions synergistically with other stimuli.

**Geotaxis**

Cameron and Carlile (1980a) found that zoospores of several *Phytophthora* species tend to swim upwards towards the surface, exhibiting negative geotaxis. This was due to the cellular arrangement of the *Phytophthora* zoospore and was not a sensory transduction system. It could be explained by the fact that the point of
flagellar insertion is closer to the anterior than to the posterior of the zoospore, so that the centre of gravity is displaced, causing a tendency for “upwards” swimming. In the rhizosphere the highest frequency of rootlets is to be found towards the soil surface. Geotaxis would therefore lead the zoospore to the area with the greatest frequency of susceptible tissue.

**Rheotaxis**

Rheotaxis is the movement of a zoospore against a water current and has been reported for *P. capsici* (Katsura and Miyata 1971). In natural systems water tends to percolate through the soil from the surface under the influence of gravity. A cell that can perceive and swim against this current flow would be able to locate the soil surface and the area of greatest frequency of susceptible host tissue. The processes of rheotaxis and geotaxis both could serve to get the zoospore towards the surface.

Phototaxis and aerotaxis have been suggested as mechanisms by which zoospores could reach the soil surface, however no firm evidence exists to support this suggestion for *Phytophthora* zoospores.

**Chemotaxis**

Chemotaxis is the movement of an organism in response to a chemical stimulus. A diverse range of chemicals have been shown to induce a chemotactic responses in *Phytophthora* species e.g. ions, sugars, amino acids and volatile compounds such as ethanol (Ho and Hickman, 1967; Zentmyer, 1970; Carlile, 1980a; Deacon and Donaldson, 1993).

Many species of *Phytophthora* show attraction towards, and accumulation on, plant roots when these are placed in the zoospore suspension. The attraction is usually
greatest at the zone of cell elongation (Royle and Hickman, 1964a; Zentmyer, 1971; Hinch and Clarke, 1980; Mitchell and Deacon 1986; Longman and Callow, 1987; Jones et al., 1991). Zoospores are also attracted to the zone of root hair emergence and wound sites.

In these cases, chemotaxis is likely to be to the low molecular weight compounds such as amino acids and sugars released by the root. These compounds diffuse through the soil setting up a concentration gradient which the zoospores can respond to. The areas of maximum nutrient release and therefore steepest concentration gradient are those areas which are most attractive to zoospores and also to which bacteria such as *Rhizobuma* and *Agrobacterium* are attracted (Gaworzewska and Carlile, 1982). The amount of nutrients released by the roots is dependent upon the age and species of the plant as well as environmental conditions such as soil moisture and temperature (Rovira, 1969).

Most of this work has been carried out *in vitro*. However Mehrotra (1970, 1972) grew safflower and soyabeans so that the roots grew through the soil next to a cellulose acetate window. When zoospores of *P. dreschsleri* and *P. megasperma* were added on top of the membrane they accumulated on regions of the membrane overlying the roots. In a further experiment plants were grown in pots containing soil and a zoospore suspension was introduced. On staining the roots with calcoflour white clear localised accumulation of zoospores on the roots was seen.

The attraction of *Phytophthora* zoospores to plant roots is generally nonspecific with regard to the type of plant. However Zentmyer (1970) reported that *P. cinnamomi* accumulates more frequently on host than on non-host roots. Mitchell and Deacon (1986) demonstrated that the graminicolous *Pythium* species showed significantly higher levels of accumulation and encystment on the Gramineae compared to dicotyledonous plants, although this difference was negated when the roots were wounded. Goldberg *et al.* (1989) reported that *Pythium dissotocum* was strongly attracted to cotton roots whereas *Pythium catenulatum* was not attracted to
these roots even after wounding. The zoospores of both fungi respond to the same compounds \textit{in vitro}, so Donaldson and Deacon (1993b) postulated that the reported differential attraction to plant roots might be explained if all plants release attractants such as amino acids but if some plants additionally, release chemoattractant "blockers" which have different effects on different fungi. Some evidence for blockade of chemotaxis was found \textit{in vitro} (Donaldson and Deacon 1993b)

In order to understand the mechanism of chemoattraction, and the chemicals involved, taxis towards individual chemicals and chemical mixtures has to be investigated.

1.8.1 Chemotaxis towards pure compounds

Responses to individual chemicals and chemical mixtures have been assessed \textit{in vitro} using a model capillary system.

Ho and Hickman (1967b) isolated root exudates from pea plants and found that the addition of these exudates significantly shortened the period of motility of zoospores of \textit{P. megasperma} var \textit{sojae}. When pea root exudate was fractionated the amino-acid-containing cationic fraction was highly attractive to zoospores of \textit{Py. aphanidermatum}. The neutral sugar-containing fraction also showed strong attraction whereas the anionic fraction was only slightly attractive (Ho and Hickman1967b).

Khew and Zentmyer (1973) and Halsall (1976) showed that individual amino acids and sugars were attractive to a wide range of \textit{Phytophthora} spp. All \textit{Phytophthora} and \textit{Pythium} species tested showed attraction to one or more of L-glutamate, L-aspartate, L-asparagine and L-glutamine, which together represent more than half the amino acid content of root exudate (Rovira, 1969; Kraffczyk \textit{et al.}, 1984). Donaldson and Deacon (1993) found that \textit{Py catenulatum}. \textit{Py dissotocum} and \textit{Py aphanidermatum} were only attracted to the L-isomers of amino acids. Khew and
Zentmyer (1973) reported that a background of one amino acid can block chemotaxis to another, normally attractive amino acid, indicating that competition between attractants might occur. *In vitro* attraction is generally greatest when zoospores are exposed to a combination of amino acids. Carlile (1983) noted that the ability to respond to several amino acids would ensure that the zoospore reaches the root surface even if one of the receptors became blocked or saturated.

Allen and Newhook (1973, 1974) reported that zoospores of *P. cinnamomi* showed attraction to capillary tubes filled with ethanol, and as the zoospores moved up the concentration gradient the frequency of random turning was suppressed. *Phytophthora palmivora* also shows attraction towards ethanol (Cameron and Carlile, 1978). Plants that are growing in waterlogged soil will have roots that are exposed to anaerobic conditions. In anaerobic conditions these roots will undergo alcoholic fermentation, so that taxis towards ethanol would bring the zoospore into proximity of the root, which would perhaps have reduced resistance to infection due to anaerobiosis.

Some *Phytophthora* zoospores show host-specific taxis in which very low concentrations of host-specific compounds elicit a chemotactic response. For example, zoospores of *P. palmivora* have a chemotactic threshold of 1μM for isovaleraldehyde (although this compound has not been shown to be produced by roots). Experiments to try and replace isovaleraldehyde from the zoospore surface by additions of other compounds indicated that isovaleraldehyde had a very high binding affinity and could only be displaced partly by chemically related compounds (Cameron and Carlile 1978, 1981). *Phytophthora sojae* has shown taxis, *in vitro*, to the host (soybean) isoflavones daidzein and genistein (Morris and Ward, 1992). It appears that zoospores are like bacteria in that some of them have a high affinity, host specific system, superimposed on a more general chemotaxis system (Ashby et al., 1988; Caetano-Anollos et al., 1988).
1.8.2. The mechanism of chemotaxis

In eukaryotes chemosensory transduction appears to be initiated at the membrane surface by interaction of an external stimulus with a receptor molecule and subsequent transduction into an intracellular message (Van Houten, 1979). In zoospores the plasma membrane extends over the entire cell including the flagella. Differences in the binding specificity of monoclonal antibodies to the anterior and posterior flagella suggest that these chemosensory receptors may be located on the flagella (Hardham, 1995).

The receptor molecules in bacterial chemotaxis, and the mechanism by which they regulate flagellar activity have been thoroughly investigated (Alder, 1975; Armitage, 1992; Stock and Surette, 1996). In Escherichia coli and Salmonella thyphimurium normal swimming involves “runs” of 1.0s interspaced with tumbles lasting 0.1s. When the cell is swimming towards an attractant source or away from a repellent the frequency of tumbles is reduced biasing the random walk in the preferred direction (Berg and Brown, 1972). In order for the cells to respond to a concentration gradient they must be capable of temporal sensing.

In E.coli and S. typhimurium the receptor molecules are a family of transmembrane proteins that are regulated by methylesterification and are therefore termed methyl accepting chemotactic proteins (MCPs). Each MCP has an extracellular sensory domain that conveys stimuli, via transmembrane sequences, to an intracellular signalling domain (Ames and Parkinson, 1988). The MCPs interact with a family of six cytoplasmic signal proteins that are products of the genes CheA, CheB, CheR, CheW, CheY, and CheZ.

The MCPs signal through a complex with the CheA and CheW proteins. CheA functions as a protein kinase, and autophosphorylates, with the phosphoryl group then being passed on to the CheY protein. The CheY protein binds to the flagellar motor causing tumbling swimming. The CheZ protein is then responsible for the
dephosphorylation of the CheY protein. Attractants binding to the MCPs inhibit kinases activity, favouring smooth swimming. The methylation of the MCP also controls kinase activity (Doetsch, 1972).

In *Paramecium* the membrane potential regulates not only the turning frequency but also the swimming speed, with action potentials causing a transient reversal of beating, initiating a change in direction (Van Outen, 1979). In *Phytophthora* negative chemotaxis to cations is due to the general mechanism of interaction of the cation with the negatively charged membrane. A change in the charge at the cell surface will, provided the internal charge is unaltered, cause a change in transmembrane potential causing changed flagellar activity and turning (Cameron and Carlile, 1980). When zoospores of *P. cinnamomi* encounter an ethanol gradient hyperpolarisation of the membrane occurs preventing spontaneous depolarisation and flagellar reorientation (Allen and Newhook, 1973). Calcium and cAMP also appear to be involved in regulating axonemal activity by controlling the level of protein phosphorylation (Bloodgood, 1991).

### 1.9 Encystment

During zoospore encystment the cell changes from a motile protoplast to a spherical cell with a microfibrillar wall below a glycoprotein coat. These changes have been extensively reviewed for *Phytophthora* (Hemmes and Hohl, 1971; Tokunaga and Bartnicki-Garcia, 1971; Bimpong and Hickman, 1975; Hardham et al., 1991) and for *Pythium* (Grove and Bracker, 1978).

During encystment the flagella are either retracted or shed and large-scale rearrangement of the cytoplasmic organelles occurs. The elucidation of the factors and processes involved in encystment has been aided by the ability to induce entire
populations of zoospores to encyst synchronously by both physical and chemical means.

1.9.1 Factors that induce encystment

Factors that induce encystment can be divided into two main groups- chemical or physical- although it should be noted that some treatments that induce encystment prevent subsequent germination. Among the physical treatments that have shown to induce encystment are agitation, contact with a host surface, extremes of temperature, pH, temperature and excessive dilution of a zoospore suspension (Tokunaga and Bartnicki-Garcia, 1971; Held, 1972; Ho and Hickman, 1967a). Generally it appears that treatments likely to increase the frequency of collisions between zoospores are also capable of causing encystment. Chemical factors capable of causing encystment include amino acids (Dill and Fuller, 1971; Donaldson and Deacon, 1993b), calcium modulators (Irving and Grant, 1984), colchicine (Held, 1972), ions (Grant et al., 1986; Iser et al., 1991), lectins (Hardham and Suzaki, 1986; Longman and Callow, 1987), monoclonal antibodies (Hardham and Suzaki, 1987) and phosphatidic acid (Zhang et al., 1992) as well as a range of host-specific factors.

1.9.2 Host surface factors involved in encystment

*In vivo* encystment appears to involve recognition of host factors, as first shown for zoospores of the mycoparasitic chytrid *Rozolla allomycis* which shows taxis to both host and non-host plants but it only encysts on host fungi (Held, 1974). As the vast majority of zoospores of phytopathogenic root-infecting fungi encyst at the zone of root elongation it seems sensible that if host factors are present then they will be located here.
Hinch and Clarke (1980) looked at the binding of zoospores of *P. cinnamomi* on the roots of maize. The area of maximal zoospore binding was at the elongation zone, and this was also the area of maximal binding for a fucose-specific lectin. The zoospore binding could be prevented by pretreatments that either removed or bound these fucose residues. Similar results were achieved by Longman and Callow (1987) with *Py. aphanidermatum* on cress roots, but as well as fucose residues, D-manosyl, D-glucosyl or D-galactosyl residues were also implicated. When root mucilage was fractionated the fucose-containing fraction caused as much zoospore encystment as did the unfractionated root mucilage (Estrada Garcia *et al.*, 1990). Zoospores of *P. cinnamomi* only bound to glass beads when they were covered with maize root mucilage or with mcoidan (sulphonated fucose polymer) (Kelleher *et al.*, 1990). As well as root mucilage, other host factors such as poly-D-galacturonic acid, pectin, alginate and host flavonoids have all been found to induce encystment in *Pythium* and *Phytophthora* spp. (Irving and Grant, 1984; Grant *et al.*, 1986; Zhang *et al.*, 1990; Jones *et al.*, 1991; Donaldson and Deacon, 1993 c).

It has been shown that applied treatments which cover the root (or other host) surface do not block attraction but only block attachment (Held, 1974; Mitchell and Deacon, 1986; Donaldson and Deacon, 1993). Jones *et al.* (1991) reported that the attraction and attachment of zoospores of *Py. aphanidermatum* was unaffected even when the roots were covered with a double layer of calcium alginate gel. It therefore appears that, at least in certain cases, the inducement of localised encystment is due solely to localised taxis, and encystment may therefore be due to the high level of nutrients present in localised zones.

1.9.3 Ultrastructural changes associated with encystment

When *Phytophthora* and *Pythium* zoospores encyst they round up, shed or retract their flagella, and secrete a thin cyst coat (Ho and Hickman, 1967; Grove and Bracker, 1978).
Within the zoospore, pre-formed peripheral vesicles fuse with the plasma membrane and deposit a glycoprotein coat on the spore surface (Sing and Bartnicki-Garcia, 1975 a, b). Ca\(^{2+}\) binding proteins have also been visualised along with the peripheral cisternae and peripheral vesicles by fixing encysting zoospores in Ca\(^{2+}\) containing glutaraldehyde (Hemmes and Pinto da Silva, 1980). They are believed to be involved in triggering membrane fusion and exocytosis. Within 3-4 minutes of the induction of encystment a wall develops under the cyst coat (Sing and Bartnicki-Garcia, 1975 a).

Recently, monoclonal antibodies and lectins have been used to characterise the vesicle contents and study their movements during encystment (Hardham et al., 1986; Gubler and Hardham, 1988). Monoclonal antibodies termed Cpa-2, Cpw-1, Lpv-1 and Vsv-1 were raised against components of spores of *P. cinnamomi* and were specific for each of the types of peripheral vesicle present in this species.

Cpw-1 labels the vesiculated peripheral cisternae at the early stages of encystment but then binds to the cyst wall once it appears at the surface. Cpa-2 and Vsv-1 label small peripheral vesicles that predominate beneath the spore surface on the dorsal and ventral side of the cell respectively. Both of these types of vesicles are exocytosed rapidly upon the induction of encystment.

Material from the ventral vesicles covers about one third of the cyst; the contents of these vesicles are believed to function as an adhesive material which mediates attachment to an adjacent surface. Material from the dorsal vesicles covers the rest of the cyst, these glycoproteins are believed to form the cyst coat. Lpv-1 labels the large peripheral vesicles, which do not undergo exocytosis but instead, about 5-10 minutes after the induction of encystment, move away from the plasmamembrane and become dispersed throughout the cytoplasm (Gubler and Hardham, 1988). This behaviour has also been observed in *P. nicotianae* and *P. parasitica*. These large vesicles were present in germlings 2h after germination but
had disappeared by 4h and are thought to serve as a protein store for early germling growth (Dearnaley et al., 1996). These three types of vesicle are not present in normally rapidly growing hyphae and only appear under conditions that induce sporangial formation. The process of encystment requires neither RNA or protein synthesis (Soll and Sonnerburn, 1971) although it appears that the synthesis of new proteins is required for cyst germination (Leaver and Lovett, 1974) and it is most likely that this \textit{de novo} synthesis depends upon endogenous precursors (Pennington et al., 1989) (Figure 1.7).

As well as the changes associated with the re-positioning of vesicles during encystment, the nucleus becomes rounded and assumes a central position in the cell, the anchoring system of rootlets disappears and the large water expulsion vacuole and associated membranes and vesicles disappear.
Figure 1.7 Simplified diagram of a zoospore in transverse section showing the ultrastructural changes that occur during encystment.
1.10 Cyst adhesion

Newly formed cysts are naturally adhesive and show non-specific attachment due to the release of an adhesive. Once the cysts have adhered they are not readily dislodged (Sing and Bartnicki-Garcia, 1975a)

The adhesive appears to be a glycoprotein, and immunological labelling suggests that the material is released from the ventral vesicles (Hardham and Gubler, 1990, Gubler and Hardham, 1991). Due to the orientation of the zoospore upon encystment, with the ventral region next to the host surface (Mitchell and Deacon, 1986) the adhesive is released into the small area between the encysting zoospore and the host surface. Once adhered to the host surface, adhesion is effectively irreversible.

The innate adhesiveness of cysts is rapidly lost and may disappear completely within 4 minutes after the induction of encystment if zoospores are induced to encyst way from a surface, although the glycoprotein coat (the presumed adhesive) is still present (Gubler et al., 1989). This loss of adhesiveness could be mimicked in young cysts by the chelation of Ca\(^{2+}\) by the addition of EGTA, before the cyst had contacted a host surface. Conversely, the loss of the adhesiveness of older cysts could be restored by the addition of Ca\(^{2+}\) (Gubler et al., 1989). This phenomenon has also been demonstrated in *Pythium* (Donaldson and Deacon, 1992).

*Ph. palmivora* releases up to 30 % of its intracellular Ca\(^{2+}\) within 2 minutes of the induction of encystment (Irving et al., 1984). Gubler et al. (1989) proposed that this released Ca\(^{2+}\) was required to fix the glycoprotein adhesive to the surface and that it dispersed if the cell had not contacted a surface in the early stages of encystment. An exogenous Ca\(^{2+}\) supplement was then required to restore this adhesiveness.
1.11 Zoospore cyst germination

The adhesion and germination of cysts is closely linked and findings indicate a central role of Ca\(^{2+}\) in this sequence (Donaldson and 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. Such cysts required exogenous Ca\(^{2+}\) or amino acids (if low Ca\(^{2+}\) was present) to induce germination.

From these findings Donaldson and Deacon (1992) proposed a simple model for germination in vivo. Zoospores dock precisely on the host so that their fixed point of germination (the ventral groove) is adjacent to the host (Hardham and Gubler, 1990; Jones et al., 1991) and the released glycoprotein adheres the cyst in that position. An initial release of Ca\(^{2+}\) during encystment (Irving et al., 1984) was suggested to lead to Ca\(^{2+}\) entrapment with the adhesive, next to the surface on which encystment occurred followed within 2 minutes by re-uptake of a large amount of Ca\(^{2+}\) (Iser et al., 1989). The initial release of Ca\(^{2+}\) interacts with the adhesive fixing the cyst to the host. The adhesive pad prevents the dispersion of the released Ca\(^{2+}\) enabling it to be rapidly reabsorbed, triggering germination. Due to the cyst orientation, germination leads directly to attempted host penetration.

This hypothesis is consistent with many in vitro studies which show that Ca\(^{2+}\) can trigger the germination of Phytophthora spp. (e.g. Bryt et al., 1982; Grant et al., 1986; Von Brombsen and Deacon, 1996; Deacon and Saxena, 1997) and Pythium (Donaldson and Deacon, 1992). This model envisions that the zoospore has a natural reporter system to signal that it has docked correctly. The nucleus of the motile zoospore extends as a beak and microtubules connect this to the base of the flagellar apparatus (Grove and Bracker, 1978), providing a possible cytoskeleton system that could transmit the signal for cyst germination.
The extreme base of the anterior flagellum has high concentrations of locally exposed calmodulin (Ca$_{2}^{+}$ binding protein), and centrin (another Ca$_{2}^{+}$ binding protein) is present on the basal body connecting fibres and microtubules of the anterior microtubule rootlet (Gubler et al., 1990; Hardham, 1992). This is in the area of future germ tube growth and it would seem that this is a likely area for the germination control centre to be located.

Recently the work of Warburton and Deacon (1998) attempted to test this model of calcium-mediated germination of zoospore cysts. Zoospores of *P. parasitica* were induced to encyst in the presence of a membrane-impermeable calcium-sensitive dye (Fluor -3) so that changes in the Ca$_{2}^{+}$ concentration surrounding the encysting zoospore could be detected by fluorimetry. Contrary to the assumptions based on previous reports, the zoospores were found to exhibit a large net influx of Ca$_{2}^{+}$ in the early (1-2 min) stages following an encystment stimulus, then progressively released Ca$_{2}^{+}$ over a period of 20-30 min, until the external Ca$_{2}^{+}$ concentration greatly exceeded the pre-encystment level (i.e. there was a progressive net Ca$_{2}^{+}$ efflux). The use of Ca$_{2}^{+}$ channel inhibitors showed that the initial large net Ca$_{2}^{+}$ influx was essential for encystment. The use of an intracellular Ca$_{2}^{+}$ inhibitor which blocks Ca$_{2}^{+}$ release from intracellular stores, showed that the subsequent progressive net Ca$_{2}^{+}$ efflux was necessary for cyst germination. Warburton and Deacon (1998) thus modified the original model proposed by Donaldson and Deacon (1992). They showed clearly that the sequence from encystment to cyst germination was associated with large transmembrane Ca$_{2}^{+}$ fluxes. However, they proposed that the progression towards cyst germination involves release of Ca$_{2}^{+}$ which, if a cell is adhered to a surface, could be reabsorbed from the zone of adhesion to trigger cyst germination.
Role of organic compounds in cyst germination

Although calcium has been shown to play a central role in cyst germination, specific organic compounds can trigger germination in vitro. Phytophthora spp. germinate in response to citrus pectin, glucose, sucrose and several amino acids (Bryt et al., 1982; Irving and Grant, 1984). Py. aphanidermatum germinates in response to certain L-amino acids and some sugars (Jones et al., 1991; Donaldson and Deacon, 1993b). For Py. aphanidermatum the amino acids had no effect in the presence of EGTA, suggesting that some Ca\(^{2+}\) uptake was required. Germination could also be blocked by Ca\(^{2+}\) channel blockers (La\(^{3+}\)) but this suppression could be partly overcome by specific amino acids. The amino acids enable Ca\(^{2+}\) to be taken up even when channels are blocked, perhaps by interaction with ligand gated Ca\(^{2+}\) channels (Donaldson and Deacon, 1992).

In vivo it appears that the host exudates would reinforce the autonomous Ca\(^{2+}\) signal. This would explain the rapid and almost 100% cyst germination found on roots (Jones et al., 1991) compared to the maximum 70% germination more commonly found in vitro (Donaldson and Deacon, 1992).

After germination, cysts require new protein synthesis (Hemmes and Hohl, 1971; Pennington et al., 1989). The energy for germination appears to be supplied by the large peripheral vesicles not exocytosed during encystment (Gubler and Hardham, 1990). These vesicles supply the germling with nutrients until it is able to take up nutrients from the host. Pennington et al. (1989) showed that net uptake of a labelled amino acid or sugar by P. palmivora only occurred once the cyst germtube had developed; up to this point, all development seems to depend on endogenous nutrient reserves.
1.12 Germtube tropism

Although the site of germination is fixed, the germ tube can subsequently change direction by tropism. *Achlya* spp. can respond in this way to protein digests or mixtures of amino acids, although Musgrave *et al.* (1977) found that *A. bisexualis* only showed tropism to individual amino acids when there was a background of casein hydrolysate.

Germtubes of *Phytophthora* and *Pythium* can respond to amino acids and in the case of *Pythium* to several alcohols (Mitchell and Deacon, 1986; Jones *et al.*, 1991). Tropic responses of germtubes enable these fungi to locate penetration sites on a host surface.

1.13 Appresorium formation and host penetration

When a zoospore germinates on a leaf to produce a germling the germ tube stays in close association with the leaf surface, In *P. palmivora* the adhesion of the germtube to the leaf is mediated by glycoprotein adhesives (Hohl, 1991). Before *P. infestans* can penetrate the leaf it must form an appresorium. Once an appresorium has formed the cyst and germtube are drained of protoplasm and penetration of the leaf occurs. The process from the release of zoospores to leaf penetration by *P. infestans* can take as little as two hours (Pristou and Gallegly, 1954).

The majority of the germinating cysts penetrate in the region of the stomata (Wilson and Coffey, 1980; Gees and Hohl, 1988). This phenomenon has also been reported by Lin and Edwards (1974) and Johnson *et al.* (1979) for the powdery mildew fungus *Erisyphe graminis* on barley. In *P. infestans* this increased frequency of penetration at the stomatal complex is not due to preferential growth towards the complex but due to more successful attack of these cells (Gees and Hohl, 1988). However differences in the CO₂ concentration and pH around the stomatal complex
(Schloss, 1983) and elevated ion concentrations due to peristomatal transpiration (Maier-Maercker, 1983) may contribute to successful penetration.

1.13.1 Induction of appresorial formation

Appresorium formation in phytopathogenic fungi may be influenced by a variety of physical and chemical signals (Hoch and Staples, 1991; Read et al., 1992; Mendgen and Deising, 1993). The growing hyphal tip is an extremely dynamic structure and is supplied by a continuous stream of vesicles (Wessels, 1993). The movement of these vesicles is controlled by the cytoskeleton (Robertson, 1992). A wide range of leaf surface structures may disrupt the hyphal tip and lead to appressorium formation (Chapela et al., 1991; Hoch and Staples, 1991).

Appressoria are firmly attached to the substrate and adhesion seems to be mediated by a mucilaginous substance covering all fungal appressoria (Read et al., 1992). The material is gel-like. It can form a thin layer on top of the appressorium, accumulates laterally, and tapers off below the appressorium (Mims and Richardson, 1989). The affinity of binding to lectins changes during maturation of many appressoria (O’Connell, 1991) and this may be due to the addition of new wall layers or through the addition of new components precluding lectin access (Mendgen and Deising, 1993).

In Gymnosporangium junipere virginiane, as the appressorium matures cytoplasmic vesicles of various sizes gather at the penetration pore, where the tip of the penetration hypha will develop (Mims and Richardson, 1989) although their role is not yet fully known. A large increase in turgor also occurs in the appressoria of M. grisea (Howard & Ferrari, 1989). This is in part due to a reduction in the porosity of the wall of the appressoria, due to the laying down of melanin, and in part due to the conversion of the internal glycogen stores to osmotically active glycerol. This process can lead to an internal pressure of 8.0 MPa, and with the penetration pore emerging
from the base of the appressorium, these pressures are sufficient to cause penetration of the leaf surface.

Gees and Hohl (1988) reported that there was no preferential surface recognition of *P. infestans* on leaves that would induce appressorium formation at the stomatal complex. However Lapwood (1968) reported that surface recognition did play a role in appressorium formation. When *P. infestans* was artificially inoculated on susceptible potato cultivars only a short germ tube was formed before an appressorium was produced, whereas in resistant cultivars the length of germtube was much longer and frequently no appressorium was produced.

In *P. palmivora* there appears to be a topographical signal created by abrupt changes in surface topography that induces appressorium formation (Bircher and Hohl, 1997a). These signals are not as tightly defined as in the case of the rust fungi (Read *et al.*, 1992) but it appears that these signals would lead to appressorium formation on the stomatal complex and the periclinal wall. It is at these regions that *P. infestans* most commonly produces appressoria. It seems probable that *P. infestans* would have a similar topographical sensing system and it is for this reason, not ease of penetration, that *P. infestans* typically infects via the stomatal complex. The germtubes of *P. palmivora* adhere to the leaf surface via glycoprotein adhesins (Bircher and Hohl 1997b) and the presence of these and other uncharacterised glycoproteins enables the germling, in the absence of an inductive topographical signal and in conditions of low nutrients, to respond to surface hydrophobicity.
1.13.2. Penetration of the leaf

It appears that mechanical pressure produced by an infection peg (hypha) protruding from the appressorium is responsible for leaf penetration by Phytophthora spp. The infection hypha initially grows in the periclinal wall of the epidermal cell, and at the point of entry into the host the hyphae are restricted in their diameter. After penetration into the host cell an infection vesicle is formed (Shimony and Friend, 1975). When the epidermal cell wall is breached the infection vesicle becomes restricted in diameter and an intracellular hypha grows into the underlying palisade mesophyll cell. The cytoplasm of the epidermal cell eventually becomes necrotic. When the intracellular hyphae contact the walls of host cells they may form haustoria, the absence of nuclei is the only characteristic difference between these and the intracellular hyphae (Coffey and Wilson, 1983). Although the light use efficiency of an infected potato leaf is not affected (Van Oijen, 1991) there is a higher rate of photosynthesis in infected tissue compared to uninfected tissue (Farrell, 1971). In compatible reactions it appears that the fungus is able to modify the photosystem-2 electron transport chain, so that a large reserve of assimilates and oxygen is produced which can then fuel rapid hyphal growth (Koch et al., 1994)

1.14. Plant Defence mechanisms against pathogenic attack

Plants respond to pathogenic attack by the rapid deployment of a multicomponent defence response. The individual components of this include the hypersensitive response (HR), chemical weapons such as phytoalexins and hydrolytic compounds, and physical barriers such as callose formation. Signals for the activation of these various defences are thought to be initiated in response to recognition of pathogen avirulence determinants by plant receptors. The plant’s response may be induced specifically or non-specifically (Dixon et al., 1994).
Compatible versus incompatible reactions

When *Phytophthora* spp. challenge a plant there are two possible outcomes, a compatible or an incompatible reaction depending upon what combination of virulence and resistance genes are involved.

In *P. infestans* there are indications that two genes in the host govern resistance towards a certain (physiologic) race; and two genes in the pathogen match with the corresponding resistance genes (Spielman *et al.*, 1990). This gene-for-gene relationship envisages that dominant resistance genes interact with fungal avirulence genes, which usually results in a hypersensitive response. Hence, biochemically, every resistance gene in the host could code for a receptor molecule that would directly or indirectly activate a HR after interaction with an avirulence gene from the pathogen. In these reactions, hypersensitive cell death limits fungal invasion to the necrotic area (Flor, 1971).

In *P. infestans* virulence is dominant and encounters between the complementary genotypes of host and pathogen result in a compatible reaction and disease progression (Spielman *et al.*, 1990). One of the earliest events in the hypersensitive response is the production of active oxygen intermediates- O$_2^-$, H$_2$O$_2$ and OH$^-$ (Doke, 1983; Chai and Doke, 1987). These compounds are directly antimicrobial, reducing pathogen viability and preventing the germination of fungal spores (Keppler and Novacky, 1987; Peng and Kuc, 1992). As well as this directly antimicrobial activity the oxidative burst drives oxidative cross linking of proline-rich proteins in the cell wall and acts as a signal inducing defence genes (Bradley *et al.*, 1992).

Cuypers & Hahlbrock (1988) used different races of *P. infestans* on the same host cultivar to obtain both susceptible and resistant responses. They observed that the invaded host epidermal cell reacted hypersensitively and produced a callose-like
reaction at the same time in both susceptible and resistant interactions. In the resistant reaction, however, adjacent epidermal and mesophyll cells become necrotic within 5h of inoculation. Over a 12 - 24 h period the compatible interaction did not exhibit this necrotic host response. This seems to be consistent with the observations of Chai and Doke (1987), in which the generation of active oxygen was characterised as a two-stage process. A rapid non-specific primary response is rapidly replaced with a longer-term, more specific reaction but only in the case of incompatible reactions.

Freytag et al. (1994) used video microscopy to investigate the interaction between *P. infestans* and detached potato leaflets. Six classes of interaction were described based on the presence of a haustorium, local wall browning, autofluorescence and cell death. It appears that the potato leaf cell perceives the first signal, triggering the defence response during or just after perforation of the anticlinal wall by the infection peg. In the majority of cases an apposition of phenolic substances and callose acting as a highly localised physical barrier is sufficient to halt fungal penetration. If a cell successfully halts fungal penetration it can return to its normal intracellular arrangements; it is only when the initial barriers are breached that the hypersensitive response is induced.

Calcium seems to play a central role in the regulation of plant defence responses, including the accumulation of phytoalexins and the induction of the oxidative burst (Kurosaki et al., 1987; Schwacke and Hayer, 1992). The capacity of potato leaves to produce an oxidative burst can be enhanced by pre-treating leaves with CaCl₂ (Chai and Doke, 1987). Calcium also plays a key role in the pathogenicity of *P. infestans*, with the level of calmodulin mRNA increasing five-fold during compatible interactions with potato leaflets (Pieterse et al., 1993).

The general conclusion from cytological studies is that the host cell, in resistant interactions, responds more rapidly to *P. infestans* by triggering the hypersensitive response. Less frequently, earlier and more extensive formation of papillae and encystments may occur, particularly in resistant host tissue (Coffey & Gees, 1991).
Recognition of non-self

As the vast majority of plant species are only susceptible to a few pathogens they must be able to recognise some portion of the pathogen as non-self and induce a defence response to combat it.

In fungi the compounds that induce a defence response are termed elicitors, and they are only capable of inducing a defence response in a plant that is resistant to the elicitor-producing pathogen. These may be general components of the fungal cell such as β1-6,1-3 linked β glucans or wall lipids such as arachidonic acid (AA) (Waldmuller et al., 1992; Coquos et al., 1995) or more specific proteins termed elicins (Ricci et al., 1989). Elicitins appear to be species specific and function as avirulence factors. Nurnburger et al. (1997) reported that P. megasperma produces a 42 KDa protein that is a powerful elicitor of the defence response in cultured parsley cells. Further characterisation revealed that a 13 amino acid oligopeptide was responsible for the reaction, suggesting a highly specific receptor system in the parsley cells.

1.15. Genetics of Phytophthora infestans

*P. infestans* is normally diploid with either 9 or 10 chromosomes, although many isolates are triploid, tetraploid or aneuploid (Shaw 1991). The nuclear genome is typically $2.5 \times 10^8$ bp in size (Tooley and Thierrien, 1987) and there have been reports of extrachromosomal material in the form of ds RNA and virus-like material (Tooley et al., 1989; Corbett and Styer, 1976). It is not know whether this material has any influence on the virulence of *P. infestans* as has been reported in the case of *Cryphonectria parasitica*, the causative agent of chestnut blight (Nuss, 1992).

*P. infestans* is heterothallic, requiring both the $A_1$ and $A_2$ mating types in order to undergo sexual recombination. Only $A_1$ mating types were known before the
1950’s when the A2 mating type was discovered in the Toluca valley in the highlands of central Mexico (Neiderhauser, 1956), leading to the possibility of a sexually reproducing population.

Since the early 1980’s the A2 mating type has been recorded in Europe (Hohl and Islen, 1984; Shattock et al., 1990) and the United States and Canada (Fry et al., 1992) and is now considered to be endemic wherever potatoes are cultivated (Day and Shattock, 1997). Goodwin et al. (1994) reported that the A2 mating type accounted for 61% of all isolates examined in certain areas of the United States in the early 1990’s. This compares to around 10% in England and Wales (Shattock et al., 1990) and 6% in Northern Ireland (Cooke et al., 1995).

Both the A1 and A2 mating type can be considered bisexual in the sense that they can both produce oogonia and antheridia, and under field conditions selfing has been shown to occur in response to stimulation by other species of Phytophthora. The production of oospores poses new problems to the grower due to the potential generation of new variation and due to the ability of oospores to function as a source of inoculum for new infections. The A1 and A2 mating types are determined by heterozygosity (Mm) and homozygosity (mm) respectively at the mating type locus (Judleson, 1997). It also now appears that far from increasing in frequency the A2 isolates are declining in frequency. Reports of 60% occurrence of the A2 type (Goodwin et al., 1995) may be due to the variety of potato that is grown in these regions. Cooke et al., (1995) noted that elevated levels of A2 isolates occurred on specific potato cultivars such as Cara, and in neighbouring regions in which these cultivars were not present the level of A2 was significantly lower.
116. Control of late potato blight

The two main mechanisms currently employed for controlling potato blight are breeding for resistant potato cultivars and the application of chemicals. Chemical control has been greatly facilitated by the development of late-blight forecasting systems, especially computer-based packages such as BLITECAST (Fry and Doster, 1991).

Chemical control

Until the late 1970’s the fungicides commonly used for the control of late blight were copper-based treatments such as Bordeaux mixture, although these could show phytotoxic effects when applied at high concentrations. However since then potent, Phytophthora-specific, systemic phenylamide fungicides, such as metalaxyl, have been developed. Metalaxyl is water soluble and can be applied to the soil where it is taken up by the roots and translocated upward into new areas of growth (Bruck et al., 1980; Zaki et al., 1981).

Schwinn and Staub (1995) compared the activity of local systemic fungicides on the life cycle of P. infestans on potato and found that metalaxyl was most effective at preventing leaf penetration and the early growth of the fungus through the host tissue, and could, to a degree prevent the progression of the disease once infection was established. Concentrations as low as 10ppm applied to the potato foliage inhibit lesion formation, lesion expansion and the sporulation of the fungus (Bruck et al., 1980). Although metalaxyl can normally control the disease in the early stages of infection, Platt (1985 a,b) reported than under heavy inoculum pressure and ideal conditions for disease progression, metalaxyl even in combination with a protectant fungicide was unable to prevent the establishment of an epidemic.
Metalaxyl inhibits the activity of RNA polymerase 1, preventing rRNA synthesis, ribosome formation and eventually protein synthesis (Davidse et al., 1981). It is perhaps not surprising that due to a single site of action, resistance to metalaxyl soon appeared.

Metalaxyl-insensitive strains were first reported in the Netherlands and Ireland in 1980 (Davidse et al., 1981; Dowley and O'Sullivan, 1981) and quickly spread. Insensitivity to metalaxyl is one of the characteristics of the new clonal lineages that are starting to predominate in Europe (Staub et al., 1996) and it appears that agricultural practice has led to the selection of metalaxyl-insensitive isolates.

Metalaxyl-insensitive isolates have a much lower binding affinity to metalaxyl, and insensitivity to metalaxyl also confers insensitivity to all fungicides of similar phenylamide composition (Davidse et al., 1991). The inheritance of metalaxyl insensitivity appears to be governed by a single nuclear locus which exhibits incomplete dominance. The persistence of metalaxyl-insensitive isolates depends on the presence of a positive selection pressure. The use of metalaxyl on its own was banned in Ireland and the Netherlands from 1981-1984 (Staub, 1991) and it should only be applied now in conjunction with a broader spectrum protectant fungicide with a different mode of action. Adherence to this protocol should prevent the appearance of more resistant isolates and the application of multiple fungicide treatments should be carried out in future.

Breeding for host resistance

The second major mechanism of disease control is the production of resistant potato cultivars. Host resistance can take one of two forms, specific (vertical) resistance or general (horizontal) resistance.
Specific resistance relies on the introduction of specific R genes into *Solanum tuberosum* from the closely related *Solanum demissum*. Pathological races of *P. infestans* are identified according to the host R genes they are able to overcome (Black *et al.*, 1953). An isolate that causes disease on a potato cultivar with R gene 1 is labelled race 1 (R1). When a potato cultivar carrying R3 (for example) is attacked by an isolate of *P. infestans* that does not carry the corresponding gene the lesions on leaves are limited to small flecks with no development of the lesion over time. This has the effect of preventing an epidemic by lowering the inoculum level and due to the lack of disease this form of host resistance is often referred to as immunity (Vanderplank, 1971).

Since specific resistance is usually controlled by a single gene mutation, mutations rapidly produce new races of *P. infestans* that are capable of infecting plants carrying specific R genes. In the 1960's, a new potato cultivar Pentland Crown was developed which contained three R genes and was resistant to all the local populations of *P. infestans*, but when the cultivar was ready for commercial release compatible isolates of *P. infestans* had been selected for and the cultivar was no longer resistant (Malcolmson, 1969). The appearance of complex races of *P. infestans* carrying multiple R genes has led to the abandonment of breeding for specific resistance; instead breeding strategies are being directed towards the development of potatoes with improved general resistance (Wastie, 1991).

General resistance limits the rate of lesion expansion and is less effective than specific resistance at preventing disease development. However general resistance is controlled by multiple genes and has therefore proved more durable (Umaerus, *et al.*, 1983).

Umaerus (1970) recognised three components of general resistance in *P. infestans*-potato interactions: resistance to penetration, resistance to the growth of the fungus in the host, and reduction of the capacity of the fungus to sporulate. Resistant potato cultivars such as Pimpernel appear to resist penetration more effectively
although the mechanism of this resistance has still to be elucidated. Caten (1974) showed that strains of *P. infestans* within a race can become differentially adapted to cultivars with general resistance from which they have been isolated, and this could possibly lead to an erosion of resistance.

Future control strategies will increasingly rely on a multicomponent system of improved host resistance and the application of fungicide mixtures to prevent the development of resistant populations of *P. infestans*.

**1.17 Aims and objectives of the thesis**

Today, more than 150 years after it first caused devastation of potato crops in Europe and the USA, *P. infestans* continues to pose a major threat to potato cultivation. The major aim of the work in this thesis was, therefore, to explore the possibility of developing new methods of control of this fungus, based on a fuller understanding of its basic biology. Specifically, attention was focused on the factors that control germination of both sporangia and zoospore cysts of *P. infestans*, based on much previous evidence that implicated a central role of calcium in mediating the infection sequence from zoospores of *Pythium* and *Phytophthora* spp.

The specific objectives were:

1. To investigate the effects of calcium-modulating treatments on germination of sporangia by hyphal outgrowth or zoospore release.

2. To investigate the effects of calcium-modulating compounds on zoospore cyst germination by *P. infestans*, and to compare these effects with those on *P. palmivora*.

3. To investigate the effect of divalent cations uronic acids and other simple non-phytotoxic compound on the sporangial germination of *P. infestans*.
4. To investigate the durability on both cysts and sporangia, of those treatments that appear most promising, with respect to time of addition and susceptibility to nutrient post-treatments.
Chapter Two

Materials and Methods
### Chemicals used in this work

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<tr>
<th>Chemical</th>
<th>Description</th>
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<td>Xylose</td>
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55
All the chemicals used were supplied by ICN Biomedicals (England) except those marked*.

2.1 Culture of Phytophthora infestans

*Phytophthora infestans strain* EUPi1 was supplied by Dr. M Chamberlain (Royal Botanic Gardens, Edinburgh, Scotland, UK) and isolates 81/1/1 and E1 3 A2 were supplied by Dr. D Shaw (University College of North Wales, Bangor, Wales, UK). All isolates were obtained from infected potato plants and maintained on rye grain infusion agar (see below). Isolate EUPi1 was grown at 17°C and was the main isolate used in the experiments; isolates 81/1/1 and E1 3 A2 were grown at 21°C.

The preparation of the rye grain infusion agar was based on the method of Caten and Jinks (1968). Organically produced, fungicide-free rye grain (60g) was soaked for between 24 and 36h in distilled water before being boiled for 1h. After the rye had cooled it was macerated in an electric food blender and supplemented with Oxoid N03 agar (15g l⁻¹) agar, and sucrose (20g l⁻¹). The medium was made up to a final volume of 1l by the addition of distilled water (DW).

2.1 Induction of indirect germination (zoosporogenesis) in *P. infestans*

For the production of zoospores, cultures between 10 and 14 days old are required. A plate was flooded by the addition of 20 ml of chilled (4°C) sterile distilled water (SDW) and the sporangia were dislodged by scraping with a sterile glass plate spreader. The resulting suspension was filtered through sterile nylon mesh to remove large hyphal debris. The resulting filtrate was collected in a chilled sterile Universal bottle (25ml) then filtered through Whatman No3 filter paper, using a chilled Buchner funnel attached to a compressor pump. The sporangia adhering to the filter paper were dislodged by washing with 3-4 ml of chilled SDW and collected in a fresh chilled Universal bottle, before being incubated at 12°C for 90 minutes.
After 90 minutes the released zoospores tended to accumulate near the surface of the water, where zoospore numbers typically were $2 \times 10^5$ / ml. The final concentration was adjusted to $2 \times 10^4$/ml by dilution with SDW using a haemocytometer to count the spores.

2.3 Induction of direct germination in *P. infestans*

For the induction of direct germination, cultures between 10 and 14 days old were required. A culture plate was flooded by the addition of 20 ml SDW and sporangia were dislodged by scraping with a sterile glass plate spreader. The resulting suspension was filtered through sterile nylon mesh and filter paper as described above. The sporangia adhering to the filter paper were dislodged by washing with 3-4 ml SDW, collected in a fresh sterile Universal bottle and incubated at 21°C for 16h.

2.4 Culture of *Phytophthora palmivora*

*Phytophthora palmivora* (Butler) Butler strain P3765, was originally isolated from cocoa and was supplied by Professor N.A.R. Gow (Aberdeen University, Aberdeen, Scotland, U.K.). It was maintained on modified V8 agar, composed of 20% V8 juice (Campbell’s Soup Corporation) 3g CaCO$_3$, 15g agar (Oxoid No3) and 30mg cholesterol (predissolved in hot 70% ethanol) and made up to 1l with DW.

2.5 Induction of indirect germination (zoosporogenesis) in *P. palmivora*

Cultures were grown in darkness for 3-4 days at 27°C before being transferred to the light for 2-3 days (2x20W “liteguard” warm white, [Osram, England] placed at 20cm above the cultures). The plates were flooded with 20ml of chilled (4°C) SDW and the sporangia dislodged by scraping with a sterile glass plate spreader. The plate
containing water was then incubated at 4°C for 30 minutes. Then the sporangial suspension was transferred to an empty Petri dish and incubated at room temperature (21°C) for 30 minutes. Zoospore numbers were typically 1x10^6/ml and were adjusted to a final concentration of 2x10^4/ml using SDW; counts were made using a haemocytometer.

2.6 Induction of direct germination in *P. palmivora*

Cultures were grown as above (3-4 days at 27°C in darkness, followed by 2-3 days under lights) then flooded with a 8% (w/v) dextran solution (pH 7.0, 27°C) and the sporangia were dislodged by scraping with a sterile glass plate spreader. The sporangial suspension was transferred to a empty Petri dish and incubated at 27°C for 5 h.

The dextran (ICN Biomedicals) was prepared in DW then clarified by centrifugation at 4000 rpm for 10 minutes using a MSE Mistral 1000 benchtop centrifuge (incomplete solubility due to impurities in the material as supplied).

2.7 Induction of encystment

Motile zoospores were induced to encyst synchronously by transferring aliquots of fresh zoospore suspension to sterile 1.5ml microcentrifuge tubes and holding them against a vortex mixer (Fisons Scientific Apparatus, England) for 70s.
2.8 Responses of pre-encysted zoospores to Ca\textsuperscript{2+} modulators, nutrients and cations

2.8.1 The effect of varying concentrations of Ca\textsuperscript{2+} modulators and cations on the germination of pre-encysted zoospores of *P. infestans* (EUPi1) and *P. palmivora*

A 1.0 ml aliquot of motile zoospores (either *P. infestans* or *P. palmivora*) was vortex encysted and adjusted so that the final cell density (after addition of test solutions) would be 2\(\times\)10\textsuperscript{4} zoospores/ml. After 10 minutes, aliquots of the cyst suspension were transferred to further 1.5 ml microcentrifuge tubes containing various test solutions so that the final volume was 100 \(\mu\)l. SDW was used as a control treatment; other treatments included caffeine (ICN Biomedicals), calmidazolium (ICN Biomedicals), dibucaine (ICN Biomedicals), lanthanum chloride (ICN Biomedicals), trifluoperazine hydrochloride (ICN Biomedicals), verapamil hydrochloride (ICN Biomedicals), ethylene glycol-bis (\(\beta\) amino ethyl ether) N,N,N',N' tetraacetic acid (EGTA) (ICN Biomedicals), 1,2-bis (2-aminophenoxy) ethane-N,N,N',N' tetraacetic acid (BAPTA, tetra potassium salt) (ICN, Biomedicals), 3,4,5-trimethoxybenzoic acid 8-(diethylamino) octyl ester (TMB-8) (ICN Biomedicals), 5-(N-methyl-n-isobutyl)- amiloride (amiloride) (ICN Biomedicals), barium chloride (ICN Biomedicals), calcium chloride (ICN Biomedicals) and magnesium chloride (ICN Biomedicals). Aliquots (50\(\mu\)l) from the microcentrifuge tubes were transferred to clean microscope slides, incubated in moist chambers, in the dark, for 1.5 h (16-21\(^\circ\)C). Germination was assessed microscopically (X100 magnification). Cysts were considered to have germinated when the germ tube length equalled or exceeded the cyst diameter. Five replicates were used, with at least 100 spores being counted per replicate. Replicate treatments were carried out over several days using one replicate of each treatment on each day.
2.8.2 The effect of time of addition of ions and Ca^{2+} modulators on the germination of pre-encysted zoospores of *P. infestans* strain Eupil

A 1 ml suspension of *P. infestans* zoospores was vortex encysted and at various times after the commencement of vortexing the following treatments were added (at the final concentrations shown): BAPTA (2.5mM), EGTA (10mM), Ba^{2+} (BaCl₂ 10mM), Ca^{2+} (CaCl₂ 10mM) Mg^{2+} (MgCl₂ 10mM) and SDW as a control. The spores were incubated in microcentrifuge tubes for 90 minutes before being transferred to microscope slides for assessment of germination.

2.8.3 The effect of time of addition of ions and Ca^{2+} modulators on the germination of pre-encysted zoospores of *P. palmivora*

A 1 ml suspension of *P. palmivora* zoospores were vortex encysted and at various times after the commencement of vortexing. The following treatments were added (at the final concentrations shown): EGTA (10mM), Ba^{2+} (BaCl₂ 10mM), Ca^{2+} (CaCl₂ 10mM) Mg^{2+} (MgCl₂ 10mM) and SDW as a control. The spores were incubated in microcentrifuge tubes for 90 minutes before being transferred to microscope slides for assessment of germination.

2.8.4. The effects of Ca^{2+} modulator pre-treatments on the germination of pre-encysted zoospores of *P. infestans* Eupil1 in the presence or absence of nutrient post-treatments

A 1 ml suspension of *P. infestans* zoospores was vortex encysted and incubated in a microcentrifuge tube for 10 minutes. A 500µl aliquot was then added to a 1.5 ml microcentrifuge tube containing either 500µl SDW (as a control) or 500µl of one of
the following treatments (final concentration given): amiloride (400 μM), BAPTA (2.5 mM), caffeine (4 mM), calmidazolium (4μM), dibucaine (250 μM), EGTA (10mM), Gd³⁺ (70 μM as GdCl₃), La³⁺ (70 μM as LaCl₃), trifluoperazine (45μM) or TMB-8 (500μM). The tubes were incubated for a further 10 minutes. After this time (t₂₀) the cysts were subsequently treated with various sugars (10mM final concentration), L-amino acids (10mM final concentration) or di-valent cations (10mM final concentration), malt extract with peptone (each at 1% concentration), or amino acids or sugars (10 mM final concentration) supplemented with divalent cations (10 mM final concentration). These post-treatments were applied by transferring 90 μl (or 80 μl in the case of dual treatments) of both the SDW and other pre-treated cysts to microcentrifuge tubes that already contained 10 μl of the relevant post-treatment. The spores were incubated for 80 minutes before being transferred to microscope slides. Germination was then assessed; cysts were considered to have germinated when the length of the germ tube at least equalled cyst diameter.

2.8.5 The effect of Ca²⁺ modulator pretreatments on the germination of pre-encysted zoospores of *P. palmivora* in the presence or absence of nutrient post treatments

The experiments were performed as described above (section 2.8.4) except that the concentrations of the pre-treatments were as follows. Amiloride (800 μM), BAPTA (2.5 mM), caffeine (10 mM), calmidazolium (1.2μM), dibucaine (200 μM), EGTA (10mM), La³⁺ (LaCl₃ 400 μM), trifluoperazine (15μM) or TMB-8 (500μM).

2.9 Responses of sporangia to calcium modulator and ion treatments
2.9.1 The effect of varying concentrations of pectin, inorganic phosphates, Ca\(^{2+}\) modulators and cations on the sporangial germination of *P. infestans*

A 1 ml aliquot of freshly prepared sporangial suspension (*P. infestans* strains EUPi1, 81/1/1 and E3i.A2) was adjusted to give a final concentration of 1x10\(^5\) sporangia / ml after treatments had been applied. After 10 minutes the sporangial suspension were transferred to 1.5ml microcentrifuge tubes containing various test solutions so that the final volume was 100\(\mu\)l. SDW was used as a control; other treatments included caffeine, dibucaine, Na\(_2\)HPO\(_4\), LaCl\(_3\), citrus pectin, NaH\(_2\)PO\(_4\), trifluoperazine hydrochloride, verapamil hydrochloride, EGTA ethylenediaminetetraacetic acid (EDTA) (ICN Biomedicals), BAPTA, TMB-8, amiloride, barium chloride, calcium chloride and magnesium chloride. The sporangial suspensions were then incubated in the microcentrifuge tubes, either at 12\(\degree\)C (for indirect germination) or 21\(\degree\)C (for direct germination in the case of isolate EUPi1). Assessment of germination was made after 16 h, although indirect germination was initiated within 1h and direct germination within 4 h. To score germination, 50\(\mu\)l aliquots were transferred to microscope slides and examined at X100 magnification. Indirect germination was assessed as the number of empty sporangia with a dissolved discharge papillum, and direct germination as the number of sporangia with a hyphal outgrowth at least equal to the sporangium length. Five replicates were used with at least 100 sporangia being counted per replicate treatment. Replicate treatments were carried out over several days, with one replicate of each treatment being performed on each day.

2.9.2 The effect of time of addition of pectin, inorganic phosphate, Ca\(^{2+}\) modulators and cations on the sporangial germination of *P. infestans*
A 1 ml aliquot of freshly prepared sporangial suspension (*P. infestans*, isolate EUPi1,) was adjusted to give a final concentration of $1 \times 10^4$ sporangia / ml after treatments had been applied. A range of chemical treatments were applied at various times, starting at 5 minutes after the sporangial suspension had been prepared: Na$_2$HPO$_4$ (20mM), NaH$_2$PO$_4$ (20mM), EDTA (2mM), EGTA (2mM), Pectin (0.1%), BaCl$_2$ (10mM), CaCl$_2$ (10mM) and MgCl$_2$ (10mM). The sporangia were then incubated in the microcentrifuge tubes, either at 12° C (for indirect germination) or 21°C (for direct germination). Other experimental details were as described in section 2.9.1.

### 2.9.3 The effects of cation post treatments on the germination of *P. infestans* sporangia that had been pre-treated with pectin, inorganic phosphate or Ca$^{2+}$ modulators

As before a 1 ml aliquot of freshly prepared sporangial suspension (*P. infestans*, EUPi1, 81/1/1 and Ei3j A2) was adjusted to a final concentration of $1 \times 10^4$ / ml and transferred to microcentrifuge tubes. After 10 minutes, the sporangia were pre-treated with the following (final concentrations given): EGTA (2mM), NaH$_2$PO$_4$ (20mM) and pectin (0.1%). At various times after the addition of the pre-treatments either CaCl$_2$ or BaCl$_2$ (both at 10mM final concentration) were added as post treatments. The sporangia were then incubated in the microcentrifuge tubes, either at 12° C (indirect germination) or 21°C (direct germination strain EUPi1 only). Assessment of germination were made after 16 h, as described above.
2.9.4 The effects of varying concentrations of pectin, inorganic phosphate, Ca\(^{2+}\) modulators and cations on the sporangial germination of *P. palmivora*.

The experiments were performed as previously described (section 2.9.1) but using varying concentrations of the following (with SDW controls) caffeine, dibucaine, Na\(_2\)HPO\(_4\), LaCl\(_3\), citrus pectin, NaH\(_2\)PO\(_4\), trifluoperazine hydrochloride, verapamil hydrochloride, EGTA, EDTA, BAPTA, TMB-8, amiloride, BaCl\(_2\), CaCl\(_2\) and MgCl\(_2\).

2.9.5 The effect of time of addition of inorganic phosphate, Ca\(^{2+}\) modulators and ions on sporangial germination of *P. palmivora*

A 1 ml aliquot of freshly prepared sporangial *P. palmivora* was adjusted to give a final concentration of 1x10\(^4\) sporangia / ml after treatments had been applied. A range of chemical treatments were applied at various times, starting at 5 minutes after the sporangial suspension had been prepared: EGTA (2mM), NaH\(_2\)HPO\(_4\) (20mM), Ca\(^{2+}\) (10mM) and Mg\(^{2+}\) (10mM). The sporangia were then incubated in the microcentrifuge tubes, either at 12\(°\)C (for indirect germination) or 21\(°\)C (for direct germination). Other experimental details were as described in section 2.9.4.

2.10 The effect of continuous elution on sporangial germination of *P. infestans*

Aliquots (50\(\mu\)l) of freshly harvested sporangial suspension (strains EUPi1, 81/1/1 and E\(_3\)A\(_2\)) were prepared with a final concentration of 1x10\(^4\) sporangia / ml. They were placed as droplets on 10x10mm squares of polycarbonate membrane (Cyclopore\textsuperscript{TM}, track etched, pore size 2\(\mu\)m; Whatman, Maidstone, Kent) 10 minutes after the sporangial suspension had been prepared. The membranes were placed on the surface of a 5mm thick layer of acid-washed glass beads (Ballontini, 40 mesh,
diameter 0.4mm, approximate volume 100ml) in a 15cm diameter Petri dish. The Petri dish had been constructed to have glass inlet and outlet tubes; the inlet was attached to a 11 reservoir, and the outlet to a collecting vessel. A peristaltic pump (MP3, Eyela, Tokyo Riakakku, Tokyo, Japan) was placed between the reservoir and the Petri dish to control the flow rate of liquid through the bed of glass beads. The system was pre-leached at maximum pump speed for 30 min (flow rate approximately 10 ml min$^{-1}$) using either SDW or CaCl$_2$ as the leachate. The squares of membrane bearing the sporangia were placed on the glass beads and exposed to leaching treatments for varying times (figure 2.1). After treatment, the membrane squares were removed and incubated either at 12°C (for indirect germination) or 21°C (for direct germination). Assessment of germination was made after 16 h, although indirect germination was initiated within 1h, and direct germination within 4h. For assessment the membrane squares were placed on a microscope slide and stained with Calcoflour (fluorescent brightener, ICN Biomedicals; 100μg ml$^{-1}$ final concentration) and Nile red (ICN Biomedicals; 50μg ml$^{-1}$ final concentration). Microscopic observations were made under epifluorescence using a Lietz orthoplan microscope equipped with a ploempak 2.1 fluorescent vertical illuminator, with light supplied from an HBO-200 mercury vapour lamp. Filter block A of this microscope incorporated a 330-385nm exciting filter (ultraviolet), TK400 dichroic beam splitting mirror and K400 suppression filter. Filter block H incorporated a BP 390-490nm exciting filter (violet and blue), TK 455 dichroic beam splitting mirror and K460 suppression filter. Microscopic observations of fluorescence were made at X250 magnification. Indirect germination was assessed as the number of sporangia that fluoresced blue with Calcofluor (indicating the presence of a sporangial wall) but showed no fluorescence with Nile red, owing to the lack of sporangial contents. Direct germination was assessed as the number of sporangia with a hyphal outgrowth at least equal to the sporangium length when viewed with Calcofluor. Five replicate membrane squares were used with at least 100 sporangia being counted per replicate treatment.
**Figure 2.1** Glass Petri dish set up to be used as a continuous flow chamber, showing the placement of the polycarbonate membranes on the glass bead matrix below the point of liquid entry.
Chapter Three

The effect of calcium modulators, ions and nutrients on the germination of Phytophthora zoospore cysts
3.1 Introduction

Ca²⁺ fluxes have vital importance in the encystment, adhesion and subsequent germination of *Phytophthora* zoospores. Irving *et al.* (1989) reported that zoospores of *P. palmivora* released up to one third of their intracellular Ca²⁺ early in the encystment process, concurrent with this is the ability rapidly to reabsorb radiolabelled Ca²⁺. The release of Ca²⁺ has been implicated in fixing the glycoprotein adhesive in *Phytophthora* and *Pythium* zoospores to a surface (Gubler *et al.*, 1989; Donaldson and Deacon, 1992). The normal adhesiveness of young cysts could be abolished by the addition of the Ca²⁺ chelator EGTA and the lack of adhesiveness of older cysts or EGTA-treated cysts could be restored by the addition of exogenous Ca²⁺.

Several studies have investigated the effects of ions and Ca²⁺ modulators on zoospore encystment and germination (Grant *et al.*, 1984; Grant *et al.*, 1986; Von Broembsen and Deacon, 1996; Deacon and Saxena, 1997). The findings of these studies not only implicate Ca²⁺ as a component of signal transduction but also as an autonomous signal that enables the rapid germination of cysts.

Experiments in this chapter are designed to investigate the effects of Ca²⁺ modulating treatments and ion supplements on the germination of pre-encysted zoospores of *P. infestans* strain EUPi1 and *P. palmivora*, with particular reference to the durability of the treatment, and the time at which zoospores are most sensitive to their addition.

3.2 The effects of varying concentrations of Ca²⁺ modulators on the germination of pre-encysted zoospores of *P. infestans* and *P. palmivora*.

As described in the Materials and Methods (section 2.8), zoospores of *P. infestans* and *P. palmivora* were vortex-encysted and adjusted to a final concentration of 1 X 10⁴ zoospores per ml. After 10 minutes incubation in microcentrifuge tubes, the calcium modulators were added and the cyst suspension
was immediately transferred to a clean microscope slide. The treated cysts were incubated in moist chambers in the dark for 1.5h before germination was assessed microscopically.

Five main types of calcium modulator were used: calcium channel blockers (La\(^{3+}\), Gd\(^{3+}\) and verapamil), calcium chelators (EGTA and BAPTA), calmodulin antagonists (Dibucaine, TFP (trifluopenazine) and calmidazolium), a Na\(^{+}/Ca\(^{2+}\) flux inhibitor (amiloride) and intracellular calcium regulators (caffeine and TMB-8).

BAPTA, a calcium chelator, significantly reduced the cyst germination of *P. infestans* (Figure 3.1) and *P. palmivora* (Figure 3.2) when added at concentrations in excess of 1mM. When concentrations exceeded 2mM very low levels of germination (<1%) were observed, although no obvious cell death was evident.

The other chelator used, EGTA, had a broadly similar effect with *P. infestans* (Figure 3.3) showing reduced germination with increasing concentration of EGTA and at the highest concentration applied (10mM) germination was less than 1%. *P. palmivora* (Figure 3.4) showed the same general response, but at 10mM EGTA germination still occurred at an appreciable level, although a 10 fold reduction from the control values was observed.

Lanthanum chloride, an inorganic inhibitor of plasma membrane channels, reduced the germination of *P. infestans* to approximately 20% of control values, at 50μM and completely inhibited germination at concentrations above 100μM (Figure 3.5). In contrast cysts of *P. palmivora* (Figure 3.6) were not as sensitive to La\(^{3+}\) and germination was only reduced to 20% of control levels at concentrations in excess of 400μM although 600μM La\(^{3+}\) did completely inhibit germination by this fungus. Gd\(^{3+}\) was also added to cysts of *P. infestans* (Figure 3.7) and produced a very similar response to that of La\(^{3+}\); germination was reduced to 20% of control levels by 50μM Gd\(^{3+}\) and was completely inhibited by 100μM Gd\(^{3+}\).

\(^{1}\) Continued on page 79
Figure 3.1 The effect of varying concentrations of BAPTA on the germination of pre-encysted zoospores of *P. infestans* strain EUPi1.

* Data are means ± se for at least three replicate treatments. Germination was assessed after 1.5h incubation and counts are based on at least 100 spores for each replicate.
Figure 3.2 The effect of varying concentrations of BAPTA on the germination of pre-encysted zoospores of *P. palmivora*.

*Data are means ± se for at least three replicate treatments. Germination was assessed after 1.5h incubation and counts are based on at least 100 spores for each replicate.*
Figure 3.3 The effect of varying concentrations of EGTA on the germination of pre-encysted zoospores of *P. infestans* strain EUPi1.

* Data are means ± se for at least three replicate treatments. Germination was assessed after 1.5h and counts were based on at least 100 spores for each replicate.
Figure 3.4 The effect of varying concentrations of EGTA on the germination of pre-encysted zoospores of *P. palmivora*

* Data are means ± se for at least three replicate treatments. Germination was assessed after 1.5h incubation and counts are based on at least 100 spores for each replicate.
Figure 3.5 The effect of varying concentrations of La$^{3+}$ on the germination of pre-encysted zoospores of *P. infestans* strain EUPi1.

*Data are means ± se for at least three replicate treatments. Germination was assessed after 1.5h incubation and counts were based on at least 100 spores per replicate.*
Figure 3.6. The effect of varying concentration of La$^{3+}$ on the germination of pre-encysted zoospores of *P. palmivora*.

*Data are means ± se for at least three replicate treatments. Germination was assessed after 1.5h incubation and counts were based on at least 100 spores per replicate.*
Figure 3.7. The effect of varying concentrations of Gd\(^{3+}\) on the germination of pre-encysted zoospores of *P. infestans* strain EUPi1.

*Data are means ± se for at least three replicate treatments. Germination was assessed after 1.5h incubation and counts were based on at least 100 spores per replicate.*
Figure 3.8. The effect of varying concentrations of verapamil on the germination of pre-encysted zoospores of \( P. \) infestans strain EUPi1.

*Data are means ± se for at least three replicate treatments. Germination was assessed after 1.5h incubation and counts were based on at least 100 spores per replicate.
**Figure 3.9.** The effect of varying concentrations of verapamil on the germination of pre-encysted zoospores of *P. palmivora*.

* Data are means ± se for at least three replicate treatments. Germination was assessed after 1.5h incubation and counts were based on at least 100 spores per replicate.
Verapamil hydrochloride, an organic Ca\(^{2+}\) channel blocker, was less effective at reducing the germination of *P. infestans* (Figure 3.8) than was La\(^{3+}\); 150\(\mu\)M verapamil was required to reduce germination to 20% of the control levels and 300\(\mu\)M to completely inhibit germination. Conversely *P. palmivora* (Figure 3.9) was more sensitive to verapamil than to La\(^{3+}\), being completely inhibited by verapamil concentrations of 60\(\mu\)M or above.

Calmidazolium, an organic calmodulin antagonist, had no noticeable effect on the germination of *P. infestans* (Figure 3.10) until a threshold concentration of 2\(\mu\)M was exceeded. Beyond this level, germination was inhibited with complete inhibition occurring at 5\(\mu\)M. The germination of *P. palmivora* (Figure 3.11) was significantly inhibited at 1\(\mu\)M and no germination occurred above 2.5\(\mu\)M calmidazolium.

The other two calmodulin antagonists employed, dibucaine and TFP, showed no difference in their effects on *P. infestans* and *P. palmivora*. *Phytophthora infestans* was completely inhibited (0% germination) by 200\(\mu\)M dibucaine or 20\(\mu\)M TFP (Figures 3.12, 3.13). *P. palmivora* was completely inhibited by 250\(\mu\)M dibucaine or 20 \(\mu\)M TFP (Figures 3.14, 3.15).

The Na\(^+/Ca\(^{2+}\) flux inhibitor, amiloride reduced the germination of *P. infestans* cysts to 20% of control values at 300\(\mu\)M (Figure 3.16) and completely inhibited germination at 500\(\mu\)M. When amiloride was added to *P. palmivora* (Figure 3.17) the germination was reduced to 20% of control values by 400\(\mu\)M. However, none of the concentrations tested (up to 800\(\mu\)M) was able to further significantly reduce the level of germination.

TMB-8, an intracellular Ca\(^{2+}\) antagonist, reduced the level of germination of *P. infestans* cysts to 20% of control values at 400\(\mu\)M and completely inhibited

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Figure 3.10 The effect of varying concentrations of calmidazolum on the germination of pre-encysted zoospores of *P. infestans* strain EUPi1.

*Data are means ± se for at least three replicate treatments. Germination was assessed after 1.5h incubation and counts are based on at least 100 spores for each replicate.*
The effect of varying concentrations of calmidazolium on the germination of pre-encysted zoospores of *P. palmivora*.

* Data are means ± se for at least three replicate treatments. Germination was assessed after 1.5h incubation and counts are based on at least 100 spores for each replicate.
Figure 3.12 The effect of varying concentrations of dibucaine on the germination of pre-encysted zoospores of *P. infestans* strain EUPi 1.

* Data are means ± se for at least three replicate treatments. Germination was assessed after 1.5h incubation and counts are based on at least 100 spores for each replicate.
Figure 3.13 The effect of varying concentrations of TFP on the germination of pre-encysted zoospores of *P. infestans* strain EUPi 1

* Data are means ± se for at least three replicate treatments. Germination was assessed after 1.5h incubation and counts are based on at least 100 spores for each replicate.
Figure 3.14 The effect of varying concentrations of dibucaine on the germination of pre-encysted zoospores of *P. palmivora*.

* Data are means ± se for at least three replicate treatments. Germination was assessed after 1.5h incubation and counts are based on at least 100 spores for each replicate.
Figure 3.15 The effect of varying concentrations of TFP on the germination of pre-encysted zoospores of *P. palmivora*.

* Data are means ± se for at least three replicate treatments. Germination was assessed after 1.5h incubation and counts are based on at least 100 spores for each replicate.
Figure 3.16 The effect of varying concentrations of amiloride on the germination of pre-encysted zoospores of *P. infestans* strain EUPI1.

*Data are means ± se for at least three replicate treatments. Germination was assessed after 1.5h incubation and counts are based on at least 100 spores for each replicate.*
**Figure 3.17** The effect of varying concentrations of amiloride on the germination of pre-encysted zoospores of *P. palmivora*.

* Data are means ± se for at least three replicate treatments. Germination was assessed after 1.5h incubation and counts are based on at least 100 spores for each replicate.
Figure 3.18 The effect of varying concentrations of TMB-8 on the germination of pre-encysted zoospores of *P. infestans* strain EUPi1

* Data are means ± se for at least three replicate treatments. Germination was assessed after 1.5h incubation and counts are based on at least 100 spores for each replicate.
Figure 3.19 The effect of varying concentrations of TMB-8 on the germination of pre-encysted zoospores of *P. palmivora*.

* Data are means ± se for at least three replicate treatments. Germination was assessed after 1.5h incubation and counts are based on at least 100 spores for each replicate.
Figure 3.20 The effect of varying concentrations of caffeine on the germination of pre-encysted zoospores of *P. infestans* strain Eupil.

* Data are means ± se for at least three replicate treatments. Germination was assessed after 1.5h incubation and counts are based on at least 100 spores for each replicate.
**Figure 3.21** The effect of varying concentration of caffeine on the germination of pre-encysted zoospores of *P. palmivora*.

* Data are means ± se for at least three replicate treatments. Germination was assessed after 1.5h incubation and counts are based on at least 100 spores for each replicate.
germination at 800µM (Figure 3.18) although at concentrations greater than 400µM germination occurred at very low levels. Phytophthora palmivora appeared to be more susceptible to TMB-8 than was P. infestans (Figure 3.19) with germination being completely suppressed by the addition of 250µM TMB-8.

Caffeine, an organic compound that reduces the level of stored intracellular Ca²⁺, completely inhibited the germination of P. infestans at 4-5mM concentration (Figure 3.20). Although 7mM was able to reduce the germination of P. palmivora (Figure 3.21) to 20% of the control values, none of the concentrations used was able to completely inhibit germination by this fungus.

3.3 The effect of divalent cations on the germination of pre-encysted zoospores of P. infestans and P. palmivora.

The effect of di-valent cations was investigated as described in section 2.8.1. The three cations used were Ba²⁺, Ca²⁺ and Mg²⁺; these ions were added as chloride salts to the freshly prepared cysts 10 minutes after the induction of encystment.

Ca²⁺ did not significantly reduce the germination of P. infestans (Figure 3.22) until it was applied at concentrations of 5mM or higher; however the germination of P. palmivora (Figure 3.23) was significantly reduced by the addition of even 2mM Ca²⁺ (LSD 6.2) with the degree of inhibition increasing with Ca²⁺ concentration.

The addition of Ba²⁺ did not cause a significant reduction in the germination of P. infestans (Figure 3.24) until at least 8mM Ba²⁺ was applied. In contrast, P. palmivora (Figure 3.25), was significantly inhibited when Ba²⁺ was present at 4mM or above.
Mg$^{2+}$ significantly reduced the germination of *P. infestans* (Figure 3.26) when present at 6mM whereas *P. palmivora* (Figure 3.27) is significantly inhibited by concentrations as low as 4mM.

In many of these comparisons of the responses of *P. infestans* and *P. palmivora* to ions it was notable that the variation between the replicates was much more pronounced for *P. infestans* than for *P. palmivora*.
Figure 3.22 The effect of varying concentrations of Ca$^{2+}$ on the germination of pre-encysted zoospores of *P. infestans* strain EUPi1.

* Data are means ± se for at least three replicate treatments. Germination was assessed after 1.5h incubation and counts are based on at least 100 spores for each replicate.
Figure 3.23 The effect of varying concentrations of Ca\(^{2+}\) on the germination of pre-encysted zoospores of \textit{P. palmivora}.

* Data are means ± se for at least three replicate treatments. Germination was assessed after 1.5h incubation and counts are based on at least 100 spores for each replicate.
Figure 3.24 The effect of varying concentrations of Ba$^{2+}$ on the germination of pre-encysted zoospores of *P. infestans* strain EUPi1.

* Data are means ± se for at least three replicate treatments. Germination was assessed after 1.5h incubation and counts are based on at least 100 spores for each replicate.
Figure 3.25 The effect of varying concentrations of Ba$^{2+}$ on the germination of pre-encysted zoospores of *P. palmivora*.

* Data are means ± se for at least three replicate treatments. Germination was assessed after 1.5h incubation and counts are based on at least 100 spores for each replicate.
Figure 3.26 The effect of varying concentrations of Mg$^{2+}$ on the germination of pre-encysted zoospores of *P. infestans* strain Eupil.

* Data are means ± se for at least three replicate treatments. Germination was assessed after 1.5h incubation and counts are based on at least 100 spores for each replicate.
Figure 3.27 The effect of varying concentrations of Mg$^{2+}$ on the germination of pre-encysted zoospores of *P. palmivora*.

* Data are means ± se for at least three replicate treatments. Germination was assessed after 1.5h incubation and counts are based on at least 100 spores for each replicate.
3.4 The effect of time of addition of ions and Ca\(^{2+}\) chelators on the germination of pre-encysted zoospores of \(P.\) infestans and \(P.\) palmivora.

The experiments were carried out as described in sections 2.8.2 and 2.8.3, the three ions used (Ca\(^{2+}\), Ba\(^{2+}\), and Mg\(^{2+}\)) were added at 10mM final concentration. In the following, \(T_0\) indicates that the treatments were present in the zoospore suspension at the time of vortex induced encystment. \(T_{11}\) indicates that the treatments were added as soon as the encystment procedure ended (after 70s vortexing).

The effect of time of addition of Ca\(^{2+}\) to pre-encysted zoospores of \(P.\) infestans is shown in Figure 2.28. There is seen to be an initial trend trend in which the early addition of Ca\(^{2+}\) caused a reduction in cyst germination compared with that in the SDW controls. Later addition of Ca\(^{2+}\) seemed to have progressively less effect on germination. However when a two-way ANOVA was carried out there was found to be no significant differences. In contrast, the addition of Ca\(^{2+}\) to \(P.\) palmivora (Figure 3.29) produced a significant reduction in germination even when Ca\(^{2+}\) was added 60 minutes after the induction of encystment. However the effect was most pronounced when Ca\(^{2+}\) is added early after (or during) encystment.

The addition of Ba\(^{2+}\) to \(P.\) infestans (Figure 3.30) resulted in a significant reduction in the level of germination only if Ba\(^{2+}\) was added in the early stages of encystment. If Ba\(^{2+}\) was added after 20 minutes then no significant difference between the germination of Ba\(^{2+}\) treated cysts and the control was observed. When Ba\(^{2+}\) was applied to \(P.\) palmivora (Figure 3.31) there is a significant difference between the Ba\(^{2+}\) treated cysts and the controls and also a significant effect of the time of addition of the treatments.

The addition of Mg\(^{2+}\) (Figure 3.32) to \(P.\) infestans resulted in significant differences in germination as compared to the SDW controls, with the effect of time of addition also being significant. Even when Mg\(^{2+}\) was added 60 minutes after the

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Continued on page 107
Figure 3.28 The effect of time of addition of 10mM Ca\(^2+\) on the germination of pre-encysted zoospores of *P. infestans* strain EUPi1, with data for the corresponding SDW controls.

* Data are means ± se for at least three replicate treatments. Germination was assessed after 1.5h incubation and counts are based on at least 100 spores for each replicate. \(T_0\) indicates that the treatment was present during encystment.
Figure 3.29 The effect of time of addition of 10mM Ca\(^{2+}\) on the germination of pre-encysted zoospores of *P. palmivora*, with data for the corresponding SDW controls.

* Data are means ± se for at least three replicate treatments. Germination was assessed after 1.5h incubation and counts are based on at least 100 spores for each replicate. T₀ indicates that the treatment was present during encystment.
Figure 3.30 The effect of time of addition of 10mM Ba\(^{2+}\) on the germination of pre-encysted zoospores of *P. infestans* strain, Eupil with data for the corresponding SDW controls.

* Data are means ± se for at least three replicate treatments. Germination was assessed after 1.5h incubation and counts are based on at least 100 spores for each replicate. T\(_o\) indicates that the treatment was present during encystment.
Figure 3.31 The effect of time of addition of 10mM $\text{Ba}^{2+}$ on the germination of pre-encysted zoospores of *P. palmivora*, with data for the corresponding SDW controls.

* Data are means ± se for at least three replicate treatments. Germination was assessed after 1.5h incubation and counts are based on at least 100 spores for each replicate. $T_0$ indicates that the treatment was present during encystment.
Figure 3.32 The effect of time of addition of 10mM Mg\(^{2+}\) on the germination of pre-encysted zoospores of *P. infestans* strain, EUPi1 with data for the corresponding SDW controls.

* Data are means ± se for at least three replicate treatments. Germination was assessed after 1.5h incubation and counts are based on at least 100 spores for each replicate. T\(_0\) indicates that the treatment was present during encystment.
Figure 3.33 The effect of time of addition of 10mM Mg²⁺ on the germination of pre-encysted zoospores of *P. palmivora*, with data for the corresponding SDW controls.

* Data are means ± se for at least three replicate treatments. Germination was assessed after 1.5h incubation and counts are based on at least 100 spores for each replicate. T₀ indicates that the treatment was present during encystment.
induction of encystment a significant difference from the control was apparent. The addition of \( Mg^{2+} \) to \( P. \) palmivora also produced a significant difference as compared to the controls, with the time of addition also being significant with respect to germination.

When the \( Ca^{2+} \) chelator EGTA was added to \( P. \) infestans (Figure 3.34) and \( P. \) palmivora (Figure 3.35) at various times after the induction of encystment there was an almost complete, inhibition of germination when EGTA was added early after encystment. When EGTA was added later, (30 min or more) the inhibition was diminished but there was still a significant effect on both fungi when EGTA was added after 60 min. In both cases all the time points differ from their corresponding SDW controls. When BAPTA was added to \( P. \) infestans (Figure 3.36) it caused a pronounced inhibition of germination when added soon after encystment and even caused 50% inhibition of germination when the added 60 min after the induction of encystment. As with EGTA, germination at all the time points differed significantly from the corresponding SDW controls.

### 2.8.4 The rescue of \( Ca^{2+} \) modulator-treated cysts of \( P. \) infestans and \( P. \) palmivora by sugar, amino acid and cation post-treatments

Freshly prepared zoospore suspensions were vortex encysted and exposed to a range of \( Ca^{2+} \) modulators. The modulator treatments were added at 10 minutes after the induction of encystment, then post-treatments were added after a further 10 minutes. All the post-treatments were added to a final concentration of 10mM except for malt extract plus peptone, which was added at 1% final concentration. Corresponding SDW controls were also used, in which SDW was added as the pre-treatment and nutrient post-treatments added 10 minutes later.

The addition of 1.75mM BAPTA reduced the germination of pre-encysted zoospores of \( P. \) infestans (Table 3.1) from a control level of 53% (non treated

Continued on page 111
Figure 3.34 The effect of time of addition of 10mM EGTA on the germination of pre-encysted zoospores of *P. infestans* strain EUPi1, with data for the corresponding SDW controls.

*Data are means ± se for at least three replicate treatments. Germination was assessed after 1.5h incubation and counts are based on at least 100 spores for each replicate. T5 indicates that the treatment was added 5 minutes after encystment.*
Figure 3.35 The effect of time of addition of 10mM EGTA on the germination of pre-encysted zoospores of *P. palmivora*, with data for the corresponding SDW controls.

*Data are means ± se for at least three replicate treatments. Germination was assessed after 1.5h incubation and counts are based on at least 100 spores for each replicate. T5 indicates that the treatment was added 5 minutes after encystment.*
Figure 3.36 The effect of time of addition of 2.5mM BAPTA on the germination of pre-encysted zoospores of *P. infestans* strain EUP1, with data for the corresponding SDW controls.

*Data are means ± se for at least three replicate treatments. Germination was assessed after 1.5h incubation and counts are based on at least 100 spores for each replicate. T₅ indicates that the treatment was added 5 minutes after encystment.*
controls) to 0.5% (SDW post-treatment). The addition of Ca$^{2+}$ or Ba$^{2+}$ as post-treatments was able to partially overcome the inhibition caused by BAPTA. The presence of Ca$^{2+}$ together with another nutrient treatment was also able to overcome this inhibition. In the corresponding controls in which SDW was added instead of BAPTA as a pre-treatment, the subsequent addition of Ca$^{2+}$ or Ba$^{2+}$ resulted in a significant reduction in germination of the cysts, but none of the other post-treatments significantly enhanced or reduced cyst germination compared with the level in the untreated controls. The addition of EGTA (Table 3.1) to *P. infestans* caused marked inhibition of germination. However, this inhibition was completely overcome by post-treatment with Ca$^{2+}$, Ba$^{2+}$, glucose + Ca$^{2+}$, glutamine + Ca$^{2+}$ and malt extract plus peptone. In contrast the maximum level of rescue achieved for BAPTA treated cells was 46% of control values.

The effects of channel blockers on cysts of *P. infestans* are shown in Table 3.2. The rescue from inhibition of germination of La$^{3+}$ and Gd$^{3+}$ treated cysts could only be achieved by the addition of MEP. None of the other post-treatments, including Ca$^{2+}$ and Ba$^{2+}$ had a significant effect. However, these ions did partially inhibit the germination of non pre-treated cysts. Pre-treatment with verapamil also markedly reduced cyst germination, but this inhibition was largely overcome when the cysts were post-treated with Ca$^{2+}$, Ba$^{2+}$, glucose + Ca$^{2+}$, glutamine + Ca$^{2+}$ or malt extract plus peptone.

All three calmodulin antagonists, when applied as "pre-treatments", inhibited cyst germination by *P. infestans* (Table 3.3). In the case of TFP and calmidazolium there was no significant reversal of this inhibition by any of the applied post-treatments. However, the inhibitory effect of dibucaine was partially reversed by subsequent addition of Ca$^{2+}$ or of glucose + Ca$^{2+}$ (though not by glucose + Ca$^{2+}$), and was completely reversed by subsequent addition of malt extract plus peptone.

Amiloride at the concentration used (400μM) caused a marked reduction but not complete inhibition of cyst germination but not complete inhibition of cyst germination (Table 3.4). This inhibitory effect was partly overcome by subsequent
addition of Ca$^{2+}$, Ba$^{2+}$, glucose + Ca$^{2+}$ or glutamine + Ca$^{2+}$. However it was notable
that there was no "rescue" effect of MEP. On the contrary, the post-treatment with
MEP further reduced the level of germination caused by amiloride, as did post-
treatment with L-asparagine.

The intracellular Ca$^{2+}$ antagonists caffeine and TMB-8 (Table 3.5) significantly
reduced the levels of *P. infestans* cyst germination when applied as a (pre-treatment).
The inhibitory effect of caffeine could not be overcome although the inhibitory effect
of TMB-8 could be partially overcome by the subsequent addition of Glucose + Ca$^{2+}$
or MEP.

Corresponding experiments to those above were performed with zoospore
cysts of *P. palmivora* (Tables 3.6-3.10). All of the pre-treatments with calcium
modulators significantly reduced cyst germination by *P. palmivora*, as they had done
with *P. infestans*. However, the responses of the cysts of *P. palmivora* to post-
treatments were sometimes markedly different than the effects for *P. infestans*. These
differences are summarised in Table 3.11.

Comparisons of the responses of the two fungi are hampered to some degree
because different concentrations of calcium modulator-treatments were used. The
calcium-modulator concentrations used were the lowest concentration that reduced
the level of cyst germination to less than 10% of the control values. Nevertheless,
Table 3.11 shows that *P. infestans* cysts could be more easily rescued from inhibition
by EGTA than was true for *P. palmivora* cysts. However the opposite seemed to true
for rescue from the effects of BAPTA: *P. palmivora* cysts germinated in response to a
wide range of post-treatments, including glucose, sucrose, L-glutamine and L-
asparagine, whereas the cyst of *P. infestans* never responded to these post-treatments.
It is notable that these compounds above, when applied after 20 minutes, caused no
significant enhancement of germination of control (non pre-treated) cysts, so their
effect was in some specific way overcoming the inhibition of *P. palmivora* caused by

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verapamil (but not by La$^{3+}$), whereas the verapamil treated cysts of *P. infestans* did not respond to post-treatment with L-glutamine or L-asparagine. \(^1\)

\(^1\) Continued on page 125
Table 3.1

Germination of *P. infestans* cysts (strain EUPii) treated with the Ca²⁺ chelators EGTA or BAPTA and then treated 10 minutes later with nutrients or divalent cations. Corresponding controls (column headed SDW) received only the "post-treatment" shown.

<table>
<thead>
<tr>
<th>Post-treatment</th>
<th>Glucose</th>
<th>Sucrose</th>
<th>Xylose</th>
<th>Ca²⁺</th>
<th>Ba²⁺</th>
<th>L-glutamine</th>
<th>L-asparagine</th>
<th>Glucose + Ca²⁺</th>
<th>Glutamine + Ca²⁺</th>
<th>MEP</th>
<th>SDW</th>
<th>SDW</th>
<th>SDW</th>
<th>SDW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BAPTA (1.75mM)</td>
<td>1.1</td>
<td>1.9</td>
<td>2.4</td>
<td>16.7*</td>
<td>10.9*</td>
<td>0</td>
<td>0</td>
<td>24.2*</td>
<td>14.6*</td>
<td>1.1</td>
<td>1.1</td>
<td>2.4</td>
<td>43.5*</td>
<td>40.9*</td>
</tr>
<tr>
<td>EGTA (10mM)</td>
<td>1.2</td>
<td>1.2</td>
<td>1.2</td>
<td>8.2</td>
<td>8.2</td>
<td>0</td>
<td>0</td>
<td>18.2</td>
<td>18.2</td>
<td>1.2</td>
<td>1.2</td>
<td>1.2</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td>Non-treated controls</td>
<td>53.0</td>
<td>59.1</td>
<td>68.7</td>
<td>69.3</td>
<td>69.3</td>
<td>69.3</td>
<td>69.3</td>
<td>69.3</td>
<td>69.3</td>
<td>69.3</td>
<td>69.3</td>
<td>69.3</td>
<td>69.3</td>
<td>69.3</td>
</tr>
</tbody>
</table>

Data are means of at least three replicate treatments. Germination was assessed after 1.5 h incubation and counts are based on at least 100 spores for each replicate treatment. * data for post-treatments differ significantly from the corresponding SDW post-treatment. Overall, for the first two columns of data the 5% LSD was 6.6; for the second two columns the LSD was 7.6. MEP = malt extract (1%) plus peptone (1%).

For the second two columns, the LSD was 7.6. MEP = malt extract (1%) plus peptone (1%)
**Table 3.2** Germination of *P. infestans* (strain EUPil) cysts treated with the Ca²⁺ channel blockers La³⁺, Gd³⁺ and verapamil and then treated 10 minutes later with nutrients or divalent cations. Corresponding controls (columns headed SDW) received only the "post-treatments" shown.

Data are means of at least three replicates. Germination was assessed after 1.5h incubation and counts are based on at least 100 spores for each replicate.

<table>
<thead>
<tr>
<th>Initial Treatment</th>
<th>Glucose</th>
<th>Sucrose</th>
<th>Xylose</th>
<th>Ca²⁺</th>
<th>Ba²⁺</th>
<th>L Glutamine</th>
<th>L Asparagine</th>
<th>Glucose and Ca²⁺</th>
<th>Ca²⁺ and Glutamine and Ca²⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>La³⁺ (50uM)</td>
<td>1.4</td>
<td>2.1</td>
<td>1.6</td>
<td>3.8</td>
<td>7.1</td>
<td>1.7</td>
<td>7.8</td>
<td>6.2</td>
<td>38.9*</td>
</tr>
<tr>
<td>SOW</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Gd³⁺ (50uM)</td>
<td>0.6</td>
<td>1.6</td>
<td>0.3</td>
<td>2.3</td>
<td>1.5</td>
<td>1.2</td>
<td>0.3</td>
<td>9.6</td>
<td>4.5</td>
</tr>
<tr>
<td>MEP</td>
<td>57.4</td>
<td>57.3</td>
<td>49.8</td>
<td>39.1*</td>
<td>39.9*</td>
<td>52.5</td>
<td>54.1</td>
<td>49.8</td>
<td>56.1</td>
</tr>
<tr>
<td>Verap. (250uM)</td>
<td>4.8</td>
<td>3.3</td>
<td>6.5</td>
<td>51.4*</td>
<td>33.4*</td>
<td>0.8</td>
<td>0.2</td>
<td>55.8*</td>
<td>54.0*</td>
</tr>
<tr>
<td>MEP</td>
<td>75.7</td>
<td>70.9</td>
<td>75.8</td>
<td>75.2</td>
<td>77.2</td>
<td>80.3</td>
<td>80.2</td>
<td>77.2</td>
<td>79.7</td>
</tr>
</tbody>
</table>

* Data for post-treatments differ significantly from the corresponding SDW post-treatment overall. For the first two columns of data the 5% LSD was 7.3; for the second two columns the LSD was 11.1; and for the last two columns of data the 5% LSD was 8.6.

Data are means of at least three replicate treatments. Germination was assessed after 1.5h incubation and counts are based on at least 100 spores for each replicate. For the second two columns the LSD was 11.1. ! and for the last two columns of data the 5% LSD was 7.3.

Data are means of at least three replicate treatments. Germination was assessed after 1.5h incubation and counts are based on at least 100 spores for each replicate. For the second two columns the LSD was 11.1. ! and for the last two columns of data the 5% LSD was 7.3.
Table 3.3
Germination of *P. infestans* cysts (strain EUPi1) treated with the calmodulin antagonists dibucaine, TFP and calmidazolium and then treated 10 minutes later with nutrients or divalent cations. Corresponding controls (column headed SDW) received only the "post-treatment" shown.

<table>
<thead>
<tr>
<th>Initial Treatment</th>
<th>SDW</th>
<th>MEP</th>
<th>Dibucaine (250µM)</th>
<th>TFP (25µM)</th>
<th>Calmidazolium (4µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>39.7</td>
<td>0.3</td>
<td>17.6*</td>
<td>35.7*</td>
<td>11.7*</td>
</tr>
<tr>
<td>Sucrose</td>
<td>39.5</td>
<td>0</td>
<td>5.4</td>
<td>2.0</td>
<td>0</td>
</tr>
<tr>
<td>Xylose</td>
<td>39.2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>36.9</td>
<td>0</td>
<td>17.6*</td>
<td>35.7*</td>
<td>11.7*</td>
</tr>
<tr>
<td>Ba²⁺</td>
<td>36.9</td>
<td>0</td>
<td>5.4</td>
<td>2.0</td>
<td>0</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>37.4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>L-asparagine</td>
<td>37.2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Glucose + Ca²⁺</td>
<td>39.2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Glutamine + Ca²⁺</td>
<td>39.2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Data are means of at least three replicate treatments. Germination was assessed after 1.5h incubation and counts are based on at least 100 spores for each replicate treatment. * data for post-treatments differ significantly from the corresponding SDW post-treatment: overall, for the first two columns of data the 5% LSD was 8.8, for the second two columns the LSD was 21.7 and for the last two columns the LSD was 26.5.

Non-treated con.

<table>
<thead>
<tr>
<th>Glucose</th>
<th>33.0</th>
<th>40.3</th>
<th>33.4</th>
<th>40.2</th>
<th>53.7</th>
<th>53.7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>39.7</td>
<td>0.3</td>
<td>17.6*</td>
<td>35.7*</td>
<td>11.7*</td>
<td>11.7*</td>
</tr>
<tr>
<td>Xylose</td>
<td>39.2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TFP</td>
<td>36.9</td>
<td>0</td>
<td>17.6*</td>
<td>35.7*</td>
<td>11.7*</td>
<td>11.7*</td>
</tr>
<tr>
<td>Calmidazolium</td>
<td>39.2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

For the second two columns the LSD was 21.7 and for the first two columns 8.8. Corresponding controls (column headed SDW) received only the "post-treatment" shown.
Table 3.4 Germination of *P. infestans* cysts (strain EUPil) treated with the Na⁺ / Ca²⁺ flux inhibitor amiloride and then treated 10 minutes later with nutrients or divalent cations. Corresponding controls (column headed SDW) received only the post treatments shown.

<table>
<thead>
<tr>
<th>Initial Treatment</th>
<th>Post-Treatment</th>
<th>Non-Treated Control</th>
<th>SDW</th>
</tr>
</thead>
<tbody>
<tr>
<td>59.2</td>
<td>57.4</td>
<td>7.9</td>
<td>6.2</td>
</tr>
<tr>
<td>59.2</td>
<td>6.9</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>62.8</td>
<td></td>
<td>33.3 + Ca²⁺</td>
<td></td>
</tr>
<tr>
<td>53.2</td>
<td>35.8 + Ca²⁺</td>
<td></td>
<td></td>
</tr>
<tr>
<td>53.2</td>
<td>34.8 + Ca²⁺</td>
<td></td>
<td></td>
</tr>
<tr>
<td>59.4</td>
<td>0.0</td>
<td>1.0</td>
<td>6.2</td>
</tr>
<tr>
<td>56.2</td>
<td>18.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>51.2</td>
<td>37.1 + Glut</td>
<td></td>
<td></td>
</tr>
<tr>
<td>44.3</td>
<td>39.4 + Glut</td>
<td></td>
<td></td>
</tr>
<tr>
<td>52.5</td>
<td>20.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>54.3</td>
<td>11.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>57.4</td>
<td>10.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose + Ca²⁺</td>
<td>Glutamine + Ca²⁺</td>
<td>MEP</td>
<td>Mep</td>
</tr>
<tr>
<td>Glucose + Ca²⁺</td>
<td>L-Asparagine</td>
<td>Glutamine + Ca²⁺</td>
<td></td>
</tr>
<tr>
<td>L-Asparagine</td>
<td>Glutamine</td>
<td>Glutamine + Ca²⁺</td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>Glutamine</td>
<td>Glutamine + Ca²⁺</td>
<td></td>
</tr>
</tbody>
</table>

Data are means of at least three replicate treatments. Germination was assessed after 1.5h incubation and counts are based on at least 100 spores for each replicate treatment. * data for post-treatments differ significantly from the corresponding SDW post-treatment; overall, for the two columns of data the 5% LSD was 14.1. MEP = malt extract (1%) plus peptone (1%).
Table 3.5 Germination of *P. infestans* cysts (strain Eupil) treated with the intracellular calcium modulators caffeine and TMB-8 and then treated 10 minutes later by nutrients or divalent cations. Corresponding controls (columns headed SDW) received only the "post-treatments" shown.

<table>
<thead>
<tr>
<th>Initial Treatment</th>
<th>Post-treatment</th>
<th>Non-treated control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>55.4</td>
<td>49.0</td>
</tr>
<tr>
<td>Sucrose</td>
<td>62.9</td>
<td>49.0</td>
</tr>
<tr>
<td>Xylose</td>
<td>75.1</td>
<td>36.3</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>5.7</td>
<td>4.3</td>
</tr>
<tr>
<td>Ba&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>3.7</td>
<td>57.9</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>2.1</td>
<td>63.2</td>
</tr>
<tr>
<td>L-asparagine</td>
<td>2.6</td>
<td>0.4</td>
</tr>
<tr>
<td>Glucose + Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>0.4</td>
<td>6.0</td>
</tr>
<tr>
<td>MEP</td>
<td>54.3</td>
<td>55.2</td>
</tr>
<tr>
<td>SDW</td>
<td>56.1</td>
<td>56.1</td>
</tr>
<tr>
<td>Caffeine (4mM)</td>
<td>55.2</td>
<td>55.2</td>
</tr>
<tr>
<td>TMB-8 (500uM)</td>
<td>53.2</td>
<td>53.2</td>
</tr>
<tr>
<td>MEP</td>
<td>63.2</td>
<td>63.2</td>
</tr>
<tr>
<td>SDW</td>
<td>57.9</td>
<td>57.9</td>
</tr>
<tr>
<td>Non-treated control</td>
<td>55.1</td>
<td>56.3</td>
</tr>
</tbody>
</table>

Data are means of at least three replicate treatments. Germination was assessed after 1.5h incubation and counts are based on at least 100 spores for each replicate treatment. * data for post-treatments differ significantly from the corresponding SDW post-treatment; overall, for the first two columns of data the 5% LSD was 51.9; for the second two columns the 5% LSD was 14.9. MEP= malt extract (1%) plus peptone (1%).
Table 3.6 Germination of *P. palmivora* treated with the Ca²⁺ chelators EGTA or BAPTA and the treated 10 minutes later with nutrients or divalent cations. Corresponding controls (columns headed with SDW) received only the "post-treatments" shown.

<table>
<thead>
<tr>
<th>Initial treatment</th>
<th>Post-treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>2.6</td>
</tr>
<tr>
<td>Sucrose</td>
<td>2.5</td>
</tr>
<tr>
<td>Xylose</td>
<td>1.5</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>1.5</td>
</tr>
<tr>
<td>Glutamine + Ca²⁺</td>
<td>5.0</td>
</tr>
<tr>
<td>Glucose + Ca²⁺</td>
<td>2.5</td>
</tr>
<tr>
<td>L-asparagine</td>
<td>1.5</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>2.0</td>
</tr>
<tr>
<td>Glutamine</td>
<td>2.0</td>
</tr>
<tr>
<td>MEP</td>
<td>2.5</td>
</tr>
<tr>
<td>SOW</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Data are means of at least three replicate treatments. Germination was assessed after 1.5h incubation and counts are based on at least 100 spores for each replicate treatment. * data for post-treatments differ significantly from the corresponding SDW post-treatment; overall, for the first two columns of data the 5% LSD was 9.8; for the second two columns the 5% LSD was 10.6. MEP= malt extract (1%) plus peptone (1%).
Table 3.7 Germination of P. palmivora treated with the channel blockers Verapamil or La³⁺ and then treated 10 minutes later with nutrients of divalent cations. Corresponding controls (columns headed SDW) received only the "post-treatments" shown. Initial treatment

<table>
<thead>
<tr>
<th>Non-Treated Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>61.9</td>
</tr>
<tr>
<td>43.2</td>
</tr>
<tr>
<td>4.9</td>
</tr>
<tr>
<td>49.3</td>
</tr>
<tr>
<td>48.4</td>
</tr>
<tr>
<td>45.4</td>
</tr>
<tr>
<td>16.1</td>
</tr>
<tr>
<td>42.6</td>
</tr>
<tr>
<td>43.3</td>
</tr>
<tr>
<td>42.4</td>
</tr>
<tr>
<td>8.8</td>
</tr>
<tr>
<td>7.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Glucose</th>
<th>Sucrose</th>
<th>Xylose</th>
<th>Ca²⁺</th>
<th>Ba²⁺</th>
<th>L-glutamine</th>
<th>L-asparagine</th>
<th>MEP</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.6</td>
<td>9.7</td>
<td>8.2</td>
<td>27.5*</td>
<td>20.4*</td>
<td>35.6*</td>
<td>27.4*</td>
<td>40.6*</td>
</tr>
<tr>
<td>64.6</td>
<td>61.8</td>
<td>61.0</td>
<td>64.0</td>
<td>63.3</td>
<td>55.4</td>
<td>57.5</td>
<td>59.0</td>
</tr>
<tr>
<td>2.2</td>
<td>1.8</td>
<td>1.9</td>
<td>8.1</td>
<td>1.7</td>
<td>2.9</td>
<td>3.0</td>
<td>9.6</td>
</tr>
<tr>
<td>7.2</td>
<td>6.3</td>
<td>6.0</td>
<td>64.0</td>
<td>27.5*</td>
<td>35.6*</td>
<td>55.4*</td>
<td>46.1</td>
</tr>
<tr>
<td>1.7</td>
<td>8.1</td>
<td>6.0</td>
<td>61.0</td>
<td>8.2</td>
<td>8.2</td>
<td>8.2</td>
<td>69.0</td>
</tr>
<tr>
<td>1.8</td>
<td>8.1</td>
<td>6.8</td>
<td>61.8</td>
<td>9.7</td>
<td>6.6</td>
<td>6.6</td>
<td>6.6</td>
</tr>
<tr>
<td>2.2</td>
<td>2.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are means of at least three replicate treatments. Germination was assessed after 1.5 h incubation and counts are based on at least 100 spores for each replicate.

For the second two columns the 5% LSD was 8.4; for the first two columns of data the 5% LSD was 8.8. * data for post-treatments differ significantly from the corresponding SDW post-treatment. Overall, for the first two columns of data the 5% LSD was 8.4 for the second two columns the 5% LSD was 11.9. MEP = malt extract (1%) plus peptone (1%).
Table 3.8 Germination of P. palmivora cysts treated with the calmodulin antagonists TFP calmidazolium or dibucaine and then treated 10 minutes later with nutrients of divalent cations. Corresponding controls (columns headed SDW) received only the post-treatments shown.

<table>
<thead>
<tr>
<th>Initial concentration</th>
<th>Post-treatment</th>
<th>Glucose</th>
<th>Fructose</th>
<th>Xylose</th>
<th>Ca²⁺</th>
<th>Ba²⁺</th>
<th>L-glutamine</th>
<th>L-asparagine</th>
<th>Glutamine + Ca²⁺</th>
<th>Glucose + Ca²⁺</th>
<th>MEP</th>
<th>SDW Non-treated control</th>
<th>TFP (20uM)</th>
<th>Calmidazolium (1.2uM)</th>
<th>Dibucaine (2000uM)</th>
<th>TFP (20uM)</th>
<th>Calmidazolium (1.2uM)</th>
<th>Dibucaine (2000uM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.2'6</td>
<td>42.6</td>
<td>9.6</td>
<td>62.6</td>
<td>6'7.8</td>
<td>42.3</td>
<td>3.9'9</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>36.2*</td>
<td>SDW</td>
<td>Non-treated control</td>
<td>TFP (20uM)</td>
<td>Calmidazolium (1.2uM)</td>
<td>Dibucaine (2000uM)</td>
<td>TFP (20uM)</td>
<td>Calmidazolium (1.2uM)</td>
<td>Dibucaine (2000uM)</td>
</tr>
<tr>
<td>4.7'3</td>
<td>41.3</td>
<td>3.3'6</td>
<td>66.3</td>
<td>1.6'1</td>
<td>48.1</td>
<td>3.6'2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>8.1</td>
<td>SDW</td>
<td>TFP (20uM)</td>
<td>Calmidazolium (1.2uM)</td>
<td>Dibucaine (2000uM)</td>
<td>TFP (20uM)</td>
<td>Calmidazolium (1.2uM)</td>
<td>Dibucaine (2000uM)</td>
<td></td>
</tr>
<tr>
<td>9.2'9</td>
<td>*</td>
<td>33.9'6</td>
<td>66.3</td>
<td>6.7'1</td>
<td>43.2</td>
<td>1.8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>8.0</td>
<td>SDW</td>
<td>Calmidazolium (1.2uM)</td>
<td>Dibucaine (2000uM)</td>
<td>TFP (20uM)</td>
<td>Calmidazolium (1.2uM)</td>
<td>Dibucaine (2000uM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.9'8</td>
<td>1.3</td>
<td>1.2</td>
<td>61.2</td>
<td>4.7</td>
<td>4.2</td>
<td>0.8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>6.9</td>
<td>SDW</td>
<td>L-asparagine</td>
<td>Dibucaine (2000uM)</td>
<td>TFP (20uM)</td>
<td>Calmidazolium (1.2uM)</td>
<td>Dibucaine (2000uM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.8'5</td>
<td>1.6</td>
<td>0.0</td>
<td>58.7</td>
<td>0.9</td>
<td>42.3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>43.8</td>
<td>SDW</td>
<td>L-glutamine</td>
<td>Dibucaine (2000uM)</td>
<td>TFP (20uM)</td>
<td>Calmidazolium (1.2uM)</td>
<td>Dibucaine (2000uM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.7'4</td>
<td>8.0</td>
<td>3.2'7</td>
<td>5.1'2</td>
<td>1.4</td>
<td>31.2</td>
<td>9.6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>48.1</td>
<td>SDW</td>
<td>Bγ'z</td>
<td>Dibucaine (2000uM)</td>
<td>TFP (20uM)</td>
<td>Calmidazolium (1.2uM)</td>
<td>Dibucaine (2000uM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.3'6</td>
<td>0.5</td>
<td>5.4</td>
<td>2.6</td>
<td>28.7</td>
<td>6.9</td>
<td>5.6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>23.6</td>
<td>SDW</td>
<td>Cα'z</td>
<td>Dibucaine (2000uM)</td>
<td>TFP (20uM)</td>
<td>Calmidazolium (1.2uM)</td>
<td>Dibucaine (2000uM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.3'4</td>
<td>1.1</td>
<td>3.9'1</td>
<td>1.2</td>
<td>2.0</td>
<td>40.2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>38.9</td>
<td>SDW</td>
<td>Xylose</td>
<td>Dibucaine (2000uM)</td>
<td>TFP (20uM)</td>
<td>Calmidazolium (1.2uM)</td>
<td>Dibucaine (2000uM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.1'6</td>
<td>0.3</td>
<td>6.4</td>
<td>0.6</td>
<td>39.8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>41.2</td>
<td>SDW</td>
<td>Sucrose</td>
<td>Dibucaine (2000uM)</td>
<td>TFP (20uM)</td>
<td>Calmidazolium (1.2uM)</td>
<td>Dibucaine (2000uM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.5'6</td>
<td>1.0</td>
<td>6.0'0</td>
<td>0.2</td>
<td>4.2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>46.2</td>
<td>SDW</td>
<td>Glucose</td>
<td>Dibucaine (2000uM)</td>
<td>TFP (20uM)</td>
<td>Calmidazolium (1.2uM)</td>
<td>Dibucaine (2000uM)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Data are means of at least three replicate treatments. Germination was assessed after 1.5 h incubation and counts are based on at least 100 spores for each replicate treatment. Data for post-treatments differ significantly from the corresponding SDW post-treatment overall. For the first two columns of data the 5% LSD was 13.8; for the second two columns the 5% LSD was 7.6; and for the final two columns the 5% LSD was 22.3. MEP = malt extract (1%) plus peptone (1%).
Table 3.9

Germination of *P. palmivora* cysts treated with the Na+/Ca2+ flux inhibitor amiloride and then treated 10 minutes later with nutrients or divalent cations. Corresponding controls (columns headed SDW) received only the "post-treatments" shown. Germination was assessed after 1.5 h incubation and counts are based on at least 100 spores for each replicate. Data are means of at least three replicate treatments. Asterisks denote values differing significantly from the corresponding SDW post-treatment. Controls received the post-treatment shown for each replicate.}

<table>
<thead>
<tr>
<th>Initial Treatment</th>
<th>Non-treated control</th>
<th>SDW</th>
<th>MEP</th>
<th>SDW</th>
<th>SDW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>38.7</td>
<td>59.6</td>
<td>58.7</td>
<td>4.52</td>
<td>8.8</td>
</tr>
<tr>
<td>Sucrose</td>
<td>3.9</td>
<td>8.8</td>
<td>8.8</td>
<td>45.2*</td>
<td>45.2</td>
</tr>
<tr>
<td>Xylose</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>Ca2+</td>
<td>8.8</td>
<td>8.8</td>
<td>8.8</td>
<td>8.8</td>
<td>8.8</td>
</tr>
<tr>
<td>Glutamine + Ca2+</td>
<td>3.4</td>
<td>3.4</td>
<td>3.4</td>
<td>3.4</td>
<td>3.4</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>3.4</td>
<td>3.4</td>
<td>3.4</td>
<td>3.4</td>
<td>3.4</td>
</tr>
<tr>
<td>L-asparginine</td>
<td>4.0</td>
<td>4.0</td>
<td>4.0</td>
<td>4.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Glucose + Ca2+</td>
<td>8.8</td>
<td>8.8</td>
<td>8.8</td>
<td>8.8</td>
<td>8.8</td>
</tr>
<tr>
<td>MEP</td>
<td>3.4</td>
<td>3.4</td>
<td>3.4</td>
<td>3.4</td>
<td>3.4</td>
</tr>
<tr>
<td>Glucose + Ca2+</td>
<td>8.8</td>
<td>8.8</td>
<td>8.8</td>
<td>8.8</td>
<td>8.8</td>
</tr>
<tr>
<td>Ba2+</td>
<td>4.4</td>
<td>4.4</td>
<td>4.4</td>
<td>4.4</td>
<td>4.4</td>
</tr>
<tr>
<td>Glucose + Ca2+</td>
<td>8.8</td>
<td>8.8</td>
<td>8.8</td>
<td>8.8</td>
<td>8.8</td>
</tr>
<tr>
<td>Sucrose</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>Glucose</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>Ammonium (800 μM)</td>
<td>58.9</td>
<td>58.9</td>
<td>58.9</td>
<td>58.9</td>
<td>58.9</td>
</tr>
</tbody>
</table>

* Data for post-treatments differ significantly from the corresponding SDW post-treatment; overall, for the two columns of data the 5% LSD was 1.2. MEP = malt extract (1%) plus peptone (1%).
Table 3.10 Germination of *P. palmivora* cysts treated with the intracellular Ca\(^{2+}\) antagonists caffeine or TMB-8 and then treated 10 minutes later with nutrients or divalent cations. Corresponding controls (columns headed SDW) received only the "post-treatments shown.

<table>
<thead>
<tr>
<th>Initial Treatment</th>
<th>Post-Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose</td>
</tr>
<tr>
<td>F.6.6</td>
<td>5.1</td>
</tr>
<tr>
<td>* 8.8</td>
<td>72.0</td>
</tr>
<tr>
<td>6.3</td>
<td>1.7</td>
</tr>
<tr>
<td>* 5.4</td>
<td>1.1</td>
</tr>
<tr>
<td>5.8.6</td>
<td>* 21.6</td>
</tr>
<tr>
<td>* 4.0.3</td>
<td>2.4</td>
</tr>
<tr>
<td>4.1</td>
<td>10.0</td>
</tr>
<tr>
<td>* 4.8.4</td>
<td>6.3</td>
</tr>
<tr>
<td>4.6.8</td>
<td>1.7.0</td>
</tr>
<tr>
<td>5.3.5</td>
<td>1.7.8</td>
</tr>
<tr>
<td>TMB-8 (250(\mu)M) SDW</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glutamine</td>
</tr>
<tr>
<td>F.6.6</td>
<td>5.1</td>
</tr>
<tr>
<td>* 8.8</td>
<td>72.0</td>
</tr>
<tr>
<td>6.3</td>
<td>1.7</td>
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<tr>
<td>* 5.4</td>
<td>1.1</td>
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<tr>
<td>5.8.6</td>
<td>* 21.6</td>
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<td>* 4.0.3</td>
<td>2.4</td>
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<td>4.1</td>
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<td>* 4.8.4</td>
<td>6.3</td>
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<tr>
<td>4.6.8</td>
<td>1.7.0</td>
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<tr>
<td>5.3.5</td>
<td>1.7.8</td>
</tr>
<tr>
<td>TMB-8 (250(\mu)M) SDW</td>
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<tr>
<td></td>
<td>Glutamine</td>
</tr>
</tbody>
</table>

Data are means of at least three replicate treatments. Germination was assessed after 1.5 h incubation and counts are based on at least 100 spores for each replicate treatment. * data for post-treatments differ significantly from the corresponding SDW post-treatment; overall, for the first two columns of data the 5% LSD was 22.3; for the second two columns of data the 5% LSD was 13.3. MEP = malt extract (1%) plus peptone (1%).
Table 3.11 Comparison of the effects of rescue treatments on germination of *P. infestans* and *P. palmivora* when zoospore cysts had previously been treated with calcium-modulating factors.

<table>
<thead>
<tr>
<th>Pre-treatment</th>
<th>Partial (+) or highly effective (++) rescue treatments.</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>P. infestans</em></td>
<td><em>P. palmivora</em></td>
</tr>
<tr>
<td>EGTA</td>
<td>Ca²⁺ (++)</td>
<td>Ca²⁺ (+)</td>
</tr>
<tr>
<td></td>
<td>Ba²⁺ (++)</td>
<td>Gluc + Ca²⁺ (+)</td>
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<td></td>
<td>Gluc + Ca²⁺ (++)</td>
<td>glu + Ca²⁺ (+)</td>
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<tr>
<td></td>
<td>glu + Ca²⁺ (++)</td>
<td>MEP (+)</td>
</tr>
<tr>
<td></td>
<td>MEP (++)</td>
<td></td>
</tr>
<tr>
<td>BAPTA</td>
<td>Ca²⁺ (+)</td>
<td>Gluc (+)</td>
</tr>
<tr>
<td></td>
<td>Ba²⁺ (+)</td>
<td>Suc (+)</td>
</tr>
<tr>
<td></td>
<td>Gluc + Ca²⁺ (+)</td>
<td>L-glu (++)</td>
</tr>
<tr>
<td></td>
<td>Glu + Ca²⁺ (+)</td>
<td>L-asn (++)</td>
</tr>
<tr>
<td></td>
<td>MEP (++)</td>
<td>Glc + Ca²⁺ (++)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glu + Ca²⁺ (++)</td>
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<td></td>
<td></td>
<td>MEP (+)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>La³⁺</td>
<td>MEP (++)</td>
<td>MEP (++)</td>
</tr>
<tr>
<td>Verapamil</td>
<td>Ca²⁺ (++)</td>
<td>Ca²⁺ (+)</td>
</tr>
<tr>
<td></td>
<td>Ba²⁺ (++)</td>
<td>Ba²⁺ (+)</td>
</tr>
<tr>
<td></td>
<td>Glc + Ca²⁺ (++)</td>
<td>L-glu (++)</td>
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<td>Glu + Ca²⁺ (++)</td>
<td>L-asn (++)</td>
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<td>MEP (++)</td>
<td>glu + Ca²⁺ (++)</td>
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<td></td>
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<td>MEP (+)</td>
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<tr>
<td>TFP</td>
<td></td>
<td>MEP (++)</td>
</tr>
<tr>
<td>Dibucaine</td>
<td>Ca²⁺ (+)</td>
<td>MEP (+)</td>
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<td></td>
<td>Gluc + Ca²⁺ (+)</td>
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<td></td>
<td>MEP (++)</td>
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<tr>
<td>Calmidazolium</td>
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<tr>
<td>Amiloride</td>
<td>Ca²⁺ (++)</td>
<td>MEP (++)</td>
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<tr>
<td></td>
<td>Ba²⁺ (++)</td>
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<td>Glc + Ca²⁺ (++)</td>
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<td></td>
<td>Glu + Ca²⁺ (++)</td>
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<tr>
<td>Caffeine</td>
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<tr>
<td>TMB-8</td>
<td>Gluc + Ca²⁺ (+)</td>
<td>L-glu (+)</td>
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<tr>
<td></td>
<td>MEP (++)</td>
<td>L-glu + Ca²⁺ (+)</td>
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<td></td>
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<td>MEP (+)</td>
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</tbody>
</table>

Partial rescue was classified as those treatments that varied significantly from the SDW control but whose germination was still less than 50% of the untreated control. Those treatments that varied significantly from the SDW control and had a germination level of more than 50% of the non-treated control value were classified as displaying highly effective rescue.
Discussion

The experiments described in this chapter investigated the effect of a range of divalent cations and calcium-modulating compounds (Godfraind et al., 1986) on the germination of pre-encysted zoospores of *P. infestans* in comparison with *P. palmivora*. Such comparative studies on different species are important for establishing common patterns of response: if *P. infestans* can be shown to behave similarly to other species which are much easier to use in laboratory studies, and which have therefore become "model organisms", then we might predict that *P. infestans* will show similar behaviour in other respects.

All the work in this chapter suggest that Ca\(^{2+}\) plays a central role in zoospore biology, and especially in zoospore cyst germination, of *P. infestans*. This parallels other work which implicates a central role of Ca\(^{2+}\) in zoospore biology of *Py. aphanidermatum* (Donaldson and Deacon, 1992, 1993a) *P. parasitica* (Von Broembsen and Deacon, 1996; Warburton and Deacon, 1998) and *Aphanomyces* species (Cerenius and Soderhall, 1985; Deacon and Saxena, 1998).

Millimolar levels of the chelators EGTA and BAPTA, strongly inhibited zoospore cyst germination of *P. infestans* and *P. palmivora* (Figs 3.1-3.4). Both of these compounds have a strong binding affinity for Ca\(^{2+}\), but they are not Ca\(^{2+}\) specific and they show even higher affinity for Zn\(^{2+}\), Mn\(^{2+}\) and Fe\(^{2+}\) (Youatt, 1993; Loukin and Kung, 1995). On this basis Youatt (1993) has criticised previous work which has used EGTA to implicate the role of Ca\(^{2+}\) in cellular responses. However it seems unlikely that the suppressive effect of these chelators on cyst germination could be explained by effects on Zn\(^{2+}\), Mn\(^{2+}\) or Fe\(^{2+}\), because these ions are unlikely to be present at millimolar levels in zoospore cyst suspensions. Previous studies such as those on the abolition of zoospore cyst adhesion by EGTA (Gubler et al., 1989; Donaldson and Deacon, 1992) and the restoration of cyst adhesion by the addition of Ca\(^{2+}\) strongly implicate an effect of EGTA (and of BAPTA) on external Ca\(^{2+}\) levels around zoospores.
Moreover recent studies (Warburton and Deacon, 1998) have shown major net influx and efflux of Ca$^{2+}$ from zoospore cysts of *P. parasitica* during the early stages of encystment and cyst germination. So it seems that chelators could affect germination by sequestering the Ca$^{2+}$ that is released from cysts during the normal course of progression towards germination.

Other evidence for a role of transmembrane Ca$^{2+}$ fluxes in zoospore cyst germination was obtained by the use of La$^{3+}$, Gd$^{3+}$ and verapamil (Figs 3.5-3.9); these substances have been reported to act as calcium channel blockers in other organisms (Godfraind *et al.*, 1986). At micromolar concentrations they strongly inhibit zoospore cyst germination by *P. infestans*. Presumably, only low levels were required, compared with the much higher levels of chelators required for suppression of germination, because the channel blockers interact with a limited number of Ca$^{2+}$ channels in the zoospore cyst membrane. It is interesting that La$^{3+}$ was more effective in suppressing cyst germination by *P. infestans* than by *P. palmivora*, whereas verapamil was more effective against *P. palmivora*. No explanation can be offered for this.

The three substances, TFP, dibucaine and calmidazolium are reported to act as calmodulin antagonists (Bereza *et al.*, 1982) and thus presumably exert their effect by interaction with this Ca-binding protein within cells. All three compounds suppressed cyst germination by *P. infestans* and *P. palmivora* at micromolar concentrations (Figs 3.10-3.15) consistent with previous reports on the effect of these compounds on zoospore cysts of *Py. aphaidermatum* (Donaldson and Deacon, 1992) and *P. parasitica* (Warburton and Deacon, 1998). Calmidazolium was the most effective of these compounds, and dibucaine the least effective, in terms of concentrations required to suppress the germination of zoospore cysts.

Amiloride also inhibited cyst germination; to a similar degree in both *P. infestans* and *P. palmivora* (Figs 3.16-3.17). This compound was suggested by
Godfraind et al. (1986) to be a Ca\(^{2+}\)/Na\(^{+}\) exchange inhibitor, but is more commonly reported to affect the H\(^{+}\)/Na\(^{+}\) antiport system of mammalian membranes. Its precise role in zoospores is unknown, although any effect on ion exchange across cell membranes could have potential secondary effects on the balance of ions within cells, because Grant et al. (1986) showed that complex ion interactions can affect zoospore cyst germination.

Two further compounds—TMB-8 and caffeine were found to inhibit cyst germination by *P. infestans* and *P. palmivora* (Figs 3.18-3.21). In other systems these compounds are reported to influence intracellular Ca\(^{2+}\) mediated responses. Caffeine is reported to deplete the levels of Ca\(^{2+}\) in intracellular stores and TMB-8 is reported to block the release of Ca\(^{2+}\) from intracellular stores (Owen and Villereal, 1982). Much higher levels of caffeine than TMB-8 were required to suppress cyst germination by *P. infestans* and *P. palmivora*, consistent with a report of Warburton and Deacon (1998) for *P. parasitica*. In a study of transmembrane Ca\(^{2+}\) flux of *P. parasitica* Warburton and Deacon (1998) showed a large initial net influx of Ca\(^{2+}\) when zoospores were induced to encyst; this was followed by a slower progressive net Ca\(^{2+}\) efflux, indicating a central role of Ca\(^{2+}\) release from intracellular stores (blockable by TMB-8) which seemed to be necessary for cyst germination.

Given that all this evidence indicates an important role of Ca\(^{2+}\) in zoospore cyst germination, it was of interest to see whether germination could be enhanced or suppressed by adding Ca\(^{2+}\) or other divalent cations (Figs 3.22-3.27). For both *P. infestans* and *P. palmivora*, it was found that Ca\(^{2+}\) supplements, up to 10mM level, tended to suppress cyst germination. Ba\(^{2+}\) supplements acted in almost exactly the same way as Ca\(^{2+}\) supplements, but Mg\(^{2+}\) supplements were usually less inhibitory to both fungi. In several other cellular systems Ba\(^{2+}\) has been shown to substitute for Ca\(^{2+}\). In detailed time-course studies (Figs 3.28-3.33) supplements of Ca\(^{2+}\), Ba\(^{2+}\) or Mg\(^{2+}\) were found to have the most inhibitory effect on cyst germination when added very early after zoospores were vortex encysted. A delay of 10-20 minutes before adding the ions had a much lesser effect on germination. Several other studies have shown that
zoospore cysts remain responsive to externally applied Ca\(^{2+}\) for only a limited time after the induction of encystment (Donaldson and Deacon, 1992; Von Broembsen and Deacon, 1996) and that high levels of Ca\(^{2+}\) supplements can suppress germination, presumably by disrupting the normal Ca\(^{2+}\) homeostasis of cells. Indeed despite its important role in cellular signalling Ca\(^{2+}\) is reported to be cytotoxic- the free Ca\(^{2+}\) level in the cytosol is maintained at a resting level of about 100-300nM, to prevent cytotoxicity (Tsien and Tsien, 1990).

The finding that divalent cation supplements were most strongly inhibitory when added to cysts in the early post-encystment period (eg. Fig 3.33) was paralleled by the finding that chelators (EGTA, BAPTA) also were most effective in suppressing germination when added relatively early after encystment (e.g. Fig 3.34). Evidently zoospore cyst germination is strongly influenced by Ca\(^{2+}\)-mediated processes that occur in the early stages following encystment. Persson and Soderhall (1986) found that the zoospore cysts of the crayfish pathogen *Aphanomyces astaci* could be induced to germinate by high Ca\(^{2+}\) supplements in the first 15-20 minutes after encystment, but germination then became non-responsive to externally supplied Ca\(^{2+}\). Yet these cysts could still respond to external Ca\(^{2+}\) supplements by secreting peptidase when Ca\(^{2+}\) was applied even 30 or 40 minutes after encystment. In explaining this, these workers, suggested that cysts can detect an external Ca\(^{2+}\) signal at anytime up to at least 45 minutes after encystment, but that this Ca\(^{2+}\) signal is only linked to the induction of germination in the initial 15-20 minutes after encystment.

In the final series of experiments in this section, attempts were made to rescue cysts of *P. infestans* and *P. palmivora* from suppression of germination caused by various calcium-modulator compounds (Tables 3.1-1.10). all the potential rescue treatments were applied 10 minutes after the calcium-modulators (which were applied 10 minutes after the induction of encystment).

For cysts of *P. infestans*, the suppression of germination caused by the chelator EGTA was completely reversed by post-application of Ca\(^{2+}\) or Ba\(^{2+}\), or by malt extract plus peptone., whereas the suppression caused by BAPTA was only partly
reversed by the application of Ca\textsuperscript{2+} or Ba\textsuperscript{2+}, and was not overcome by MEP. The partial or complete rescue of germination by the divalent cations is consistent with the suggestion that these chelators suppress germination by sequestering Ca\textsuperscript{2+} released from cysts during the normal post-encystment events (Warburton and Deacon, 1998). The role of MEP is more difficult to explain, this mixture of nutrients might contain sufficient quantities of Ca\textsuperscript{2+}, but if this is true then MEP should also have helped to rescue the inhibition of germination caused by BAPTA (Table 3.1). An alternative explanation is that MEP acted as a general nutrient trigger, overcoming the suppression of germination caused by Ca\textsuperscript{2+} chelation. MEP has been found to act as a general trigger for cyst germination by several fungi (Donaldson and Deacon, 1993b; Deacon and Saxena, 1998). For *P. palmivora* the suppression of germination caused by EGTA was much stronger than the suppression caused by BAPTA, and it was less easily overcome by post-treatments (Table 3.6). Of most interest however was the finding that chelator-induced suppression of germination of *P. palmivora* could sometimes be overcome by simple nutrient triggers such as glucose, sucrose, L-glutamine or L-asparagine. This was never seen for *P. infestans*. It suggests that *P. palmivora* might have nutrient receptors in the cyst membrane, which are absent from *P. infestans*. In this regard zoospores of *P. palmivora* are known to respond chemotactically to a range of individual organic compounds. Whereas zoospores of *P. infestans* have not been found to respond chemotactically to individual organic compounds (D.E. Grayson unpublished).

In experiments with putative Ca\textsuperscript{2+} channel blockers (Tables 3.2, 3.7) the suppression of germination caused by La\textsuperscript{3+} or Gd\textsuperscript{3+} could be overcome only by MEP. Suppression of germination by verapamil, however, was more easily overcome by MEP and by divalent cations in the case of *P. infestans* and by these treatments as well as by L-glutamine and L-asparagine in the case of *P. palmivora*. In mammalian systems there is strong evidence for the existence of nutrient-gated Ca\textsuperscript{2+} membrane channels (Gilbertson *et al.*, 1991). Such that the entry of Ca\textsuperscript{2+} into the cell is mediated by a specific nutrient receptor in the membrane. Donaldon and Deacon (1992) postulated the existence of an asparagine gated Ca\textsuperscript{2+} channel in the zoospore cyst.
membrane of *Py. aphanidermatum*. Perhaps similar Ca$^{2+}$ channels responsive to L-gln and L-asn, are present in the cyst membrane of *P. palmivora* but not *P. infestans*.

The most effective calmodulin antagonist used in this study was calmidazolium. Its effectiveness in suppressing cyst germination could not be reversed by any treatment in either *P. palmivora* or *P. infestans*. Similarly, the suppression by TFP could not be reversed by any treatment in *P. infestans*, and only partly reversed by MEP in *P. palmivora*. However, the effects of dibucaíne on *P. infestans* was partly reversed by some Ca$^{2+}$-containing treatments and by MEP, and also by MEP in *P. palmivora* (Tables 3.3,3.8). Dibucaíne is a less effective inhibitor than the other two compounds, as indicated by the relative concentrations required for inhibition of cyst germination. It also might have non-specific effects on other aspects of cellular function, in contrast to the more specific effects of TFP and calmidazolium.

The suppressive effects of caffeine on cyst germination could not be reversed by any treatments in either *P. infestans* or *P. palmivora*. The effects of TMB-8 could be reversed by MEP for both fungi, perhaps indicating a non-specific nutrient triggering effect. In addition, a mixture of glucose plus Ca$^{2+}$ caused some reversal of TMB-8 suppression in *P. infestans*. Whereas treatments containing L-glutamine caused a similar partial reversal of suppression in *P. palmivora* (tables 3.5, 3.10).

Overall, the results in this section demonstrate that zoospore cysts of *P. infestans* are affected by a similar range of calcium-mediating treatments as are zoospore cysts of *P. palmivora* and of other Oomycota that have been studied previously (Donaldson and Deacon, 1992, 1993 a,b; Von Broembsen and Deacon 1996; Deacon and Saxena, 1998; Warburton and Deacon, 1998). Also in general the effects of these treatments can be overcome partly or completely by a range of post-treatments, but with one important difference; the cysts of *P. palmivora* sometimes respond to individual nutrient triggers such as L-glu or L-asn whereas the cysts of *P. infestans* never responded to these compounds. This could be an important difference, perhaps related to differences in membrane-located nutrient receptors in these two fungal species.
Chapter Four

The effects of Ca\textsuperscript{2+} modulators, cations, orthophosphates and pectin on the germination of *Phytophthora* sporangia.
4.1 Introduction

Although zoospores are the infective propagule of potato late blight it is actually the sporangia that provide the principal means of dissemination. The sporangia of *P. infestans* can germinate either directly via a germ tube, or indirectly via zoospores, with the mode of germination being mainly determined by temperature (Crosier, 1934)

It appears that zoosporogenesis is the default mechanism of germination, with essential zoospore features such as flagella being pre-formed even before a sporangial germination trigger is received (Hemmes and Hohl, 1969). Direct germination only seems to occur when the sporangia encounter specific conditions inimical to zoospore release.

Zoospores of *P. infestans* can readily be disrupted by a range of Ca^{2+} modulators and divalent cations. The inhibition of cyst germination is most effective when the treatments are added just after encystment, and the effect diminishes with time of addition of treatment after encystment (Chapter 3). The effectiveness of the treatments could also be overcome by the addition of a range of post-treatments that were able to rescue germination. The efficacy of various rescue treatments was dependent upon the type of modulator that the cyst had been exposed to.

The experiments in this chapter were designed to investigate the effectiveness of altering the sporangial Ca^{2+} environment by the addition of Ca^{2+} modulators and other treatments in order to block sporangial germination. The experiments were designed not only to see if modulators had an effect, but also to study the durability of the effect, by varying the time of addition of treatments and also by adding appropriate post-treatments.
4.2 The effect of varying concentrations of Ca\(^{2+}\) modulators, orthophosphates, cations and pectin on the indirect germination of *P. infestans*.

Three isolates of *P. infestans* were used, *P. infestans* EUPi1, *P. infestans* 81/1/1 and *P. infestans* Ei3\(3\)i2. The sporangia were prepared as described in section 2.1 and the experiments were carried out as described in section 2.9.1. It was essential to ensure that freshly prepared sporangia were used for each experiment and that the treatments were added at a standardised 10 minutes after the sporangial suspension was prepared.

As shown in Figure 4.1, amiloride significantly inhibited the indirect (zoosporic) germination of *P. infestans* EUPi1 sporangia, with germination being reduced to 5% of the control level at amiloride concentrations of 600\(\mu\)M or above. The chelator BAPTA significantly also inhibited the indirect germination of *P. infestans* EUPi1 at concentrations in excess of 250 \(\mu\)M (Fig 4.2) and completely blocked germination at concentrations of 3\(m\)M or above.

Caffeine, significantly reduced the level of indirect germination of *P. infestans* EUPi1 at concentrations in excess of 100\(\mu\)M (Fig 4.3). Germination is reduced to 0% at caffeine concentrations of 4\(m\)M or above.

The calmodulin antagonists TFP (Fig 4.4) and dibucaine (Fig 4.5) both inhibited the indirect germination of *P. infestans* EUPi1 at micromolar levels. TFP completely blocked germination at 20\(\mu\)M whereas a dibucaine concentration of 250\(\mu\)M was required to achieve the same effect.

The Ca\(^{2+}\) channel blocker La\(^{3+}\) significantly inhibited indirect germination of *P. infestans* EUPi1 (Fig 4.6) at concentrations as low as 25\(\mu\)M, although La\(^{3+}\) concentrations of 1\(m\)M were required too completely inhibit germination. The channel blocker Gd\(^{3+}\), also significantly reduced indirect germination of *P. infestans* EUPi1 at

Continued on page 141
Figure 4.1 The effect of varying concentrations of amiloride on indirect germination of sporangia of *P. infestans* strains EUPi1.

Data are means ± se for at least three replicate treatments, based on counts of at least 100 sporangia per treatment. Germination was assessed microscopically after 16h incubation.
Figure 4.2 The effect of varying concentrations of BAPTA on indirect germination of sporangia of *P. infestans* strain EUPi1.

Data are means ± se for at least three replicate treatments, based on counts of at least 100 sporangia per treatment. Germination was assessed microscopically after 16h incubation.
Figure 4.3 The effect of varying concentrations of caffeine on indirect germination of sporangia of *P. infestans* strain EUPi1.

Data are means ± se for at least three replicate treatments, based on counts of at least 100 sporangia per treatment. Germination was assessed microscopically after 16h incubation.
Figure 4.4 The effect of varying concentrations of trifluoperazine on indirect germination of sporangia of *P. infestans* strain EUPi1.

Data are means ± se for at least three replicate treatments, based on counts of at least 100 sporangia per treatment. Germination was assessed microscopically after 16h incubation.
Figure 4.5 The effect of varying concentrations of Dibucaine on indirect germination of sporangia of *P. infestans* strain EUPi1.

Data are means ± se for at least three replicate treatments, based on counts of at least 100 sporangia per treatment. Germination was assessed microscopically after 16h incubation.
Figure 4.6 The effect of varying concentrations of \( \text{La}^{3+} \) on indirect germination of sporangia of \( P. \text{infestans} \) strain EUPi1.

Data are means ± se for at least three replicate treatments, based on counts of at least 100 sporangia per treatment. Germination was assessed microscopically after 16h incubation.
Figure 4.7 The effect of varying concentrations of Gd$^{3+}$ on indirect germination of sporangia of *P. infestans* strain EUPi1.

Data are means ± se for at least three replicate treatments, based on counts of at least 100 sporangia per treatment. Germination was assessed microscopically after 16h incubation.
25µM, and completely blocked germination at concentrations in excess of 800µM (Figure 4.7). The effect of the chelator EDTA on indirect germination of three isolates of *P. infestans* was investigated. (Fig 4.8-4.10). The same general trend was apparent for each isolate, with increases in EDTA concentration causing a reduction in the sporangial germination, and germination being completely inhibited at the higher concentrations. However, the concentration at which complete inhibition was achieved varied from 2mM EDTA (isolates 81/1/1 and E131A2) to 10mM (isolate EUPi1). The chelator EGTA was also applied to sporangia of the three isolates (Fig 4.11-4.13). A similar trend was observed to that seen with the addition of EDTA. Both *P. infestans* 81/1/1 and E131A2 were completely inhibited by chelator concentrations in excess of 2mM whereas a concentration in excess of 10mM was required for complete inhibition of *P. infestans* isolate EUPi1.

Di-sodium hydrogen orthophosphate also had similar effects on all three isolates of (Fig 4.14-4.16). As the concentration of orthophosphate was increased, so the indirect germination of all three isolates was reduced; concentrations of 20mM or above reduced germination to less than 5%. Only *P. infestans* isolate 81/1/1 was completely inhibited by any of the orthophosphate concentrations used (50mM) although germination of both *P. infestans* EUPi1 and 81/1/1 was reduced to less than 1% at this concentration.

Sodium di-hydrogen orthophosphate produced broadly similar inhibitory effects to di-sodium hydrogen orthophosphate for all three isolates (Fig 4.17-4.19). However, when 2mM NaH2PO4 was added to *P. infestans* isolate 81/1/1 a significant increase in germination was observed (LSD 6.6). The addition of 5mM orthophosphate gave germination equivalent to the control levels.

Citrus pectin was inhibitory to indirect germination of all three isolates of *P.* (Figures 4.20-4.22). Increasing pectin concentrations reduced the germination, and 0.2% pectin completely inhibited germination. Orthophosphates and pectin were used as they are naturally occurring compounds that have the ability to chelate Ca2+. As
these compounds are often associated with plants it is unlikely that they will prove to be phytotoxic.

\[ \text{Ba}^{2+} \] at 2mM or above significantly inhibited germination of \textit{P. infestans} EUPi1 (Fig 4.23) but 20mM \text{Ba}^{2+} was required for complete inhibition. In contrast, isolate 81/1/1 was completely inhibited by 2mM \text{Ba}^{2+} (Fig 4.24)

\[ \text{Ca}^{2+} \] completely inhibited germination of \textit{P. infestans} EUPi1 at 10mM concentration (Fig 2.25). \textit{P. infestans} isolate 81/1/1 was significantly inhibited by \text{Ca}^{2+} concentrations of 2mM or above (Fig 4.26)

The addition of \text{Mg}^{2+} to \textit{P. infestans} EUPi1 (Fig 4.27) or 81/1/1 (Fig 4.28) resulted in a concentration dependent inhibition of germination, with complete inhibition at \text{Mg}^{2+} concentrations of 10mM or above.
Figure 4.8 The effect of varying concentrations of EDTA on indirect germination of sporangia of *P. infestans* strain EUPi1.

Data are means ± se for at least three replicate treatments, based on counts of at least 100 sporangia per treatment. Germination was assessed microscopically after 16h incubation.
Figure 4.9 The effect of varying concentrations of EDTA on indirect germination of sporangia of *P. infestans* strain 81/1/1.

Data are means ± se for at least three replicate treatments, based on counts of at least 100 sporangia per treatment. Germination was assessed microscopically after 16h incubation.
Figure 4.10 The effect of varying concentrations of EDTA on indirect germination of sporangia of *P. infestans* strain E$_{131A2}$.

Data are means ± se for at least three replicate treatments, based on counts of at least 100 sporangia per treatment. Germination was assessed microscopically after 16h incubation.
Figure 4.11 The effect of varying concentrations of EGTA on indirect germination of sporangia of *P. infestans* strain EUP11.

Data are means ± se for at least three replicate treatments, based on counts of at least 100 sporangia per treatment. Germination was assessed microscopically after 16h incubation.
Figure 4.12 The effect of varying concentrations of EGTA on indirect germination of sporangia of *P. infestans* strain 81/1/1.

Data are means ± se for at least three replicate treatments, based on counts of at least 100 sporangia per treatment. Germination was assessed microscopically after 16h incubation.
Figure 4.13 The effect of varying concentrations of EGTA on indirect germination of sporangia of *P. infestans* strain E_{131A2}.

Data are means ± se for at least three replicate treatments, based on counts of at least 100 sporangia per treatment. Germination was assessed microscopically after 16h incubation.
Figure 4.14 The effect of varying concentrations of Na$_2$HPO$_4$ on indirect germination of sporangia of *P. infestans* strain EUPi1.

Data are means ± se for at least three replicate treatments, based on counts of at least 100 sporangia per treatment. Germination was assessed microscopically after 16h incubation.
Figure 4.15 The effect of varying concentrations of Na$_2$HPO$_4$ on indirect germination of sporangia of *P. infestans* strain 81/1/1.

Data are means ± se for at least three replicate treatments, based on counts of at least 100 sporangia per treatment. Germination was assessed microscopically after 16h incubation.
Figure 4.16 The effect of varying concentrations of Na$_2$HPO$_4$ on the indirect germination of *P. infestans* $E_{13}A_2$.

Data are means ± se for at least three replicate treatments with at least 100 sporangia per treatment being scored. Germination was assessed microscopically after 16h incubation.
Figure 4.17 The effect of varying concentrations of NaH$_2$PO$_4$ on the indirect germination of *P. infestans* EUPi1.

Data are means ± se for at least three replicate treatments with at least 100 sporangia per treatment being scored. Germination was assessed microscopically after 16h incubation.
Figure 4.18 The effect of varying concentrations of NaH$_2$PO$_4$ on the indirect germination of *P. infestans* 81/1/1.

Data are means ± se for at least three replicate treatments with at least 100 sporangia per treatment being scored. Germination was assessed microscopically after 16h incubation.
Figure 4.19 The effect of varying concentrations of NaH$_2$PO$_4$ on the indirect germination of *P. infestans* E$_{131}$A$_2$.

Data are means ± se for at least three replicate treatments with at least 100 sporangia per treatment being scored. Germination was assessed microscopically after 16h incubation.
Figure 4.20 The effect of varying concentrations of pectin on the indirect germination of *P. infestans* EUPi1.

Data are means ± se for at least three replicate treatments with at least 100 sporangia per treatment being scored. Germination was assessed microscopically after 16h incubation.
Figure 4.21 The effect of varying concentrations of pectin on the indirect germination of *P. infestans* 81/1/1.

Data are means ± se for at least three replicate treatments with at least 100 sporangia per treatment being scored. Germination was assessed microscopically after 16h incubation.
Figure 4.22 The effect of varying concentrations of pectin on the indirect germination of *P. infestans* E131A2.

![Graph showing the effect of varying concentrations of pectin on indirect germination](image)

Data are means ± se for at least three replicate treatments with at least 100 sporangia per treatment being scored. Germination was assessed microscopically after 16h incubation.
Figure 4.23 The effect of varying concentrations of Ba$^{2+}$ on the indirect germination of *P. infestans* EUPi1.

Data are means ± se for at least three replicate treatments with at least 100 sporangia per treatment being scored. Germination was assessed microscopically after 16h incubation.
Figure 4.24 The effect of varying concentrations of Ba\(^{2+}\) on the indirect germination of *P. infestans* 81/1/1.

Data are means ± se for at least three replicate treatments with at least 100 sporangia per treatment being scored. Germination was assessed microscopically after 16h incubation.
Figure 4.25 The effect of varying concentrations of \(\text{Ca}^{2+}\) on the indirect germination of \textit{P. infestans} EUPi1.

Data are means ± se for at least three replicate treatments with at least 100 sporangia per treatment being scored. Germination was assessed microscopically after 16h incubation.
Figure 4.26 The effect of varying concentrations of Ca$^{2+}$ on the indirect germination of *P. infestans* 81/1/1.

![Graph showing the effect of varying Ca$^{2+}$ concentrations on germination percent]  

Data are means ± se for at least three replicate treatments with at least 100 sporangia per treatment being scored. Germination was assessed microscopically after 16h incubation.
Figure 4.27 The effect of varying concentrations of Mg\textsuperscript{2+} on the indirect germination of *P. infestans* EUPII.

Data are means ± se for at least three replicate treatments with at least 100 sporangia per treatment being scored. Germination was assessed microscopically after 16h incubation.
Figure 4.28 The effect of varying concentrations of Mg$^{2+}$ on the indirect germination of *P. infestans* 81/1/1.

Data are means ± se for at least three replicate treatments with at least 100 sporangia per treatment being scored. Germination was assessed microscopically after 16h incubation.
4.3 The effect of time of addition of chelators, cations and orthophosphates on the indirect germination of *P. infestans* isolate EUPi1.

Some of the inhibitory treatments investigated earlier in this chapter (section 4.2) were applied to sporangial suspensions at different times, using the method detailed in section 2.9.2

Both Ba$^{2+}$ (10mM) and Ca$^{2+}$ (10mM) were strongly inhibitory to indirect germination of sporangia of *P. infestans* EUPi1 when added at any time between 5 and 60 minutes after the sporangial suspension was prepared (Fig 3.29 and 3.30). This was also true for EGTA (2mM), EDTA (2mM), Na$_2$HPO$_4$ (20mM) and NaH$_2$PO$_4$ (20mM) (Fig 4.31-4.34).
Figure 4.29 The effect of time of addition of 10mM Ba$^{2+}$ on indirect germination of sporangia of *P. infestans* strain EUPi1.

Data are means ± se for at least three replicate treatments, based on counts of at least 100 sporangia per treatment. Germination was assessed microscopically after 16h incubation.
Figure 4.30 The effect of time of addition of 10mM Ca\(^{2+}\) on indirect germination of sporangia of *P. infestans* strain EUPi1.

Data are means ± se for at least three replicate treatments, based on counts of at least 100 sporangia per treatment. Germination was assessed microscopically after 16h incubation.
Figure 4.32 The effect of time of addition of 2mM EDTA on indirect germination of sporangia of *P. infestans* strain EUPi1.

Data are means ± se for at least three replicate treatments, based on counts of at least 100 sporangia per treatment. Germination was assessed microscopically after 16h incubation.
Figure 4.31 The effect of time of addition of 2mM EGTA on indirect germination of sporangia of *P. infestans* strain EUPi1.

Data are means ± se for at least three replicate treatments, based on counts of at least 100 sporangia per treatment. Germination was assessed microscopically after 16h incubation.
Figure 4.32 The effect of time of addition of 20mM Na$_2$HPO$_4$ on indirect germination of sporangia of *P. infestans* strain EUPi1.

Data are means ± se for at least three replicate treatments, based on counts of at least 100 sporangia per treatment. Germination was assessed microscopically after 16h incubation.
Figure 4.33 The effect of time of addition of 20mM NaH$_2$PO$_4$ on indirect germination of sporangia of *P. infestans* strain EUPi1.

Data are means ± se for at least three replicate treatments, based on counts of at least 100 sporangia per treatment. Germination was assessed microscopically after 16h incubation.
4.4 The effect of Ca\textsuperscript{2+} modulators, cations and orthophosphates on the direct germination of *P. infestans* strain EUPi1 and *P. palmivora*

Although indirect germination results in the majority of infections in the potato / pathosystem direct germination can lead to the infection of potato tubers via lenticel. So, the effects of treatments on direct germination were studied, by the methods in sections 2.91 and 2.9.4. Only one isolate of *P. infestans* (EUPi1) was used as this was the only isolate that could be induced to germinate directly. As shown in Figure 4.35 it showed the highest percentage of direct germination in suspensions incubated at about 22°C, whereas it showed maximal indirect germination at about 12°C. So 20-22°C was used for all experiments on direct germination.

Amiloride strongly reduced direct germination at concentrations as low as 25μM (Fig 4.36), at completely inhibited germination at 200μM or above. Caffeine reduced germination at 250μM, and completely inhibited germination at 5mM concentration (Fig 4.42)

Both Gd\textsuperscript{3+} (Fig 4.38) and La\textsuperscript{3+} (Fig 4.39) reduced germination at a concentration as low as 10μM, and blocked germination at 200μM

The chelators BAPTA (Fig 4.40), EDTA (Fig 4.41) and EGTA (Fig 4.42) also reduced germination, but concentrations of 2mM or higher were required for complete inhibition. EGTA significantly reduced the direct germination of *P. palmivora* (Fig 4.43) at concentrations of 2mM or above, although even at the highest concentration tested here (10mM) germination is not completely inhibited.

Both the calmodulin antagonists TFP (Fig 4.44) and dibucaine (Fig 4.45) caused a concentration dependent inhibition of germination. In *P. infestans* EUPi1 a TFP concentration of 40μM was sufficient to completely block germination whereas dibucaine required a final concentration of 250μM to achieve the same effect.

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Figure 4.35 Temperature profile for direct and indirect germination of sporangia *P. infestans* strain EUPi1

Data are means ± se for at least three replicate treatments, based on counts of at least 100 sporangia per treatment. Germination was assessed microscopically after 16h incubation.
Figure 4.36 The effect of varying concentrations of amiloride on direct germination of sporangia of *P. infestans* strain EUPi1.

Data are means ± se for at least three replicate treatments, based on counts of at least 100 sporangia per treatment. Germination was assessed microscopically after 16h incubation.
Figure 4.37 The effect of varying concentrations of caffeine on direct germination of sporangia of *P. infestans* strain EUPi1.

Data are means ± se for at least three replicate treatments, based on counts of at least 100 sporangia per treatment. Germination was assessed microscopically after 16h incubation.
Figure 4.38 The effect of varying concentrations of Gd$^{3+}$ on direct germination of sporangia of *P. infestans* strain EUPi1.

Data are means ± se for at least three replicate treatments, based on counts of at least 100 sporangia per treatment. Germination was assessed microscopically after 16h incubation.
Figure 4.39 The effect of varying concentrations of La\(^{3+}\) on direct germination of sporangia of *P. infestans* strain EUPil.

Data are means ± se for at least three replicate treatments, based on counts of at least 100 sporangia per treatment. Germination was assessed microscopically after 16h incubation.
Figure 4.40 The effect of varying concentrations of BAPTA on direct germination of sporangia of *P. infestans* strain EUPi1.

Data are means ± se for at least three replicate treatments, based on counts of at least 100 sporangia per treatment. Germination was assessed microscopically after 16h incubation.
Figure 4.41 The effect of varying concentrations of EDTA on direct germination of sporangia of *P. infestans* strain EUPi1.

Data are means ± se for at least three replicate treatments, based on counts of at least 100 sporangia per treatment. Germination was assessed microscopically after 16h incubation.
Figure 4.42 The effect of varying concentrations of EGTA on direct germination of sporangia of *P. infestans* strain EUPi1.

Data are means ± se for at least three replicate treatments, based on counts of at least 100 sporangia per treatment. Germination was assessed microscopically after 16h incubation.
Figure 4.43 The effect of varying concentrations of EGTA on direct germination of sporangia of *P. palmivora*.

Data are means ± se for at least three replicate treatments, based on counts of at least 100 sporangia per treatment. Germination was assessed microscopically after 16h incubation.
Figure 4.44 The effect of varying concentrations of TFP on direct germination of sporangia of *P. infestans* EUPi1.

Data are means ± se for at least three replicate treatments, based on counts of at least 100 sporangia per treatment. Germination was assessed microscopically after 16h incubation.
Figure 4.45 The effect of varying concentrations of dibucaine on direct germination of sporangia of *P. infestans* strain EUPi1.

Data are means ± se for at least three replicate treatments, based on counts of at least 100 sporangia per treatment. Germination was assessed microscopically after 16h incubation.
The divalent cations Ca\(^{2+}\) (Fig 4.46), Ba\(^{2+}\) (Fig 4.47) and Mg\(^{2+}\) (Fig 4.48) have strikingly different effects on sporangia of *P. infestans* EUPi1. Only Ba\(^{2+}\) showed any significant effect on direct germination (*P* = 3.09\(^{-11}\) 5% LSD = 4.5). Barium concentrations as low as 2mM caused a significant reduction in germination. In contrast a small (but significant) effect of Ca\(^{2+}\) was only seen at 10mM concentration. Mg\(^{2+}\) also caused a significant reduction of germination at 4mM concentration, but higher concentrations, up to 10mM, had no further effects.

Na\(_2\)HPO\(_4\) caused a strong, concentration-dependent reduction of direct germination of sporangia of *P. infestans* EUPi1 (Fig 4.49). Concentrations as low as 2mM were sufficient to reduce significantly germination below that of the control, and concentrations above 10mM almost completely inhibited germination. NaH\(_2\)PO\(_4\) had similar effects (Fig 4.50). However the same treatments applied to sporangia of *P. palmivora* (Fig 4.51, 4.52) were much less effective: only 50mM levels achieved a significant reduction in direct germination and even at this concentration 18% - 33% of the sporangia are able to germinate.

The addition of pectin to *P. infestans* EUPi1 (Fig 4.53) produced a concentration-dependent reduction in germination, with concentrations as low as 0.001% having a significant effect. Germination was completely inhibited by 0.1-0.2% pectin.

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Figure 4.46 The effect of varying concentrations of Ca$^{2+}$ on direct germination of sporangia of *P. infestans* strain EUPi1.

Data are means ± se for at least three replicate treatments, based on counts of at least 100 sporangia per treatment. Germination was assessed microscopically after 16h incubation.
Figure 4.47 The effect of varying concentrations of Ba\(^{2+}\) on direct germination of sporangia of *P. infestans* EUPi1.

Data are means ± se for at least three replicate treatments, based on counts of at least 100 sporangia per treatment. Germination was assessed microscopically after 16h incubation.
Figure 4.48 The effect of varying concentrations of $\text{Mg}^{2+}$ on direct germination of sporangia of \textit{P. infestans} strain EUPi1.

Data are means ± se for at least three replicate treatments, based on counts of at least 100 sporangia per treatment. Germination was assessed microscopically after 16h incubation.
Figure 4.49 The effect of varying concentrations of Na$_2$HPO$_4$ on direct germination of sporangia of *P. infestans* strain EUPi1.

Data are means ± se for at least three replicate treatments, based on counts of at least 100 sporangia per treatment. Germination was assessed microscopically after 16h incubation.
Figure 4.50 The effect of varying concentrations of NaH$_2$PO$_4$ on direct germination of sporangia of *P. infestans* strain EUPI1.

Data are means ± se for at least three replicate treatments, based on counts of at least 100 sporangia per treatment. Germination was assessed microscopically after 16h incubation.
Figure 4.51 The effect of varying concentrations of Na$_2$HPO$_4$ on direct germination of sporangia of *P. palmivora*.

Data are means ± se for at least three replicate treatments, based on counts of at least 100 sporangia per treatment. Germination was assessed microscopically after 16h incubation.
Figure 4.52 The effect of varying concentrations of NaH$_2$PO$_4$ on direct germination of sporangia of *P. palmivora*.

Data are means ± se for at least three replicate treatments, based on counts of at least 100 sporangia per treatment. Germination was assessed microscopically after 16h incubation.
Figure 4.53 The effect of varying concentrations of pectin on direct germination of sporangia of *P. infestans* strain EUPi1.

Data are means ± se for at least three replicate treatments, based on counts of at least 100 sporangia per treatment. Germination was assessed microscopically after 16h incubation.
4.5 The effects of time of addition of treatments on the direct germination of *P. infestans* EUPi1.

For a selected range of treatments (Ba$^{2+}$, NaH$_2$PO$_4$, Na$_2$HPO$_4$ and EDTA), Figures 4.54-4.57 show that a delay of up to one hour in the addition of inhibitory substances was as effective as their early application in inhibiting direct sporangial germination of *P. infestans*. The same effect was seen for EGTA on direct germination of *P. palmivora* sporangia (Fig 4.58).
Figure 4.54 The effect of time of addition of 10mM Ba$^{2+}$ on the direct germination of sporangia of *P. infestans* strain EUPi1 with corresponding SDW controls.

Data are means ± se for at least three replicate treatments, based on counts of at least 100 sporangia per treatment. Germination was assessed microscopically after 16h incubation.
Figure 4.55 The effect of time of addition of 20mM NaH$_2$PO$_4$ on direct germination of sporangia of *P. infestans* strain EUPi1 with corresponding SDW controls.

Data are means ± se for at least three replicate treatments, based on counts of at least 100 sporangia per treatment. Germination was assessed microscopically after 16h incubation.
Figure 4.56 The effect of time of addition of 20mM Na$_2$HPO$_4$ on direct germination of sporangia of *P. infestans* EUPi1 with corresponding SDW controls.

Data are means ± se for at least three replicate treatments, based on counts of at least 100 sporangia per treatment. Germination was assessed microscopically after 16h incubation.
Figure 4.57 The effect of time of addition of 2mM EDTA on direct germination of sporangia of *P. infestans* strain EUPi1 with corresponding SDW controls.

Data are means ± se for at least three replicate treatments, based on counts of at least 100 sporangia per treatment. Germination was assessed microscopically after 16h incubation.
Figure 4.58 The effect of time of addition of 10mM EGTA on direct germination of sporangia of *P. palmivora* with corresponding SDW controls.

Data are means ± se for at least three replicate treatments, based on counts of at least 100 sporangia per treatment. Germination was assessed microscopically after 16h incubation.
4.6 The effect of cation post-treatments on the germination of sporangia of *P. infestans* pre-treated with pectin, EGTA or orthophosphate.

Sporangia of three isolates of *P. infestans* were pre-treated with pectin, EGTA or NaH$_2$PO$_4$, then post-treated with 10mM Ca$^{2+}$ or Ba$^{2+}$ after different times as described in section 2.9.6

As shown in Table 4.1, 0.05% pectin significantly reduced sporangial germination of all three isolates of *P. infestans*. This was true for both direct and indirect germination of isolate EUPi1, but isolates 81/1/1 and Ei3/A2 only germinated indirectly, not by hyphal outgrowth. Ca$^{2+}$ (10mM) or Ba$^{2+}$ (10mM) alone also reduced germination below the levels of the SDW controls, but in several cases the simultaneous addition of Ca$^{2+}$ or Ba$^{2+}$ with pectin caused some reduction of the pectin induced suppression of germination. A delayed addition of Ca$^{2+}$ or Ba$^{2+}$ to pectin-treated sporangia had little or no effect in overcoming the pectin-induced suppression.

EGTA (2mM) strongly suppressed germination by all three isolates of *P. infestans* (Table 4.2). This suppression was partly overcome by simultaneous addition of Ca$^{2+}$ (10mM) and sometimes by simultaneous addition of Ba$^{2+}$ (10mM). A smaller reversal of EGTA induced suppression was sometimes achieved by adding Ca$^{2+}$ or Ba$^{2+}$ 15 minutes after EGTA was applied. However there was no significant effect of Ca$^{2+}$ or Ba$^{2+}$ added 30 minutes after EGTA.

Essentially similar results to those above were found when NaH$_2$PO$_4$ (20mM) was applied to sporangia (Table 4.3). This caused strong suppression of germination by all three isolates, and the suppression could be partially overcome by addition of Ca$^{2+}$ or Ba$^{2+}$ in the early stages of incubation.

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Table 4.1: The effects of divalent cation post-treatment (10mM) on germination of pectin (0.05%) pre-treated sporangia of three isolates of *P. infestans* (EUPi; 81/1/1; E3-A2).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Isolate EUPi1 Direct germination</th>
<th>Isolate EUPi1 indirect germination</th>
<th>Isolate 81/1/1 indirect germination</th>
<th>Isolate E131A2 indirect germination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SDW</td>
<td>Pectin</td>
<td>Pectin + Ca²⁺ simultaneous</td>
<td>Pectin + Ca²⁺ 15 min</td>
</tr>
<tr>
<td>SDW</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.8</td>
<td>3.6</td>
<td>5.9*</td>
<td>12.6*</td>
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<td></td>
<td>1.7</td>
<td>0.5</td>
<td>2.7</td>
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</tr>
</tbody>
</table>

Data are means ± se for at least three replicates, based on assessment of at least 100 sporangia per replicate. Germination was assessed microscopically after 16h incubation; * denotes a significant difference from treatment with pectin alone.

Data from: Table 4.1. The effects of divalent cation post-treatment (10mM) on germination of pectin (0.05%) pre-treated sporangia of three isolates of *P. infestans*.
Table 4.2: The effects of divalent cation post-treatments (10mM) on the germination of EGTA (2mM) pre-treated sporangia of three isolates of *P. infestans* (EUPil; 81/1/1; E3/A2).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Isolate EUPil Direct germination</th>
<th>Isolate EUPil indirect germination</th>
<th>Isolate 81/1/1 indirect germination</th>
<th>Isolate E3/A2 indirect germination</th>
</tr>
</thead>
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<tr>
<td>SDW</td>
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<td>34.8</td>
<td>41.8</td>
<td>46.2</td>
</tr>
<tr>
<td>EGTA</td>
<td>3.7</td>
<td>2.8</td>
<td>1.7</td>
<td>0.7</td>
</tr>
<tr>
<td>Ca(^{2+}) simultaneous</td>
<td>18.3</td>
<td>9.5</td>
<td>3.0</td>
<td>1.4</td>
</tr>
<tr>
<td>EGTA + Ca(^{2+}) 15 min</td>
<td>10.8*</td>
<td>10.1*</td>
<td>4.3*</td>
<td>3.2*</td>
</tr>
<tr>
<td>EGTA + Ba(^{2+}) simultaneous</td>
<td>6.4</td>
<td>8.3</td>
<td>4.9</td>
<td>6.7</td>
</tr>
<tr>
<td>EGTA + Ba(^{2+}) 15 min</td>
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<td>7.0</td>
<td>6.9</td>
<td>7.0</td>
</tr>
<tr>
<td>EGTA + Ba(^{2+}) 30 min</td>
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<td>6.4</td>
<td>7.0</td>
<td>7.0</td>
</tr>
<tr>
<td>EGTA + Ba(^{2+}) 45 min</td>
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<td>6.4</td>
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<td>7.0</td>
</tr>
<tr>
<td>LSD 3.2</td>
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<td>2.8</td>
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<tr>
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<tr>
<td>LSD 1.9</td>
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Note: Data are means ± se for at least three replicates, based on assessment of at least 100 sporangia per replicate. Germination was assessed microscopically after 16h incubation. * denotes a significant difference from treatment with EGTA alone.
Table 4.3: The effects of divalent cation post-treatments (10mM) on the germination of NaH₂PO₄ (20mM) pre-treated sporangia of three isolates of *P. infestans* (EUPi1; 81/1/1; E131A2).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Isolate EUPi1 direct germination</th>
<th>Isolate EUPi1 indirect germination</th>
<th>Isolate 81/1/1 indirect germination</th>
<th>Isolate E131A2 indirect germination</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDW</td>
<td>34.8</td>
<td>3.5</td>
<td>17.4</td>
<td>13.5*</td>
</tr>
<tr>
<td>NaH₂PO₄</td>
<td>3.5</td>
<td>2.4</td>
<td>1.9</td>
<td>1.5</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>17.4</td>
<td>6.6</td>
<td>3.4</td>
<td>2.4</td>
</tr>
<tr>
<td>NaH₂PO₄ + Ca²⁺ simultaneous</td>
<td>10.3*</td>
<td>6.6</td>
<td>3.4</td>
<td>2.4</td>
</tr>
<tr>
<td>NaH₂PO₄ + Ca²⁺ 15 min</td>
<td>6.6</td>
<td>3.4</td>
<td>2.4</td>
<td>2.4</td>
</tr>
<tr>
<td>NaH₂PO₄ + Ba²⁺ simultaneous</td>
<td>10.3*</td>
<td>6.6</td>
<td>3.4</td>
<td>2.4</td>
</tr>
<tr>
<td>NaH₂PO₄ + Ba²⁺ 15 min</td>
<td>6.6</td>
<td>3.4</td>
<td>2.4</td>
<td>2.4</td>
</tr>
</tbody>
</table>

LSD 6.4 are means ± se for at least three replicates, based on assessment of at least 100 sporangia per replicate. Germination was assessed microscopically after 16h incubation. * denotes a significant difference from treatment with NaH₂PO₄ alone.
4.7 Assessment of sporangial viability of *P. infestans* after treatment with pectin, other uronates, inorganic phosphate, ion chelators and other calcium-modulating substances

Experiments was done to assess the reduction of sporangial viability in response to treatments that were found to suppress sporangial germination. Full details are given in the publication (Hill, Grayson & Deacon, 1998) appended to this thesis. The work was done in conjunction with A. E. Hill, an undergraduate student working in the laboratory of Dr J. W. Deacon. The author’s (D. Grayson’s) contribution to this work was to supervise the design and execution of the experiments, and to contribute to the analysis and publication of the results.

Sporangial viability was assessed by the number of sporangia with coagulated or disrupted contents, in contrast to healthy sporangia which exhibited normal protoplasmic fluidity. These assessments were made at x700 magnification, using a video camera attachment to the microscope [Jones, Donaldson and Deacon, 1991] and slides with strips of tape to prevent the coverslip from crushing the sporangia.

The treatments for the various experiments included EGTA, BAPTA, citrus pectin (Sigma), sodium alginate (BDH Chemicals), D-galacturonic acid (Sigma), Na₂HPO₄, LaCl₃ (both BDH Chemicals), trifluoperazine, verapamil and caffeine (all ICN Biomedicals). All the experiments were performed on sporangia of *P. infestans* isolate EUP1, incubated at either 12°C (for indirect germination) or 20°C (for direct germination).

4.7.1. Effects of trifluoperazine, lanthanum and verapamil

Sporangia incubated at either 12° or 20° with La³⁺ (1 mM), verapamil (30 μM) or TFP (5 μM) were sampled periodically for microscopic assessment (Figure 4.59; Fig. 3 in Hill *et al.*, 1998). In all cases a significant proportion of sporangia were assessed as being irreversibly damaged after incubation for 30 or 45 minutes, whereas fewer than 10% of non-treated sporangia showed damage at any time.
The data in Figure 4.59, when transformed to probits of cell death against logarithm of incubation time (+ 1 min), gave highly significant straight-line relationships, with estimated times for 50% sporangial death ranging from 20-30 min (TFP) to an extrapolated value of more than 3 h for La$^{3+}$ at 20°C (Table 4.4; Table 2 in Hill et al., 1998).

4.7.2. Effects of pectin, other uronates, Na$_2$HPO$_4$, EGTA and BAPTA

The effects of pectin (0.1%), sodium alginate (0.1%), D-galacturonic acid (0.1%), Na$_2$HPO$_4$ (5 mM), EGTA (5 mM) and BAPTA (5 mM) on sporangial death were compared by periodic sampling of sporangia incubated at 12° and 20° (Figure 4.60; Fig. 5 in Hill et al., 1998). Control sporangia incubated in water showed consistently low death (usually < 10%), whereas all the treated sporangia exhibited at least 37% death after 120 min. However, there were major differences between the treatments, and there was evidence of interaction between treatments and temperature of incubation. At 20° (conducive to direct germination) pectin and BAPTA caused rapid onset of cell death, whereas EGTA, Na$_2$HPO$_4$, D-galacturonic acid and sodium alginate caused less death. At 12°C (conducive to indirect germination) pectin, BAPTA, EGTA and Na$_2$HPO$_4$ all caused rapid cell death, but D-galacturonic acid and sodium alginate caused less death.

The estimated times for 50% sporangial death derived from log (time + 1 min)/ probit plots (Table 4.5; Table 6 in Hill et al., 1998) show that, except for pectin and BAPTA which were the most toxic treatments, sporangia were more sensitive to disruption by treatments at 12° than at 20°. In other words, sporangia were more sensitive to disruption when induced to undergo cytoplasmic cleavage than to germinate by hyphal outgrowth.
Table 4.4. Regressions of straight-line plots of probit (percent death of sporangia) against log \( (\text{min} + 1) \) for the curves in Figure 4.59, with estimated times \((\text{min} + 1)\) for 50% death (probit value 5) of sporangia caused by calcium-modulators.

<table>
<thead>
<tr>
<th></th>
<th>Correlation coefficient</th>
<th>Probability of regression (P)</th>
<th>Regression equation</th>
<th>Estimated time (min + 1) for 50% death</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Direct germination</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lanthanum</td>
<td>0.955</td>
<td>6.25 \times 10^{-5}</td>
<td>( y = 0.760x + 3.276 )</td>
<td>185.5*</td>
</tr>
<tr>
<td>Verapamil</td>
<td>0.955</td>
<td>6.31 \times 10^{-5}</td>
<td>( y = 1.140x + 3.084 )</td>
<td>47.9</td>
</tr>
<tr>
<td>Trifluoperazine</td>
<td>0.951</td>
<td>7.95 \times 10^{-5}</td>
<td>( y = 1.219x + 3.167 )</td>
<td>31.9</td>
</tr>
<tr>
<td><strong>Indirect germination</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lanthanum</td>
<td>0.942</td>
<td>1.46 \times 10^{-4}</td>
<td>( y = 1.341x + 2.318 )</td>
<td>109.9</td>
</tr>
<tr>
<td>Verapamil</td>
<td>0.947</td>
<td>1.08 \times 10^{-4}</td>
<td>( y = 1.090x + 2.798 )</td>
<td>104.8</td>
</tr>
<tr>
<td>Trifluoperazine</td>
<td>0.986</td>
<td>1.07 \times 10^{-6}</td>
<td>( y = 1.376x + 3.160 )</td>
<td>21.7</td>
</tr>
</tbody>
</table>

* extrapolated
Table 4.5. Regressions of straight-line plots of probit (percent death of sporangia) against log (min + 1) for the curves in Figure 4.60, with estimated times (min + 1) for 50% death (probit value 5) of sporangia caused by treatments.

<table>
<thead>
<tr>
<th>Direct germination</th>
<th>Correlation coefficient</th>
<th>Probability of regression (P)</th>
<th>Regression equation</th>
<th>Estimated time (min + 1) for 50% death</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGTA</td>
<td>0.959</td>
<td>$4.54 \times 10^{-5}$</td>
<td>$y = 1.062x + 2.727$</td>
<td>138*</td>
</tr>
<tr>
<td>Alginate</td>
<td>0.945</td>
<td>$1.24 \times 10^{-4}$</td>
<td>$y = 0.394x + 3.848$</td>
<td>839*</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$</td>
<td>0.951</td>
<td>$7.96 \times 10^{-5}$</td>
<td>$y = 0.815x + 3.500$</td>
<td>69</td>
</tr>
<tr>
<td>BAPTA</td>
<td>0.942</td>
<td>$1.44 \times 10^{-4}$</td>
<td>$y = 1.076x + 3.518$</td>
<td>24</td>
</tr>
<tr>
<td>Pectin</td>
<td>0.918</td>
<td>$4.89 \times 10^{-4}$</td>
<td>$y = 1.709x + 2.881$</td>
<td>17</td>
</tr>
<tr>
<td>D-Galacturonate</td>
<td>0.838</td>
<td>$4.77 \times 10^{-3}$</td>
<td>$y = 0.548x + 3.604$</td>
<td>353*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Indirect germination</th>
<th>Correlation coefficient</th>
<th>Probability of regression (P)</th>
<th>Regression equation</th>
<th>Estimated time (min + 1) for 50% death</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGTA</td>
<td>0.882</td>
<td>$1.64 \times 10^{-4}$</td>
<td>$y = 0.935x + 4.100$</td>
<td>9</td>
</tr>
<tr>
<td>Alginate</td>
<td>0.991</td>
<td>$2.52 \times 10^{-7}$</td>
<td>$y = 1.526x + 2.022$</td>
<td>89</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$</td>
<td>0.946</td>
<td>$1.13 \times 10^{-4}$</td>
<td>$y = 1.383x + 2.993$</td>
<td>28</td>
</tr>
<tr>
<td>BAPTA</td>
<td>0.902</td>
<td>$8.71 \times 10^{-4}$</td>
<td>$y = 1.219x + 3.305$</td>
<td>25</td>
</tr>
<tr>
<td>Pectin</td>
<td>0.929</td>
<td>$2.92 \times 10^{-4}$</td>
<td>$y = 1.217x + 3.607$</td>
<td>14</td>
</tr>
<tr>
<td>D-Galacturonate</td>
<td>0.900</td>
<td>$9.52 \times 10^{-4}$</td>
<td>$y = 0.982x + 2.859$</td>
<td>151*</td>
</tr>
</tbody>
</table>

* Extrapolated
Figure 4.59. Time course of sporangial death caused by the Ca\textsuperscript{2+} modulators, trifluoperazine (TFP, 5 μM), verapamil (Ver, 30 μM) and lanthanum (1 mM), when sporangia were incubated to induce direct germination (20°C) or indirect germination (12°C). Data points are assessments of 100 sporangia at each time.
Figure 4.60. Time course of sporangial death caused by pectin (0.1%), sodium alginate (0.1%), D-galacturonic acid (0.1%), BAPTA (5 mM), EGTA (5 mM) or Na₂HPO₄ (5 mM). Sporangia were incubated to induce direct germination (20°C) or indirect germination (12°C). Data points are assessments of 100 sporangia at each time.

Points are assessments of 100 sporangia at each time.
4.8 Discussion

The experiments in this section investigated the role of Ca$^{2+}$ in the direct and indirect germination of sporangia of *P. infestans* and *P. palmivora*, with particular reference to the effect of Ca$^{2+}$ modulator treatments. There had been little previous work on the effects of calcium on sporangial germination, in contrast to the large volume of work on the role of calcium in zoospore cyst germination of *Phytophthora* and *Pythium* spp. (Grant et al., 1986; Donaldson and Deacon, 1992; Von Broembsen and Deacon, 1997). During the period of this study, Von Broembsen and Deacon (1997) reported that 50mM Ca$^{2+}$ promoted the cleavage of *P. parasitica* sporangia, but at this elevated Ca$^{2+}$ concentration the level of zoospore release from the sporangia was reduced. Also during the course of this study Hill *et al.* (1998) published some of the results of the work presented in this thesis.

Most of the isolates were done with *P. infestans* isolate Eupil, as this was the only isolate that could be induced to undergo both direct, and indirect sporangial germination. In contrast isolates 81/1/1 and Ei3;A2 could only be induced to undergo indirect germination. Temperature had been thought to be the main determinant of the mode of sporangial germination (Crosier, 1934) with indirect germination occurring at lower temperatures and direct germination at higher temperatures. This was confirmed for isolate EUPil (Figure 4.35). Observations by Hemmes and Hohl (1969, 1972) and Clerk (1972) indicated that indirect germination is the default mode for sporangia of *P. palmivora*, in which there is evidence of pre-formed flagella. Nevertheless, *P. palmivora* could be induced to germinate directly. *Phytophthora infestans* isolates 81/1/1 and Ei3;A2 may have lost the ability to germinate directly if this offers no advantage in field conditions.

Insofar as they could be compared for indirect germination, there was no evidence of any difference between the three isolates of *P. infestans* in terms of their responses to various treatments with ions, calcium modulators or other compounds. Also a limited number of experiments with sporangia of *P. palmivora* (Figures 4.43,
4.52, 4.58) showed that these are sensitive to inhibition by the ion chelator, EGTA and by Na$_2$HPO$_4$ or NaH$_2$PO$_4$. However, the concentrations of EGTA required for complete suppression of direct sporangial germination by *P. palmivora* (>10mM; Figure 4.43) was much higher than the levels required to suppress direct germination by *P. infestans* (2mM; Figure 4.42). Similarly the responsiveness of *P. palmivora* to orthophosphates (Figures 4.51, 4.52), was much less than the responsiveness of *P. infestans* (Figures 4.49, 4.50): orthophosphate concentrations as high as 50mM still enabled 15-30% of *P. palmivora* sporangia to germinate, whereas the direct germination of *P. infestans* sporangia was almost completely inhibited by orthophosphate concentrations of 20mM. Grant *et al.* (1992) had previously reported large variations in the inhibition of *Phytophthora* spp. By phosphates and phosphonates in chemically defined media. The variations between *P. infestans* and *P. palmivora* in responses of sporangial germination to different phosphate levels extend these earlier findings.

In the present study (see also Hill *et al.*, 1998) it has been shown that the germination of *P. infestans* sporangia can be suppressed by a wide range of treatments that either directly influence the availability of Ca$^{2+}$ to the cells or that influence calcium-mediated processes. For example the indirect germination of sporangia was strongly suppressed by ion chelators such as BAPTA, EDTA or EGTA (e.g. Figures 4.2, 4.8, 4.11) indicating that the sporangia require at least some calcium to be available in the external environment. However, for indirect germination the sporangia are also sensitive to an elevation of external Ca$^{2+}$ (e.g. Figure 4.25) or of other divalent cations such as Ba$^{2+}$ (figure 4.23) or Mg$^{2+}$ (Figure 4.27). Thus it seems that the external availability of Ca$^{2+}$ or possible surrogate divalent cations must be maintained within defined limits to enable the sporangia to undergo indirect germination.

Indirect germination was also suppressed by the calcium channel blockers La$^{3+}$, Gd$^{3+}$ and verapamil (Figures 4.6, 4.7; Table 1 in Hill *et al.*, 1998), by calmodulin antagonists (e.g. Figures 4.4, 4.5) amiloride (Figure 4.1) and caffeine (Figure 4.3).
indicating a central role for Ca\textsuperscript{2+} fluxes and intracellular Ca\textsuperscript{2+} mediated events in sporangial germination.

The direct germination of \textit{P. infestans} sporangia was also affected by this range of treatments- chelators, amiloride, calcium channel blockers, calmodulin antagonists and caffeine (Figures 4.36-4.45)- at broadly equivalent concentrations to those that suppressed indirect germination. However, supplements of Ca\textsuperscript{2+}, Ba\textsuperscript{2+} and Mg\textsuperscript{2+}, up to 10mM concentration, were much less suppressive to direct germination (Figures 4.46-4.48) than to indirect germination of the same \textit{P. infestans} isolate (Figures 4.25, 4.23, 4.27).

A remarkable feature revealed by this study was that sporangial germination of \textit{P. infestans} could be suppressed by even "mild" chemical treatments such as pectin (e.g. Table 4.1; Table 4 in Hill \textit{et al.,} 1998) and, to a lesser degree, alginate or D-galacturonic acid (Table 4 in Hill \textit{et al.,} 1998). These treatments completely (pectin) or partially suppressed both direct and indirect sporangial germination. They are assumed to have suppressed germination by complexing with external Ca\textsuperscript{2+}, reducing the availability of this ion to the cells. Orthophosphates presumably has the same effect by forming insoluble calcium phosphates in the sporangial suspension.

In attempts to rescue sporangial germination from the effects of these treatments, it was found that Ca\textsuperscript{2+} (Tables 4.1-4.3; Table 4 in Hill \textit{et al.,} 1998) Mg\textsuperscript{2+} (Table 4 in Hill \textit{et al.,} 1998) and Ba\textsuperscript{2+} (Tables 4.1-4.3) could partly overcome the suppression, but only when added simultaneously with the treatments or within 15 minutes of the original treatment. Moreover, rescue from suppression of direct germination was often more easily achieved than was rescue from suppression of indirect germination. This might be explained by the finding that indirect germination occurs faster than direct germination, indirect germination typically reaching the maximum level within 2-3 hours, whereas direct germination was not seen to occur until at least 4 hours. Presumably, the cellular events leading to zoosporogenesis involve an early requirement for Ca\textsuperscript{2+} or for calcium-mediated cellular events. Based
on the use of calcium-sensitive dyes, Jackson and Hardham (1996) reported that a transient rise in cytosolic free calcium is required to induce cytokinesis in the sporangia of *P. cinnamomi*; but it is not known whether this rise in cytosolic free calcium is achieved by $\text{Ca}^{2+}$ uptake or release from intracellular stores.

Perhaps the most surprising feature revealed by the present study is that the sporangia of *P. infestans* are rapidly killed by a range of treatments that might chelate extracellular calcium or block $\text{Ca}^{2+}$ uptake into the sporangia (Figures 3-5, Hill *et al.* 1998). For example, regression analysis of the time course of cell death (Tables 4.4, 4.5) indicated that 50% of sporangia incubated at 20°C (for direct germination) were killed within 17 minutes of the addition of 0.1% pectin, and 50% of sporangia incubated at 12°C were killed within 14 minutes of this treatment. In these circumstances, rapid death could only be averted by simultaneous (or early) addition of divalent cations. However, it was not possible to assess the full effectiveness of the cations in reversing the effects of pectin or other cytotoxic treatments, because the cation supplements alone partly suppress sporangial germination (Tables 4.1-4.3). Further discussion of these points is given in Hill *et al.* (1998).
Chapter 5

The effect of elution on sporangial germination of *P. infestans*.
5.1 Introduction

Sporangia of *P. infestans* have been shown to be sensitive to a wide range of simple chemical treatments that alter normal Ca$^{2+}$ levels and inhibit both direct and indirect germination (Hill et al., 1998). These treatments were generally added at specific times and at elevated concentrations. In the experiments described below, an attempt was made to test the effects of continuous addition of lower concentrations of ions, and of continuous elution with sterile distilled water.

5.2 The effect of elution with SDW and Ca$^{2+}$ on the germination of *P. infestans*

These experiments were carried out as described in section 2.10. They were based on the method originally developed by Hsu and Lockwood (1973) and Lockwood (1977), in which cells are placed on a permeable membrane overlying a bed of continuously leached glass beads. In this way nutrients can be continuously leached from the sporangial environment, or a perpetual supply of nutrients can be supplied to the sporangia. Polycarbonate membrane squares were randomly placed around the inlet pipe of the Petri dish to ensure that they were exposed to the treatments. Replicate experiments were carried out over several days to minimise the impact of variation in membrane placement and culture age. Visualisation of the sporangia on the membrane was difficult, and differentiation between sporangia that had not germinated and those that had undergone zoosporogenesis proved impossible under normal conditions. In order to differentiate between these types of sporangia a dual staining method using Calcoflour and Nile red was used. Sporangia *P. infestans* strain EUPi1 were eluted with SDW either at 12°C or 21°C in order to induce indirect germination or direct germination respectively, and to see if elution with SDW had any effect on germination. A typical sporangium stained with Calcoflour and Nile red but viewed by brightfield microscopy is shown in Figure 5.1. Similar sporangia viewed by fluorescence microscopy to show Calcoflour and Nile red fluorescence are shown in Figure 5.2.
When sporangia of *P. infestans* EUPi1 are eluted for 10, 20 or 30 minutes with SDW (21°C) at a flow rate of 3.2ml min⁻¹ through the underlying glass beads, there was no significant reduction of subsequent sporangial germination, compared with sporangia that were incubated on membranes in the absence of water flow (Figure 5.3). Similarly, there was no significant effect of continuous leaching with water for up to 30 minutes when sporangia were incubated at 12°C (Figure 5.4).

In further experiments, the sporangia were incubated at 12°C, with continuous flow of dilute CaCl₂ solutions for 10, 20 or 30 minutes before the flow was stopped and the sporangia were incubated further to assess germination (Figures 5.5-5.11).

For *P. infestans* strain EUPi1 there was no significant effect of elution with 1μM CaCl₂ (Figure 5.5). But an initial period of elution for 20 or 30 minutes with 10μM CaCl₂ caused a reduction in sporangial germination (Figure 5.6), and even an initial 10 minutes elution with 100μM CaCl₂ or 500μM CaCl₂ caused a marked suppression of sporangial germination (Figures 5.7, 5.8). Two further strains of *P. infestans* (81/1/1; E131A2) showed similar responses to cumulative time of elution and concentration of CaCl₂ (Figures 5.9-5.11).

Continued on page 226
Figure 5.1 A sporangium of *P. infestans* strain EUPi1 which has been stained with Calcoflour and Nile red, viewed using brightfield illumination.

Bar = 10μM
**Figure 5.2** (a) Sporangium showing calcofluor fluorescence of the wall, when viewed using a fluorescence microscope, using ploempak filter block A (U.V. illumination) Bar = 10µM.

**Figure 5.2** (b) Sporangium showing the presence of neutral lipids when stained with Nile red, and viewed under a fluorescence microscope using a ploempak filter block H (colour illumination). Bar = 10µM.
Data are mean ± se for at least 5 replicate treatments. Germination was assessed after 16h incubation at 12°C, with at least 100 sporangia being assessed per replicate treatment.
Figure 5.4 The effect of elution with SDW on the indirect germination of sporangia *P. infestans* EUPi1.

Data are mean ± se for at least 5 replicate treatments. Germination was assessed after 16h incubation at 12°C, with at least 100 sporangia being assessed per replicate treatment.
Figure 5.5 The effect of elution with 1μM Ca$^{2+}$ on the indirect germination of *P. infestans* EUPi1.

Data are mean ± se for at least 5 replicate treatments. Germination was assessed after 16h incubation at 12°C, with at least 100 sporangia being assessed per replicate treatment.
Figure 5.6 The effect of elution with 10μM Ca\(^{2+}\) on the indirect germination of *P. infestans* EUPi1.

Data are mean ± se for at least 5 replicate treatments. Germination was assessed after 16h incubation at 12°C, with at least 100 sporangia being assessed per replicate treatment.
Figure 5.7 The effect of elution with 100μM Ca$^{2+}$ on the indirect germination of *P. infestans* EUPi1.

Data are mean ± se for at least 5 replicate treatments. Germination was assessed after 16h incubation at 12°C, with at least 100 sporangia being assessed per replicate treatment.
**Figure 5.8** The effect of elution with 500μM Ca$^{2+}$ on the indirect germination of *P. infestans* EUPi1.

Data are mean ± se for at least 5 replicate treatments. Germination was assessed after 16h incubation at 12°C, with at least 100 sporangia being assessed per replicate treatment.
Figure 5.9 The effect of elution with 500μM Ca$^{2+}$ on the indirect germination of *P. infestans* 81/1/1.

Data are mean ± se for at least 5 replicate treatments. Germination was assessed after 16h incubation at 12°C, with at least 100 sporangia being assessed per replicate treatment.
Figure 5.10 The effect of elution with 1mM Ca$^{2+}$ on the indirect germination of *P. infestans* 81/1/1.

Data are mean ± se for at least 5 replicate treatments. Germination was assessed after 16h incubation at 12°C, with at least 100 sporangia being assessed per replicate treatment.
**Figure 5.11** The effect of elution with 500µM Ca$^{2+}$ on the indirect germination of *P. infestans* E$_{131}$A$_2$.

Data are mean ± se for at least 5 replicate treatments. Germination was assessed after 16h incubation at 12°C, with at least 100 sporangia being assessed per replicate treatment.
5.3 Discussion

The experiments in this section were designed initially to see whether the germination of *P. infestans* sporangia is sensitive to the effects of continuous withdrawal of nutrients from the environment of the sporangia. Lockwood and his co-workers (Hsu and Lockwood, 1973; Lockwood, 1977) demonstrated clearly that continuous nutrient leaching can suppress the germination of many types of fungal spore, paralleling the phenomenon known as fungistasis or mycostasis. Even spores that could germinate when incubated in distilled water were prevented from germinating when exposed to a continuous slow leaching with distilled water. The implication of that work, was that spore germination is regulated by a feedback mechanism whereby, nutrients are released from spores and provided that the nutrients are not removed from the spore’s vicinity (by nutrient leaching or microbial utilisation), then the spore in some way receives a signal to proceed towards germination. A broadly similar suggestion was made by Donaldson and Deacon (1992) for the autonomous germination of zoospore cysts—that, in this case, calcium or some triggering substance is released from the cells and its external concentration around the cysts is sensed, leading to rapid cyst germination.

In the experiments here, elution of sporangia with distilled water was tested for up to 30 min before the water flow was stopped and the sporangia were incubated in moist chambers to enable them to germinate. However, these initial leaching periods had no effect on sporangial germination, perhaps because they were not continued for long enough.

In contrast, continuous exposures of sporangia to low calcium concentrations in the first 10-30 minutes of incubation caused a substantial reduction of germination. The lowest Ca\(^{2+}\) concentration (1\(\mu\)M) had no effect, but 30 minutes exposure to a continuous flow of 10\(\mu\)M Ca\(^{2+}\) caused a significant reduction in germination, as did even 10 minutes exposure to 100\(\mu\)M Ca\(^{2+}\) (Figures 5.6, 5.7). Higher concentrations of Ca\(^{2+}\) (500\(\mu\)M or 1 mM), caused even stronger effects, which were confirmed for all
three isolates of *P. infestans*. Thus it was demonstrated that even low Ca\(^{2+}\) concentrations can be inhibitory to sporangia of this fungus if the Ca\(^{2+}\) is supplied in a continuously replenished solution. These findings may be compared with those in Figure 1 of Hill *et al.* (1998), where, in the standing solutions, concentrations of even 1 or 2mM CaCl\(_2\) had only a small inhibitory effect on the indirect germination of *P. infestans* sporangia. Figure 5.7 in this section shows that an equivalent level of inhibition to that caused by 2mM CaCl\(_2\) in standing solution was achieved by even 10 minutes initial exposure to 100\(\mu\)M CaCl\(_2\) in a continuous flow system.

The original hypothesis proposed by Hsu and Lockwood (1973) and Lockwood (1977) was that continuous leaching may lead to fungistasis. The experiments described here do not entirely support this conclusion, but they do show that sporangia of *P. infestans* are sensitive to the continual addition of very low concentrations of Ca\(^{2+}\), perhaps indicating that any possible feedback control of germination is Ca\(^{2+}\) dependent. The continual addition of Ca\(^{2+}\) may either disrupt a potential Ca\(^{2+}\) concentration gradient or "swamp" any Ca\(^{2+}\) sensing molecule released by the sporangium preventing the sporangium from responding to a potentially inductive germination signal.
Chapter 6

Concluding discussion
The work in this thesis has been directed towards the development of an alternative method of control for *P. infestans* the causative agent of potato late blight. Oomycetes have a precise host location and infection sequence in which a motile zoospores swims towards a host, encysts, adheres and germinates penetrating the host tissue. Ca$^{2+}$ has been shown to have a central role in this infection sequence being involved in motility (Donaldson and Deacon 1993 b), encystment (Bryet *et al.*, 1982) and adhesion and germination (Gubler *et al.*, 1989; Donaldson and Deacon, 1992; Von Broembsen and Deacon, 1996 and Deacon and Saxena, 1998).

Targeting this Ca$^{2+}$ signalling mechanism could possibly be used to develop a new method of control. To this end pre-encysted zoospores of *P. infestans* and *P. palmivora*, an organism which has previously been used to study Ca$^{2+}$ fluxes, (Grant *et al.*, 1985, 1986; Griffith *et al.*, 1988) were treated with a range of Ca$^{2+}$ modulators. All the modulators interfere with Ca$^{2+}$ mediated responses in a specific manner. All the modulators tried, significantly inhibited the germination of these pre-encysted zoospores although there were variations in effect on the different species. The inhibition of zoosporic germination is an important observation but perhaps more importantly when these studies were extended to the germination of three different isolates of *P. infestans* a quite remarkable inhibition of both direct and indirect germination occurred. Preventing the sporangia from germinating is in effect disabling the whole host location and infection process as without a zoospore this can’t occur.

The pre-encysted zoospores of *P. infestans* and *P. palmivora* generally displayed a concentration dependent inhibition for all the modulators tested. All the modulators added reduced the level of subsequent germination to less than 10 % of the control values and the majority of the modulators in fact reduced germination to zero.

The inhibition of germination by the modulators strengthens the case for the role of Ca$^{2+}$ in the germination process. When Ca$^{2+}$ is removed from the medium by the addition of the Ca$^{2+}$ chelators EGTA or BAPTA (Schmid and Reilley, 1957;
Tisen, 1980) germination is strongly inhibited, chelation of the Ca\(^{2+}\) prevents it being taken up by the cell triggering germination. Uptake of Ca\(^{2+}\) and subsequent germination can also be prevented by the addition of the Ca\(^{2+}\) channel blockers La\(^{3+}\) and verapamil (Godfraind et al., 1986). The effect of La\(^{3+}\) can be replicated by the mechanosensitive Ca\(^{2+}\) channel blocker Gd\(^{3+}\).

The effect of the Ca\(^{2+}\) modulators is not confined to preventing Ca\(^{2+}\) entry to the cell, once inside the cell Ca\(^{2+}\) antagonists such as TMB-8 (Owen and Villereal, 1982) are able to function and prevent the germination of the pre-encysted zoospores. Caffeine which reduces the level of Ca\(^{2+}\) stored in intracellular organelles, has a similar effect. The inhibition of germination by these intracellular modulators indicates that Ca\(^{2+}\) plays a central role in germination intracellularly as well as extracellularly. The three calmodulin antagonists applied to the cysts all prevent the germination of the cysts when applied at very low concentration. Calmodulin is one of the most important Ca\(^{2+}\) binding proteins and is apparently ubiquitous amongst eukaryotes. The level of calmodulin expression in \textit{P. infestans} is known to increase five fold during pathogenesis of potato (Pieterse et al., 1993).

The binding of Ca\(^{2+}\) induces a conformational change in calmodulin enabling it to interact with a number of target proteins including Ca\(^{2+}\) ATPases and protein kinases. Ca\(^{2+}\) / calmodulin complexes can produce a physiological response directly by the regulation of phosphorylation reactions which may lead to cell proliferation, cell cycle control and nuclear division (Anraku et al., 1991). It has been postulated by St. Leger et al., (1990) that an uneven distribution of calmodulin can lead to polarised cell growth. Calmodulin is reported to be localised at the base of the anterior flagella of \textit{P. cinnamomi} (Gubler et al., 1990) and it perhaps this highly localised concentration of calmodulin that leads to subsequent germ tube emergence from this area (Paktitis et al., 1986; Jones et al., 1991).

The addition of post-treatments to modulator treated cells was in certain instances able to overcome the inhibitory effect of the modulators. The addition of Ca\(^{2+}\) or Ba\(^{2+}\) to EGTA treated cells of \textit{P. infestans} resulted in a restoration of germination to 98% of control levels, although in \textit{P. palmivora} the restored levels of
germination were not so high. Indeed rather strangely, Ca\(^{2+}\) or Ba\(^{2+}\) could not rescue BAPTA treated cysts of *P. palmivora* although sugar and amino acids were able to.

Channel blocker treated cysts could be rescued when an alternative mode of Ca\(^{2+}\) entry was used, the most likely mechanism for this is "gated" entry of Ca\(^{2+}\) into the cell. Gated entry relies on an external molecule binding to a specific receptor which then allows the entry of Ca\(^{2+}\) into the cell. Ca\(^{2+}\) channel linked glutamate receptors are widely found in other organisms (Gilbertson *et al.*, 1991; Hollman *et al.*, 1990) and it is therefore not surprising that the post treatment that was most effective was MEP. This alternative method of Ca\(^{2+}\) entry allows the Ca\(^{2+}\) to be taken up and trigger germination. Donaldson and Deacon (1992) found that asparagine was able to overcome the effect of several modulators including channel blockers, on the germination of *Py. aphanidermatum* although this treatment had no effect on *P. infestans*. Asparagine is a strong chemoattractant for *Py. aphanidermatum* whereas *P. infestans*, displays no such chemoattraction and it is therefore possible that *P. infestans* lacks the appropriate receptor for asparagine. Verapamil may not associate as tightly with the channel as La\(^{3+}\), and the addition of an excess of Ca\(^{2+}\) or Ba\(^{2+}\) is able to overcome its effect. Amiloride appears to be functioning as a channel blocker and its rescue profile follows that of La\(^{3+}\).

Of the three calmodulin antagonists used only dibucaicne treated cells could be effectively rescued for both species. It is interesting that in order to effectively inhibit germination dibucaicne had to applied at x10 the concentration of the other calmodulin antagonists (200-250\(\mu\)M). This may indicate that the interaction between dibucaicne and calmodulin is not as stable as for TFP of calmidazolium. Calmidazolium is the only one of the calmodulin antagonists that showed propensity for rescue and was also the antagonist that was effective at the lowest concentration (1-3\(\mu\)M). Caffeine could not be rescued by any of the treatments applied indicating that its depletion of intracellular Ca\(^{2+}\) stores is irreversible.

It generally appears that modulator treated cysts of *P. palmivora* are more readily rescued by subsequent post-treatments than those of *P. infestans* and the range
of chemicals that are able to achieve rescue is also wider. The addition of MEP to the corresponding SDW controls of *P. palmivora* promotes germination to approaching 100%. This may indicate that *P. palmivora* has a wider range of receptors and is therefore more able to respond to the rescue treatments.

The effect of the modulators is not constant and a reduction in the sensitivity of the cysts is noted when the modulators are added more than 20 minutes after the induction of encystment. This reduction in the sensitivity of the cysts is mirrored in the inability of *A. euteiches* to respond to stimuli when they are applied more than 20 minutes after encystment (Deacon and Saxena, 1998) and the reduced capacity of *P. parasitica* to respond to germination triggers when applied 9-12 minutes after encystment (Von Broembsen and Deacon, 1996). When the modulator treatments are added more than 20 minutes after encystment it may be that the cysts have already perceived and responded to a germination trigger and have already undertaken steps that will lead to germination.

This reduced sensitivity to modulators and to germination triggers corresponds to the period of large scale ultrastructural changes in the zoospore (Holloway and Heath, 1977; Grove and Bracker, 1978). It is this period before the mature cyst wall is formed that the cysts are at their most sensitive.

The addition of the di-valent cations Ca\(^{2+}\), Ba\(^{2+}\) or Mg\(^{2+}\) results in a concentration dependent reduction in germination. The effect is apparent even when the treatments are added 60 minutes after the induction of encystment. This is rather surprising as di-valent cations have been shown to have a stimulatory effect on the germination of *P. palmivora* zoospores (Grant *et al.*, 1985;1986). It may be however that rather than actually blocking germination these treatments simply interfere with germ tube out growth.

In sporangia unlike zoospores (Irving *et al.*, 1984; Gubler *et al.*, 1989) no evidence exists for Ca\(^{2+}\) fluxes. The application of Ca\(^{2+}\) modulators and the subsequent inhibition of both direct and indirect germination implicates Ca\(^{2+}\) in these
processes. Hyde et al. (1991) investigated zoosporogenesis in P. cinnamomi and noted that the pattern of cleavage vesicle dispersal in the sporangium interior suggested a reliance on a cytoskeleton infrastructure. Calmodulin is a known cytoskeleton binding protein (Heath, 1994) and so regulation of zoosporogenesis via Ca\(^{2+}\) / calmodulin is possible. If Ca\(^{2+}\) is required to activate the calmodulin and hence the cytoskeleton rearrangements any treatment which affects this process will block germination. Preventing the entry of Ca\(^{2+}\) into the sporangia either via external chelation or by blocking the Ca\(^{2+}\) channels would prevent this. Disruption of the Ca\(^{2+}\) signal within the sporangium would also be expected to prevent germination. This is illustrated by the effect of caffeine and TMB-8 on the process of sporangial germination. The depletion of intracellular organelle Ca\(^{2+}\) stores by caffeine would raise the resting [Ca\(^{2+}\)] above the normal 100-300nM (Miller et al. 1990) preventing the generation of the Ca\(^{2+}\) signal as would the interaction of TMB-8 with the internal Ca\(^{2+}\).

The time of addition of the modulator treatment had no effect on the germination of P. infestans sporangia. This is rather surprising, but as the increased sensitivity of zoospores occurred during the time before the cyst wall had formed and the sporangia already has a wall it should perhaps be expected.

EGTA, orthophosphate or pectin treated sporangia can only be rescued when the Ca\(^{2+}\) or Ba\(^{2+}\) treatment is added at the same time or shortly after the pre-treatment. All these compounds appear to be functioning as Ca\(^{2+}\) chelators and the addition of exogenous Ca\(^{2+}\) is able to overcome this inhibition. Although the data is not shown preliminary studies indicate that nutrient treatments such as glucose or asparagine have no ability to rescue the modulator treated sporangia. These compounds are able to restore the germination of chelator treated cysts by permitting the entry of Ca\(^{2+}\) via nutrient gated channels. These channels are believed to be associated with receptors involved in chemotactic responses (detecting and responding to a concentration gradient) and as sporangia are sessile it is unlikely that they would have the appropriate receptors and associated nutrient gated channels, explaining the inability of these compounds to overcome the effect of chelator pre-treatments.
When divalent cations were added to sporangia of *P. infestans* an interesting trend was noted. Calcium, barium and magnesium all produced a concentration dependent inhibition of indirect germination in both the isolates tested, but when the same treatments were applied to directly germinating sporangia of *P. infestans* EUPi1 only Ba$^{2+}$ produced a similar concentration dependent inhibition of germination.
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