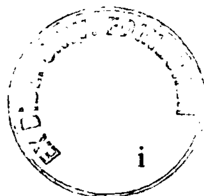
Effects of cations, natural toxins and other factors on infection-related behaviour of the zoosporic fungi *Pythium aphanidermatum* and *Phytophthora parasitica*.

Paul Wade Matthews.

A Thesis Presented for the Degree of Doctor of Philosophy.

University of Edinburgh, 2000.



Declaration.

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

Acknowledgements.

I thank Dr Jim Deacon for his supervision during this project, and particularly during the writing of this thesis. Thanks also go to Dr Graham Russell for his encouragement and 'just being there', since 1992! Many thanks go to Dr Martin McPherson and his colleagues at Horticultural Research International (Stockbridge House) who provided the irrigation solutions for the analyses performed in Chapter 4, and for spending time explaining the background and experimental set-up of their trials in North Yorkshire. I thank the Ministry of Agriculture, Fisheries and Food for funding this project and partially funding attendance of the joint annual meeting of the American Phytopathological Society and the Canadian Phytopathological Society (Montréal, 1999). I also thank the British Society of Plant Pathology for also partially funding attendance of APS/CPS99 and for the opportunity to attend the International Congress of Plant Pathology (Edinburgh, 1998).

I wish to thank Lynn Kennedy for the frequent use of her balance, Dr Seddon (Aberdeen) for the isolates of *Brevibacillus brevis*, Michael Dye (Institute of cell, animal and population biology) for the blood agar plates and Jacques de Liedekerke for assisting me with the β-escin experiments during his honour's project.

Thanks go to those who I have shared the lab with over the past few years. In particular Dave 'Crazy' Grayson (*First he was afraid, then he was...*), for his 'humour', abuse, and vast knowledge of 'news' and all things scientific - except for the solubility of $CaSO_4$, Helen 'Flops' Robinson for surviving my attempts to overcome her pyrophobia, and Elizabeth 'Petra' Cryer for Swann level seven coffees.

Thanks also go to all those non-KB types, in particularly those at Carrubbers who have been supportive, although not fully understanding what this project has researched (bananas and peanuts!). Those I wish to thank in particular are: Hywel 'Muppet' Clifford for his long-suffering friendship and wise counsel; William 'the Fish' Cumming for understanding me and his encouragement; Gary 'Gonzo' Connor for his constant humour and support; John 'Horatio Hornblower' Hawthorne for Wednesday evenings and Wall's Vienetta; James 'Marvin' MacDonell for being a thoroughly decent chap; and last but not least, Auntie Doris - Rebecca Ralley for being agony aunt *par excellence*.

Dedication.

I would like to dedicate this thesis to my parents,
Derek and Anne,
who have lovingly supported me throughout my time in Edinburgh
and all my previous years.

*"....of making many books there is no end, and much study wearies the body.
Now all has been heard; here is the conclusion of the matter:
Fear God and keep his commandments, for this is the whole duty of man."*

Ecclesiastes 12:12-13 (NIV).

Abstract.

In continuously irrigated, commercial horticultural glasshouses in Britain, the surplus mineral nutrient solutions usually enter local watercourses. Recirculation of "spent" irrigation solution is environmentally desirable, but also would recirculate pathogen propagules and thereby increase the potential for disease. With the aim of finding simple and effective disease-control measures, laboratory experiments described in this thesis tested the effects of various cations (Ca^{2+}, Mg^{2+}, K^{+}) and potentially fungitoxic compounds (saponins, gramicidin S, ethanol) on different stages of the life cycle of two pathogenic zoospore-forming fungi, *Pythium aphanidermatum* and *Phytophthora parasitica*. Also, nutrient irrigation solutions from various experimental treatments in a large tomato-cropping glasshouse trial at Horticultural Research International (HRI, Yorkshire) were tested for effects on the two fungi in laboratory conditions. Cations, fungitoxic compounds and experimental irrigation solutions were tested for effects on the following aspects of fungal behaviour: mycelial growth, production of sporangia, release of zoospores from sporangia, zoospore motility, zoospore encystment and cyst germination.

When tested on individual stages of the life cycle, high concentrations of Ca^{2+} and Mg^{2+} in nutrient broth reduced mycelial growth by *Py. aphanidermatum* but not *Ph. parasitica*. Sporangial production by *Ph. parasitica* in mineral salts solution was unaffected by supplements of Ca^{2+}, Mg^{2+} or K^{+}, but these supplements suppress the ability of sporangia to subsequently liberate zoospores into water; the exception was 5 mM Ca^{2+} which markedly enhanced subsequent zoospore release. Increasing concentrations of Ca^{2+}, Mg^{2+} and K^{+} in the solution that bathed pre-formed sporangia of either fungus reduced the number of zoospores that were released. These three cations also suppressed the proportion of zoospores that remained motile, and increased the proportion of vortex-encysted zoospores that would germinate.

When tested cumulatively on all stages of the life cycle, K^{+} was more effective than Ca^{2+} in suppressing the infection related behaviour of both fungi. These experiments suggest that increasing the ratio of K^{+} to Ca^{2+} in bathing solutions should suppress the ability of both fungi to spread and cause disease.

The trial undertaken by HRI failed to demonstrate natural suppression in a semi-commercial irrigation system, which was the principal trial objective. Consequently the analyses presented in this thesis were unable to determine at which points of the infection sequence the agents of natural suppression might act. However, it was found that the duration of zoospore motility was

greater in some irrigation solutions than in distilled water; also, in treatments where disease was observed, the associated irrigation solutions promoted the production of sporangia in laboratory experiments.

Different stages of the life cycle of both fungi were exposed experimentally to various concentrations of ethanol. Naked zoospores and germinating cysts were relatively insensitive to ethanol; mycelial growth was only slightly sensitive, but low ethanol concentrations markedly suppressed the formation of sporangia, and the release of zoospores.

The saponin, β-escin and the cyclic oligopeptide gramicidin S lysed or killed motile, naked zoospores. Gramicidin S also was toxic to zoospore cysts, but the presence of a cyst wall markedly reduced the toxic effect of β-escin. The toxicity of gramicidin S to zoospores or cysts was reduced in the presence of divalent cations (Ca^{2+}, Mg^{2+} or Sr^{2+}), but not with monovalent cations (Na^+ and K^+). The toxicity of β-escin was reduced in the presence of Ca^{2+} or K^+, but not with Mg^{2+} or Na^+. The toxicity of β-escin (but not of gramicidin S) on zoospores can be partially relieved by the addition of calcium five minutes after exposure to the toxin. When zoospore suspensions were supplemented with both β-escin and gramicidin S the combined toxicity was greater than expected, suggesting that these toxins act synergistically. The effect of 5 mM Ca^{2+} in promoting sporangiogenesis was antagonised by ethanol. Several irrigation solutions and extracts from oat tissues negated the effects of cations on various life cycle stages related to infection by the zoosporic fungi.

Overall, the work in this thesis indicates that several factors might suppress the infection-related behaviour of *Py. aphanidermatum* and, to a lesser degree, *Ph. parasitica*. Some of these factors act synergistically and might be used as combination treatments, but some antagonise each other's effects.

Table of Contents.

Title page i
Declaration. ii
Acknowledgements. iii
Dedication iv
Abstract v
Table of Contents vii
List of Figures. vii
List of Tables. xv
Abbreviations xix

Chapter 1 - Introduction 1
1.1 General Introduction. 2
1.2. Biology of the family Pythiaceae. 2
  1.2.1. General biology. 2
    1.2.1.1. Biology of *Pythium aphanidermatum* 3
    1.2.1.2. Biology of *Phytophthora parasitica.* 3
  1.2.2. Genetics. 4
  1.2.3. Taxonomy. 4
1.3. Ecology. 5
1.4. General pathology 6
1.5. Generalised life cycle for the Pythiaceae. 7
1.6. The infection sequence of zoosporic fungi. 7
  1.6.1. Vegetative growth 8
  1.6.2. Sporangiogenesis. 9
    1.6.2.1. Germination of sporangia. 11
  1.6.3. Motile zoospores. 14
  1.6.4. Zoospore encystment. 17
    1.6.4.1. Factors involved in inducing zoospore encystment. 17
    1.6.4.2. The mechanics of zoospore encystment. 18
  1.6.5. Diplanetism. 21
  1.6.6. Cyst germination. 21
  1.6.7. Infection. 23
    1.6.7.1. Factors affecting infection. 23
    1.6.7.2. Penetration of host. 24
    1.6.7.3. Host reaction to infection. 24
1.7. Glasshouse irrigation systems. 26
  1.7.1. Nutrient film technique. 27
    1.7.1.1. Construction of a recirculating NFT installation. 27
    1.7.1.2. Operation of a NFT installation. 29
1.8. Microbiology of irrigation systems. 30
  1.8.1. Susceptibility of nutrient solutions to root pathogens. 32
1.9. Disease control. 35
  1.9.1. Plant breeding. 35
  1.9.2. Chemical control. 35
  1.9.3. Biological control. 37
  1.9.4. Natural control. 38
1.10. Legislation. 40
1.11. Summary 40

1.12. Aims of the project. 42
1.13. Major findings of this project. 42

Chapter 2 - General materials and methods **44**
2.1. Media 45
2.1.1 Agars 45
2.1.2. Broths 45
2.1.3. Mineral salts solution (MSS). 46
2.1.4. Mineral nutrient solution. 46
2.1.5. Preparation of crude oat extracts. 46
2.1.5.1. Terminal root tip extract. 47
2.1.5.2. Leaf extract. 47
2.1.5.3. Seed and root complex extract. 47
2.2 Origin, maintenance and preservation of strains. 47
2.2.1 *Phytophthora parasitica*. 47
2.2.2. *Pythium aphanidermatum*. 48
2.2.3. *Brevibacillus brevis*. 48
2.3. Standard techniques. 48
2.3.1. Production of zoospores. 48
2.3.1.1. *Ph. parasitica* 48
2.3.1.2. *Py. aphanidermatum*. 48
2.3.1.3. Bulk zoospore production. 49
2.3.2. Mechanical induction of zoospore encystment. 49
2.3.3. Effects of treatments on mycelial growth. 49
2.3.4. Effects of treatments on zoosporangiogenesis. 50
2.3.5. Effects of treatments on zoospore release. 51
2.3.6. Effects of treatments on zoospore motility. 51
2.3.7. Effects of treatments on cyst germination. 52
2.4. Culturing of *Brevibacillus brevis* in various liquid media. 52
2.5. Source of reagents. 52
2.6. Statistics. 52

Chapter 3 - The effects of cations on the zoosporic infection sequence **53**
3.1. Introduction. 54
3.2. Effects of supplementing CV8 broth with cations on mycelial growth. 54
3.3. Zoosporangiogenesis and subsequent zoospore liberation in cation supplemented mineral salt solutions. 59
3.3.1. Effect on Pythium aphanidermatum. 59
3.3.2. Effect on Phytophthora parasitica. 61
3.3.2.1. Effects of treatments on sporangial density. 61
3.3.2.2. Effects of treatments on zoospore release. 63
3.3.2.3. Effects of treatments on sporangial discharge. 66
3.4. Zoospore release into various salt solutions. 69
3.5. Motility of zoospores and subsequent cyst germination in various salt solutions. 73
3.6. Germination of vortex-encysted zoospores in various salt solutions 79
3.7. Summary of effects of cations on individual stages of the zoosporic infection sequence.... 82
3.8. The compounded effects of cationic supplements on aspects of zoospore biology 85
3.8.1. Pythium aphanidermatum. 85
3.8.2. Phytophthora parasitica. 93
3.9. Summary of the effects of cations on various stages of the zoosporic infection sequence when the test supplement was constant throughout the experimental protocol. 97

3.10. The effect of zoospore density on zoospore motility....99
3.11. Modification of the potassium to calcium ratio....100
3.12. Comparison of the effects of the anions: Cl^- v NO_3^-....103
3.13. Discussion....107

Chapter 4 - The effects of recirculating horticultural irrigation solutions on the biology of zoosporic fungi....115
4.1. Introduction....116
4.2. The experimental protocol at Stockbridge House....117
4.2.1. An overview....117
4.2.2. The experimental set up of the large glasshouse....119
4.2.3. The set up of the mini-NFT systems....122
4.2.4. Assessment of plants grown in the mini-NFT systems and number of *Phytophthora* propagules recirculating....124
4.2.5. Summary of results from Stockbridge House....125
4.3. The work performed at Edinburgh....127
4.3.1. Summary of 'Edinburgh' data....127
4.4. Details of 'Edinburgh' results....129
4.4.1. Determination of the pH of irrigation solutions....129
4.4.2. The effects of irrigation solutions on the motility of zoospores of *Ph. parasitica* and subsequent cyst germination....129
4.4.3. The effects of irrigation solutions on the motility of *Py. aphanidermatum* and subsequent cyst germination....131
4.4.4. The effects of irrigation solutions on the release of zoospores from pre-formed sporangia of *Ph. parasitica*....131
4.4.5. The effects of irrigation solutions on germination of vortex-encysted zoospores of *Py. aphanidermatum*....132
4.4.6. The effects of irrigation solutions on sporangiogenesis of *Ph. parasitica*....133
4.5. The effect of pH on zoospore motility and subsequent cyst germination....145
4.5.1. Modification of irrigation solution pH....145
4.5.2. Zoospore motility and subsequent cyst germination in solutions of various pH....145
4.6. Correlations between characteristics of irrigation samples....149
4.7. Discussion....156

Chapter 5 - The effects of ethanol on *Py. aphanidermatum* and *Ph. parasitica*....161
5.1. Introduction....162
5.2. Effect on mycelial growth....162
5.3. Effect of supplementing MSS with ethanol at different times during sporangiogenesis of *Py. aphanidermatum*....163
5.4. Effect of various ethanol concentrations in MSS on zoospore release from *Py. aphanidermatum*....165
5.5. Effect of various ethanol concentrations in MSS on zoospore release from *Ph. parasitica*....165
5.6. Effect of various ethanol concentrations in the release solution on zoospore release from pre-formed sporangia of *Py. aphanidermatum* and *Ph. parasitica*....170
5.7. Effects of various ethanol concentrations on zoospore motility and cyst germination of *Py. aphanidermatum*....172
5.8. The effect of ethanol on zoospore and cyst nutrient induced germination of *Py. aphanidermatum*....173
5.9. Summary of the effects of ethanol on the biology of *Py. aphanidermatum* and *Ph. parasitica*....175

5.10. Discussion.....177

Chapter 6. - The effects of natural toxins on aspects of the infection sequences of *Py. aphanidermatum* and *Ph. parasitica*.....180
6.1. Introduction.....181
6.2. The effects of dimethy sulphoxide on aspects of biology of zoosporic fungi.....181
6.3. Effects of β-escin and gramicidin S on zoospore, immature cyst and mature cyst nutrient induced germination.....182
6.4. Visual observations of the effects of β-escin and gramicidin S on zoospores of *Py. aphanidermatum*.....189
6.5. The effects of β-escin and gramicidin S on sporangiogenesis and sporangial discharge of *Ph. parasitica*.....190
6.6. Effect of β-escin on zoospore discharge from pre-formed sporangia of *Ph. parasitica*.....193
6.7. The Effect of β-escin and gramicidin S on zoospore motility and subsequent cyst germination of *Py aphanidermatum*.....194
6.8. Effects of β-escin and gramicidin S on germination of vortex-encysted zoospores of *Py. aphanidermatum*.....196
6.9. The effects of β-escin and gramicidin S with various cationic supplements on germination of zoospores, immature cysts and mature cysts.....197
6.9.1. The effects of different times of addition of cations on the germination of zoospores of *Py. aphanidermatum* in the presence of β-escin or gramicidin S.....204
6.10. The effect of zoospore population density on susceptibility to β-escin and gramicidin S.....207
6.11. The degradation of β-escin by bacterial isolates from soil.....208
6.12. The effect of crude saponin extracts from oat seedlings on the motility of zoospores, the germination of cysts and the nutrient induced germination of zoospores.....209
6.13. Production of zoospore-toxic compounds by *Brevibacillus brevis*.....213
6.14. *Brevibacillus brevis* (wild-type) produces a haemolytic compound.....216
6.15. Discussion.....218

Chapter 7 - The effect of combining potential control techniques on aspects of the infection sequence of zoosporic fungi.....226
7.1. Introduction.....227
7.2. Synergism between β-escin and gramicidin S.....227
7.3. Synergism between natural suppression and 10 mM K^+ supplement on the motility of zoospores of *Py. aphanidermatum* and the subsequent germination of encysted zoospores.....228
7.4. Synergism between natural suppression and Ca^{2+} supplement on the release of zoospores from pre-formed sporangia of *Py. aphanidermatum*.....231
7.5. Synergism between natural suppression and Ca^{2+} supplment on the germination of vortex-encysted zoospores of *Py. aphanidermatum*.....232
7.6. Synergism between natural suppression and either 5 mM K^+ or 35 mM ethanol on the infection sequence of *Py. aphanidermatum*.....233
7.7. Synergism between natural suppression and 35 mM ethanol on sporangiogenesis of *Ph. parasitica*.....237
7.8. Antagonism between 5 mM Ca^{2+} and 35 mM ethanol on the sporangiogenesis of *Ph. parasitica*.....240
7.9. Discussion.....243

Chapter 8 - Concluding discussion **246**
8.1. Discussion 247
8.2. Further work 250

Appendices **251**

References **265**

List of Figures.

Figure 1.1. Qualitative phylogenetic placement of the Oomycetes. 5
Figure 1.2. The homing sequence of a zoosporic fungus in an irrigation system based on NFT. 8
Figure 1.3. Direct germination of a sporangium of *Phytophthora palmivora*. 12
Figure 1.4. Indirect germination (zoospore release) of a sporangium of *Phytophthora palmivora*. 12
Figure 1.5. Simplified diagram of a typical zoospore of the Oomycota. 14
Figure 1.6. Schematic diagram of the ultrastructural changes that occur in a zoospore during encystment... 20
Figure 1.7. Schematic representation of a recirculating NFT installation. 28

Figures 3.1 - 3.4. Effect of salt concentration on biomass gain of *Pythium aphanidermatum* during a 48h incubation at 23 °C in the dark. 57
Figures 3.5 - 3.8. Effect of salt concentration on biomass gain of *Phytophthora parasitica* during a 48h incubation at 23 °C in the dark. 58
Figures 3.9 - 3.12. Effect of salt type and salt concentration in MSS on the sporangial density of mats of *Phytophthora parasitica*. 62
Figure 3.13. Effect of KNO_3 concentration in MSS on the sporangial density of mats of *Phytophthora parasitica*. 63
Figures 3.14 - 3.17. Effect of salt type and salt concentration in MSS on zoospore release from mats of *Phytophthora parasitica* 65
Figures 3.18 - 3.21. Effect of salt type and salt concentration in MSS on sporangial discharge in mats of *Phytophthora parasitica* 68
Figures 3.22 - 3.25. Effect of salt type and salt concentration in release solution on zoospore release from pre-formed sporangia of *Pythium aphanidermatum*. 71
Figures 3.26 - 3.29. Effect of salt type and salt concentration in release solution on zoospore release from pre-formed sporangia of *Phytophthora parasitica*. 72
Figures 3.30 - 3.33. Effects of salt type and salt concentration on motility of zoospores of *Pythium aphanidermatum*. 75
Figures 3.34 - 3.37. Effect of salt type and salt concentration on motility of zoospores of *Phytophthora parasitica*. 76
Figures 3.38 - 3.41. Effect of salt type and salt concentration on spontaneous germination of encysted zoospores of *Pythium aphanidermatum*. 77
Figures 3.42 - 3.45. Effect of salt type and salt concentration on spontaneous germination of encysted zoospores of *Phytophthora parasitica*. 78
Figures 3.46 - 3.49. Effects of salt type and salt concentration on germination of encysted zoospores of *Pythium aphanidermatum*. 80
Figures 3.50 - 3.52. Effects of salt type and salt concentration on germination of encysted zoospores of *Phytophthora parasitica*. 81
Figures 3.53 - 3.56. Effect of salt type and salt concentration on zoospore release from sporangia of *Pythium aphanidermatum*. 88
Figures 3.57 - 3.60. Effect of salt type and salt concentration on the number of spores of *Py. aphanidermatum* observed at the bases of the Petri dishes after 2 h incubation 89
Figures 3.61 - 3.64. Effect of salt type and salt concentration on the proportion of spores of *Py. aphanidermatum* remaining motile after 2 h incubation. 90
Figures 3.65 - 3.68. Effect of salt type and salt concentration on the proportion of naturally encysted zoospores of *Py. aphanidermatum* that had germinated during 2 h incubation 91
Figures 3.69 - 3.72. Effect of salt type and salt concentration on the proportion of vortex-encysted zoospores of *Py. aphanidermatum* that had germinated during 2 h incubation 92
Figure 3.73. The effect of zoospore density on zoospore motility 99

Figure 4.1. Schematic representation of the relationship between the work performed at various locations. 118
Figure 4.2. The arrangement of rockwool blocks on rockwool slabs. 119
Figure 4.3. The arrangement of rockwool slabs in a double row. 120
Figure 4.4. Photograph of young tomato plants in the double row arrangement. 120
Figure 4.5. Diagram of the layout of the double rows (east half of the glasshouse only) and the collection of irrigation solution. 121
Figure 4.6. The set up of a single mini-NFT system. 123
Figure 4.7. Diagram of the allocation of the mini-NFT trials. 123
Figures 4.8 and 4.9. The effect of increasing $Ca(NO_3)_2$ and nitric acid concentration on zoospore motility and subsequent zoospore release. 147

Figure 5.1. The effect of supplementing CV8B with ethanol on final biomass of *Py. aphanidermatum* and *Ph. parasitica*. 163
Figure 5.2. The effect of supplementing MSS with various concentrations of ethanol on subsequent zoospore release from *Py. aphanidermatum* into SDW. 165
Figure 5.3. The effect of supplementing MSS with various concentrations of ethanol on sporangial density of *Ph. parasitica*. 166
Figure 5.4. The morphology of sporangia of *Ph. parasitica* that formed in ethanol-free MSS (100× total magnification) 167
Figure 5.5. The morphology of sporangia of *Ph. parasitica* that formed in MSS supplemented with 176 mM ethanol (100× total magnification). 167
Figure 5.6. Effect of ethanol concentration in MSS on the proportion of sporangia of *Phytophthora parasitica* that discharge their contents before and after the induction of zoospore release. 168
Figure 5.7. Effect of ethanol concentration in MSS on the zoospore release from sporangia of *Phytophthora parasitica* before and after the induction of zoospore release. 169
Figure 5.8. Effect of ethanol concentration in MSS on the specific zoospore release of zoospores from sporangia of *Phytophthora parasitica* before and after the induction of zoospore release. 170
Figure 5.9. Effect of ethanol concentration in the release solution on zoospore release from pre-formed sporangia of *Py. aphanidermatum*. 171
Figures 5.10 and 5.11. The effect of ethanol concentration in the release solution on zoospore release from pre-formed sporangia of *Ph. parasitica*. 172
Figures 5.12. and 5.13. The effect of ethanol concentration on zoospore and cyst viability of *Py. aphanidermatum*. 174

Figures 6.1 and 6.2. The effects of β-escin and gramicidin S concentration on the nutrient induced germination of zoospores of *Py. aphanidermatum*. 185
Figures 6.3 and 6.4. The effects of β-escin and gramicidin S concentration on the nutrient induced germination of zoospores of *Ph. parasitica*. 185
Figures 6.5 and 6.6. The effects of β-escin and gramicidin S concentration on the nutrient induced germination of immature cysts of *Py. aphanidermatum*. 186
Figure 6.7. The effect of β-escin concentration on the nutrient induced germination of immature cysts of *Ph. parasitica*. 186
Figures 6.8 and 6.9. The effects of β-escin and gramicidin S concentration on the nutrient induced germination of mature cysts of *Py. aphanidermatum*. 187
Figure 6.10. The effect of β-escin concentration on the nutrient induced germination of mature cysts of *Ph. parasitica*. 187
Figures 6.11 and 6.12. The effects of β-escin and gramicidin S on the appearance of non-motile cells of *Py. aphanidermatum*. 190

Figures 6.13 and 6.14. The effects of β-escin and gramicidin S concentration on sporangial density of *Ph. parasitica.* 192
Figures 6.15 and 6.16. The effects of β-escin and gramicidin S concentration on the proportion of sporangia of *Ph. parasitica* discharging their contents. 192
Figures 6.17 and 6.18. The effects of β-escin and gramicidin S concentration on the number of zoospores released from sporangia of *Ph. parasitica.* 193
Figure 6.19. The effect of β-escin on the proportion of pre-formed sporangia of *Ph. parasitica* that empty their contents. 194
Figures 6.20 and 6.21. The effects of β-escin and gramicidin S on the proportion of zoospores of *Py. aphanidermatum* that remain motile during the 2h incubation period. 195
Figures 6.22 and 6.23. The effects of β-escin and gramicidin S on the proportion of cysts of *Py. aphanidermatum* that subsequently germinate during the 2h incubation period. 196
Figures 6.24 and 6.25. The effects of β-escin and gramicidin S on the proportion of vortex-encysted zoospores of *Py. aphanidermatum* that germinate during the 2h incubation period. 197
Figures 6.26 and 6.27. The effects of increasing cation concentration in solutions of 3 μM gramicidin S on the germination of zoospores and mature cysts of *Py. aphanidermatum.* 201
Figure 6.28. The effect of increasing cation concentration in solutions of 25 μM β-escin on the germination of zoospores of *Ph. parasitica.* 202
Figures 6.29 and 6.30. The effect of increasing Ca^{2+} or Sr^{2+} concentrations in solutions of 1 μM gramicidin S or 80 μM β-escin on the germination of zoospores of *Py. aphanidermatum.* 203
Figure 6.31. The effect of increasing gramicidin S concentration in solutions of either no Ca^{2+} or 20 mM Ca^{2+} on the nutrient induced germination of zoospores of *Py. aphanidermatum.* 204
Figures 6.32 and 6.33. The effects of gramicidin S and β-escin on the germination of various population densities of zoospores of *Py. aphanidermatum.* 208
Figures 6.34 and 6.35. The effects of crude oat extracts on the nutrient induced germination of zoospores of *Py. aphanidermatum.* 213
Figures 6.36 and 6.37. The effect of diluting yeast extract broth inoculated with *B. brevis* (wild type) on the viability of zoospores of *Py. aphanidermatum.* 216
Figure 6.38. *Brevibacillus brevis* (wild-type) cultured on blood agar for 7 days. 217
Figure 6.39. *Brevibacillus brevis* (E1) cultured on blood agar for 7 days. 217

List of Tables.

Table 1.1. Infectious agents isolated from roots of hydroponically grown crops. 31
Table 1.2. Bacterial flora of a hydroponic system. 32
Table 1.3. The problems of chemical control of the Oomycetes and the reasons that underlie these problems. 37

Table 3.1. Correlation statistics and the equations for lines of best fit for the effects of $Ca(NO_3)_2$ and $Mg(NO_3)_2$ concentration on mycelial growth of *Py. aphanidermatum*. 55
Table 3.2. Effect of various supplements to Mineral Salt Solution on zoosporangiogenesis, assessed indirectly by measurement of subsequent zoospore release (zoospores.μl^{-1}) from sporangia-bearing mycelia of *Py. aphanidermatum*. 60
Table 3.3. Effect of Mineral Salt Solution supplemented with 5 mM $Ca(NO_3)_2$ on zoosporangiogenesis, assessed indirectly by measurement of subsequent zoospore release (zoospores.μl^{-1}) from sporangia-bearing mycelia of *Py. aphanidermatum*.. 60
Table 3.4. Correlation statistics and the equations for lines of best fit for the effects of KNO_3, $Mg(NO_3)_2$ and $CaCl_2$ concentration on zoospore release into MSS from sporangia of *Ph. parasitica* 64
Table 3.5. Correlation statistics and the equations for lines of best fit for the effect of KNO_3 and $CaCl_2$ concentration on pre-cold-shock sporangial discharge of *Ph. parasitica* 66
Table 3.6. Correlation statistics and the equations for lines of best fit for the effect of calcium salts on zoospore release from pre-formed sporangia of *Ph. parasitica*. 70
Table 3.7. Summary of effects of cations on individual stages of the zoosporic infection sequence. 84
Table 3.8. Effects of applications of 5 mM KNO_3 on aspects of biology of *Ph. parasitica* 95
Table 3.9. Effects of applications of 5 mM and 20 mM $Ca(NO_3)_2$ on aspects of biology of *Ph. parasitica* 96
Table 3.10. Summary of effects of cations on stages of the zoosporic infection sequence when the test supplement was constant throughout the experimental protocol 98
Table 3.11. The effect of modifying the potassium to calcium ratio on the biology of *Ph. parasitica*. 101
Table 3.12. The effects of modifying the potassium to calcium ratio on the biology of *Py. aphanidermatum* 102
Table 3.13. Direct comparison between the effect of Cl^- and NO_3^- on suppression of zoospore motility and subsequent spontaneous cyst germination for spores of *Pythium aphanidermatum* in the presence of 10 mM Ca^{2+} 103
Table 3.14. Comparison of the effects of Cl^- and NO_3^- salts of Ca^{2+} and Mg^{2+} (30 mM) on germination cysts of *Pythium aphanidermatum* 104
Table 3.15. Direct comparison between the effect of Cl^- and NO_3^- on aspects of *Phytophthora parasitica* sporangiogensis[1] and subsequent zoospore release in the presence of 5 mM and 40 mM Ca^{2+} salts 105
Table 3.16. Summary of direct comparisons between the effect of Cl^- and NO_3^- on aspects of *Phytophthora parasitica* and *Pythium aphanidermatum* zoosporic infection sequences in various concentrations of Ca^{2+} and Mg^{2+} salts 106

Table 4.1. Ranking of irrigation solutions based on theoretical suppressiveness. 128
Table 4.2. Overall theoretical suppressiveness in each treatment and associated mean solution pH. 129
Table 4.3. The pH of irrigation solutions from HRI. 135
Table 4.4. Percentage of zoospores of *Ph. parasitica* that were motile after 1 h incubation in various irrigation solutions. 136

Table 4.5. Percentage of encysted zoospores of *Ph. parasitica* that had germinated after 1 h incubation in various irrigation solutions. 137
Table 4.6. Percentage of zoospores of *Py. aphanidermatum* that were motile after 2 h incubation in various irrigation solutions. 138
Table 4.7. Percentage of encysted zoospores of *Py. aphanidermatum* that had germinated after 2 h incubation in various irrigation solutions. 139
Table 4.8. Number of zoospores (zoospores.μl^{-1}) of *Ph. parasitica* released into various irrigation solutions from pre-formed sporangia. 140
Table 4.9. ..... Percentage of vortex-encysted zoospores of *Py. aphanidermatum* that had germinated after 2 h incubation in various irrigation solutions. 141
Table 4.10. Subsequent zoospore release (Zoospores.μl^{-1}) into SDW from sporangia of *Ph. parasitica* that had formed in various irrigation solutions. 142
Table 4.11. The density of sporangia (sporangia per video monitor screen) of *Ph. parasitica* after incubation in various irrigation solutions. 143
Table 4.12. Percentage of sporangia of *Ph. parasitica* that had discharged contents after incubation in various irrigation solutions. 144
Table 4.13. Concentrations of HNO_3 and $Ca(NO_3)_2$ for the experiments on the effects of HNO_3 and $Ca(NO_3)_2$ concentration on zoospore motility and subsequent cyst germination. ... 146
Table 4.14. The data transformation method, formula of relationship and correlation statistics for lines of best fit between either $Ca(NO_3)_2$ or HNO_3 concentration and zoospore motility, cyst germination, expected pH and observed pH. 148
Table 4.15. Summary of characteristics associated with each irrigation sample. 149
Table 4.16. Correlation values for correlations between characteristics for all irrigation samples. 151
Table 4.17. Correlation values for correlations between characteristics for October irrigation samples only. 152
Table 4.18. Correlation values (*R*) and corresponding values of significance (*F*) for the major correlations between characteristics of irrigation samples based on data from all months. 153
Table 4.19. Correlation values (*R*) and corresponding significance (*F*) for the major correlations between characteristics determined in Edinburgh and the additional agronomic factors for the October irrigation samples. 155

Table 5.1. The effect of ethanol added to wash solutions at different times on sporangiogenesis of *Py. aphanidermatum* when assessed by subsequent zoospore release into SDW 164
Table 5.2. The effect of 176 mM ethanol on the proportion of zoospores of *Py. aphanidermatum* that remain motile, that subsequently germinate and the proportion of vortex-encysted zoospores that germinate 173
Table 5.3. Correlation statistics for lines of best fit for the effect of ethanol concentration on zoospore and cyst nutrient induced germination of *Py. aphanidermatum*. 174
Table 5.4. Summary of the effects of ethanol on the biology of *Py. aphanidermatum* and *Ph. parasitica* 176

Table 6.1. The effects of 28 mM DMSO on zoospore motility and subsequent cyst germination of *Py. aphanidermatum* and on sporangiogenesis of *Ph. parasitica*. 182
Table 6.2. LD_{50} values for β-escin and gramicidin S on various forms of spore of *Py. aphanidermatum* and *Ph. parasitica*. 188
Table 6.3. The effect of gramicidin S in the presence and absence of cations on germination of originally motile zoospores of *Py. aphanidermatum*. 198
Table 6.4. The effect of gramicidin S in the presence and absence of cations on the germination of mature cysts of *Py. aphanidermatum*. 199

Table 6.5. The effect of β-escin in the presence and absence of cations on the germination of originally motile zoospores of *Py. aphanidermatum.* 200
Table 6.6. The effect of timing on the ability of calcium to overcome the inhibition of gramicidin S to zoospores of *Py. aphanidermatum*........ 205
Table 6.7. The effect of timing on the ability of potassium to overcome the inhibition of β-escin to zoospores of *Py. aphanidermatum*........ 206
Table 6.8. The effect of timing on the ability of calcium to overcome the inhibition of β-escin to zoospores of *Py. aphanidermatum*........ 207
Table 6.9. The effect of axenic bacterial cultures amended with β-escin at two dilutions on the nutrient induced germination of zoospores of *Py. aphanidermatum.*........ 209
Table 6.10. The effect of crude oat extracts on the motility and subsequent germination of zoospores of *Py. aphanidermatum.* 211
Table 6.11. The effect of crude oat extracts on percentage germination of vortex-encysted zoospores of *Py. aphanidermatum.*........ 212
Table 6.12. The effects of different broth cultures of *Brevibacillus brevis* on the germination [arcsine (and %)] of zoospores. The broths were either tested alone (uninoculated) or after a wild-type (w.t.) strain of *B. brevis* had grown in them. In some cases the broths had been inoculated with a strain of *B. brevis* (E-1) that does not produce gramicidin S........ 215
Table 6.13 Summary of the effects of β-escin and gramicidin S........ 219

Table 7.1. Synergism between β-escin and gramicidin S assessed by the nutrient induced germination of zoospores of *Py. aphanidermatum.* 228
Table 7.2. Synergism between irrigation solutions and a 10 mM K^+ supplement, as assessed by the proportion of zoospores of *Py. aphanidermatum* that remained motile........ 230
Table 7.3. Synergism between irrigation solutions and a 10 mM K^+ supplement, as assessed by the proportion of spontaneously encysted zoospores of *Py. aphanidermatum* that subsequently germinated. 231
Table 7.4. Synergism between irrigation solutions and a 20 mM Ca^{2+} supplement, as assessed by the number of zoospores that are released from pre-formed sporangia of *Py. aphanidermatum*........ 232
Table 7.5. Synergism between irrigation solutions and a 20 mM Ca^{2+} supplement, as assessed by the proportion of vortex-encysted zoospores of *Py. aphanidermatum* that germinated. 233
Table 7.6. Synergism between irrigation solutions and either 5 mM K^+ or 35 mM ethanol, as assessed by the number of zoospores released from sporangia of *Py. aphanidermatum.* The effect was assessed when the treatment was applied to the whole experimental protocol. 234
Table 7.7. Synergism between irrigation solutions and either 5 mM K^+ or 35 mM ethanol, as assessed by the proportion of zoospores of *Py. aphanidermatum* that remain motile. The effect was assessed when the treatment was applied to the whole experimental protocol. 235
Table 7.8. Synergism between natural suppression and either 5 mM K^+ or 35 mM ethanol, assessed by the proportion of spontaneously encysted zoospores of *Py. aphanidermatum* that subsequently germinated. The effect was assessed when the treatment was applied to the whole experimental protocol. 236
Table 7.9. Synergism between irrigation solutions and either 5 mM K^+ or 35 mM ethanol, as assessed by the proportion of vortex-encysted zoospores of *Py. aphanidermatum* that germinated. The effect was assessed when the treatment was applied to the whole experimental protocol. 237
Table 7.10. Synergism between irrigation solutions and 35 mM ethanol, as assessed by the number of zoospores that were released prior to cold shock from sporangia of *Ph. parasitica* that had formed in the treatment........ 238

Table 7.11. Synergism between irrigation solutions and 35 mM ethanol, as assessed by the number of zoospores that were released after the cold shock from sporangia of *Ph. parasitica* that had formed in the treatment. 239
Table 7.12. Synergism between irrigation solutions and 35 mM ethanol, as assessed by the density of sporangia of *Ph. parasitica* that had formed in the treatment. 240
Table 7.13. Antagonism between 5 mM Ca^{2+} and 35 mM ethanol as assessed by aspects of sporangiogenesis of *Ph. parasitica*. 242

Abbreviations.

ANOVA	analysis of variance
ATP	Adenosine triphosphate
BA	blood agar
°C	degrees Celsius
CBS	Centraalbureau voor Schimmelcultures
CCD	charge coupled device
cfu	colony forming units
cm	centimetres
conc.	concentration
CV8A	clarified V8 agar
CV8B	clarified V8 broth
df	degrees of freedom
DMSO	dimethy sulphoxide
DNA	deoxyribonucleic acid
DW	distilled water
EC	electrical conductivity
EDTA	ethylenediaminetetraacetic acid
F	probability of correlation
f.sp.	*forma specialis*
g	grams or centrifugal force
h	hours
ha	hectares
HRI	Horticultural Research International
KB	King's Buildings (The University of Edinburgh)
kg	kilograms
kPa	kilo pascals
l	litres
L.	Linnaeus
LD	lethal dose
LSD	least significant difference
m	metres
m^2	square metres
M	molar
mbar	millibar
mg	milligrams
min	minutes
ml	millilitres
mM	millimolar
mΩ	milliohms
mol	mole
mS	milli-siemens
MSS	mineral salts solution
MPa	mega pascals
mRNA	messenger ribonucleic acid
n	number
n.a.	not applicable / not appropriate
NA	nutrient agar
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
n.d.	not determined
nS	nano-siemens
NFT	nutrient film technique
NIV	New International Version
nM	nanomolar

P	probability
Pa	pascal (1 Pa ≡ 1 $g.m^{-2}$)
Pa	*Pythium aphanidermatum*
Pc	*Phytophthora cryptogea*
PDA	potato dextrose agar
pH	hydrogen ion concentration ≡($-log_{10}[H^+]$)
Pp	*Phytophthora parasitica*
ppm	parts per million
psi	pounds per square inch (1 psi ≈ 705 kPa)
R	regression coefficient
RNA	ribonucleic acid
rpm	revolutions per minute
S	siemens
SDW	sterile distilled water
s.e.m.	standard error of mean
Suppl.	supplement
sp.	species (singular)
spp.	species (plural)
subsp.	subspecies (singular)
SVHS	super video home system
T	time
USA	United States of America
UV	ultraviolet light
var.	variety
v/v	volume to volume ratio
V8A	V8 agar
w.t.	wild-type
w/v	weight to volume ratio
λ	molar (equivalent) conductivity
μ	mean of a population
μg	micrograms
μl	microlitres
μM	micromolar
μm	micrometre
σ	standard deviation of a population
σ_d	standard deviation of the difference between any two means
ψ	matric potential
~	approximately

Chapter 1.

Introduction.

1.1 General Introduction.

The zoosporic pathogens of the family Pythiaceae are of economic importance in irrigated British glasshouse systems. Consequently there is pressure to develop methods to control these pathogens. For biological and economic reasons non-toxic chemical control is an option. An understanding of the biology of the closely related genera *Phytophthora* and *Pythium* may open up commercial methods of acceptable control.

Infection by zoospores is a multi-stage process. The resource base for zoospore production is the hyphal mycelium. Therefore, the greater the resource base the greater the potential disease pressure. Sporangia are the structures in which cytoplasm differentiates into zoospores and from where zoospores are released. Once zoospores are released into the bathing medium their helical swimming pattern is modified by exogenous factors to facilitate taxis to the host. Prior to encystment a zoospore locates a suitable infection site and orientates its fixed point of future germination relative to the infection site. After encystment and adhesion to the host surface a single germ tube is produced which is capable of tropism to the site of host penetration. The germ tube ultimately develops into a hypha, which infects the host.

1.2. Biology of the family Pythiaceae.

1.2.1. General biology.

Mycelium of the Pythiaceae is coenocytic with no or few septa. Oomycetes differ from the Eumycota in having β-1,3-glucans, β-1,6-glucans and β-1,4-glucans (cellulose) instead of chitin as the principal cell wall components (Bartnicki-Garcia & Wang, 1983). Another feature that is unique to the Oomycetes is mycolaminarin (a β-1,3-glucan) being the storage carbohydrate (Wang & Bartnicki-Garcia, 1973). The Pythiaceae require an exogenous supply of β-hydroxyl sterols for reproduction because of their inability to synthesis sterols *de novo* (Elliott, 1983). However, this peculiarity does confer insensitivity to the polyene antibiotics (Eckert & Tsao, 1962). The polyene antibiotics target sterol synthesis pathways.

1.2.1.1. Biology of *Pythium aphanidermatum*.

On agar *Py. aphanidermatum* is woolly in appearance and grows rapidly between 10 °C and 40 °C, with the optimum temperature being between 35 and 40 °C. However, zoospore production is optimal between 25 and 30 °C. *Py. aphanidermatum* is homothallic with the sexual organs, oogonia and antheridia, being produced in a ratio of one-to-one or occasionally one-to-two. Although usually found juxtaposed, the reproductive structures can originate from different hyphae. The smooth globose oogonia terminate from straight stalks and have an average diameter of 23 µm. Antheridia are usually intercalary, sometimes terminal, are broadly sac shaped and 10 to 14 µm long and wide. The resulting oospores loosely fit in the oogonium and have an average diameter of 20 µm with wall thickness being between 1 and 2 µm. An encysted zoospore is typically less than one quarter the size of an oospore (in terms of volume) with an approximate diameter of 12 µm. Sporangia, the structures that release zoospores, are terminal complexes of swollen hyphal branches of varying length and up to 20 µm wide. The morphology of sporangia is highly variable, but they are generally inflated, lobe-forming structures (Plaats-Niterink, 1981).

1.2.1.2. Biology of *Phytophthora parasitica*.

Ph. parasitica has a similar temperature tolerance range to *Py. aphanidermatum*, but the optimum temperature range is lower at between 30 and 32 °C (Waterhouse, 1956). However, the temperature range for sporangia formation is between 15 and 30 °C, with the optimum temperature being between 20 and 25°C (Ribeiro, 1983). Mycelial growth of *Ph. parasitica* is annulate on agar and not woolly. Oogonia when produced are globose, honey coloured, smooth or wrinkled structures between 18 and 25 µm in size. The resulting oospores are smooth, honey coloured, thick walled, globose structures with a diameter between 15 and 20 µm. The sporangia of *Ph. parasitica* are more consistent in morphology than of *Py. aphanidermatum* and are terminal ovoid structures with dimensions of 25 to 50 µm long by 20 to 40 µm wide. Although sporangial morphology is conservative the distribution within the hyphal mat is variable. Motile zoospores of *Ph. parasitica* are 9 to 12 µm long and 5 to 8 µm wide (Waterhouse, 1956).

1.2.2. Genetics.

Generally fungi (Ascomycetes, Basidomycetes, Zygomycetes) are haploid. For a time Oomycetes were also believed to be haploid (Dick and Win-Tin, 1973). However, it is now known that they are diploid in the vegetative state (Shaw, 1983). In the young gametangia (antheridia and oogonia), the DNA content per nucleus is twice that of a nucleus in a vegetative cell because of meiosis, that is, there are a tetrad of haploid nuclei. Meiosis occurs after karyogamy, which in turn occurs immediately after gametangial union (Shaw & Khaki, 1971; Khaki & Shaw, 1974). This high DNA content per nucleus is subsequently reduced through meiosis and the resulting gametes each have a haploid nucleus, or one quarter the DNA content of the young gametangia (Mortimer & Shaw, 1975).

Peronosporales can either be homothallic or heterothallic (Fincham *et al.*, 1979). Homothallism is probably the commonest mode of sexual reproduction in fungi as a whole (Burnett, 1975). Both *Ph. cryptogea* and *Ph. parasitica* are heterothallic, although some isolates of *Ph. parasitica* have been found to produce oogonia and oospores in single cultures (Erwin & Ribeiro, 1996).

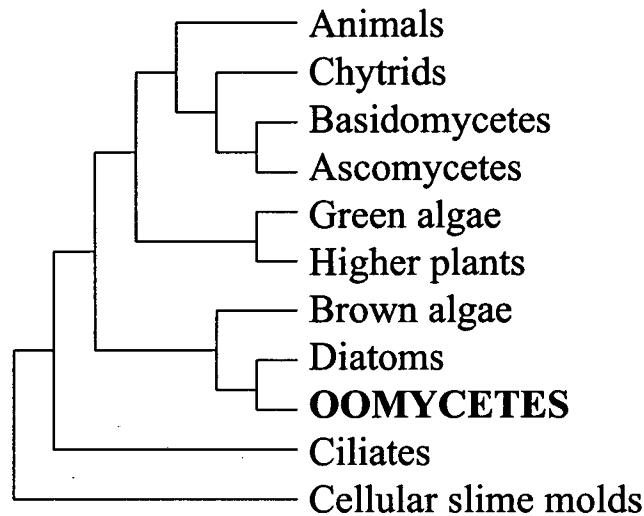
1.2.3. Taxonomy.

Both *Phytophthora* and *Pythium* genera are placed in the family *Pythiaceae* in the class Oomycota. The Oomycota are one of the five groups of fungi that produce zoospores (Alexopoulos *et al.*, 1996). Traditionally the Oomycota have been grouped into four primary orders: the Lagendiales; the Leptomitales; the Peronosporales and the Saprolegniales. However, the latter two groups have recently been elevated to form the basis of two new sub-classes, the Peronsporomycetidae and the Saprolegniomycetidae (Beakes *et al.*, 1995).

The morphological plasticity of the Oomycota causes classification, by traditional methods, to be difficult. The presence of tubular mitochondrial cristae, tripartite mastigonemes and the presence of β-1,3 or β-1,6 linked glucans in the cell wall of Oomycota are features common with the chromophyte algae, but not common to the Basidiomycetes and Ascomycetes. The sequencing of 16s RNA and of the NAD sub-unit 5 places the chromophyte algae, the diatoms and Oomycota in proximal phylogenetic groups (Bhattacharya and Medlin, 1992; Parquin *et al.*, 1995). Dick (1995), based on phylogenetic studies, placed the Oomycota in the Chromista kingdom. This kingdom, which also contains the brown algae and all the protists, is characterised by the presence of tripartite tubular hairs (mastigonemes) arranged

in two rows along the flagellar shaft or chloroplast reticulum or both (Cavalier-Smith, 1986). Therefore, the *Pythiaceae* are not phylogenetically close (Figure 1.1) to the eumycetes, but do exhibit physiology, morphology and life cycle that is consistent with the eumycetes. The genetic variability, based on nuclear ribosomal DNA, is more conservative for *Phytophthora* than *Pythium* (Lévesque *et al.*, 1999).

Figure 1.1. Qualitative phylogenetic placement of the Oomycetes.



(Based on Judleson, 1997.)

1.3. Ecology.

Pythium spp. are found in tropical, temperate and even cold regions of the world. Species have been isolated from soils of arable land, pastures, forests, nurseries, fresh water marshes, swamps and fresh water. In contrast, dry sandy places, dry forests and salt marshes are generally poor in *Pythium* spp. They occur most abundantly in the superficial rhizosphere of cultured soils, but have also been recorded at a depth of 3.55 m (Plaats-Niterink, 1981). The abundance of *Pythium* spp. is very low in non-cultivated or very acidic soils where *Trichoderma viride* is present (Barton, 1958). However, Barton (1958) did not implicate *T. virde* as a biological control agent, but concluded that the low pH was responsible for the absence of *Pythium* spp.

Pythium spp. with filamentous non-swollen sporangia have been found to be good pioneer fungi. For example, 89% of fungal isolates in 1968 from the newly reclaimed polder of Zuidelijk Flevoland (the Netherlands) were identified as *Pythium* spp. with filamentous non-swollen sporangia. After four years the number of isolates in this group had fallen to 9% (Plaats-Niterink, 1975). Further evidence of *Pythium* spp. as pioneer fungi is found in the ability to isolate *Pythium* spp. from the initial stages of marshes, but not from established marshes and swamps (Apinis, 1964).

Animals (including man) can facilitate the distribution of *Pythium* species. *Pythium* has also been isolated from the nests, intestines, feathers and droppings of free-living birds in the former Czechoslovakia and Yugoslavia (Plaats-Niterink, 1981). Thornton (1970) implicated earthworms in transporting aquatic phycomycetes. Out of 15 earthworm casts analysed three harboured species of *Pythium*. The external surfaces of earthworms are also believed to transport aquatic phycomycetes, including *Pythium* spp. In Scotland, Hutchinson and Kamel (1956) isolated *Pythium* spp. from the alimentary canal of the earthworm (*Lumbricus terrestus* Linn.)

1.4. General pathology.

Zoospores of *Pythium* spp. and *Phytophthora* spp. have been demonstrated to be propagules of disease in recirculating irrigation systems (Stanghellini *et al.*, 1996a; 1996b; von Broembsen & Deacon, 1997). The host range for both *Pythium* and *Phytophthora* is large. For *Phytophthora*, with 43 species and four varieties, over a thousand pathogen-host combinations have been cited (Gregory, 1983). The economic and social importance of these two genera is significant, with the greatest historic example being potato blight caused by *Phytophthora infestans* in Western Europe in the nineteenth century.

Py. aphanidermatum as a pathogen has an extensive host range, causing diseases of various seedlings including damping off of the Cucurbitaceae and Solanaceae families (Waterhouse and Waterston, 1964b). The host range of *Ph. parasitica* comprises of 58 families and the diseases include damping off of seedlings of tomato (*Lycopersicum esculentum*) and brown stem rot of tobacco (*Nicotiana* spp.) (Waterhouse and Waterston, 1964a)

1.5. Generalised life cycle for the Pythiaceae.

Mycelium of *Phytophthora* grows well on complex media. When the mycelium becomes stressed due to environmental changes one of three possible reproductive structures can be produced. Which structures form is dependent on which environmental factors have altered.

If mycelia are starved then zoosporangia are produced. However, production of sporangia requires light, high humidity and sterols, and is inhibited by sugars and ionic copper. When subjected to a cool solution with a high matric potential sporangia will germinate (Elliott, 1983). Direct sporangial germination (the production of germ-tubes directly from the sporangium) occurs in environments with high nutrition (particularly sugars and amino acids) and at the optimal temperature for vegetative growth. Germ tubes will subsequently grow with the support of exogenous nutrition. However, if germinating sporangia are subject to sub-optimal temperature and lack of nutrients (typical of an irrigation system based on nutrient film technique – see Section 1.7.1) then zoospores are produced (indirect germination) and subsequently liberated. Zoospores after a period of motility will encyst and germinate (Elliott, 1983).

Chlamydospore production occurs when mycelia are subject to low nutrition, aeration and the presence of sterols. Light and temperature also govern chlamydospore production. In the presence of amino acids chlamydospores germinate. If the germinating spore is in an environment high in nutrients - namely sugars and a suitable nitrogen source - then a germ tube forms which will subsequently develop into a mycelium. Otherwise, under low nutrition, the chlamydospore produces a germ tube and a sporangium, which under the influence of temperature and cations produce encysted zoospores (Elliott, 1983).

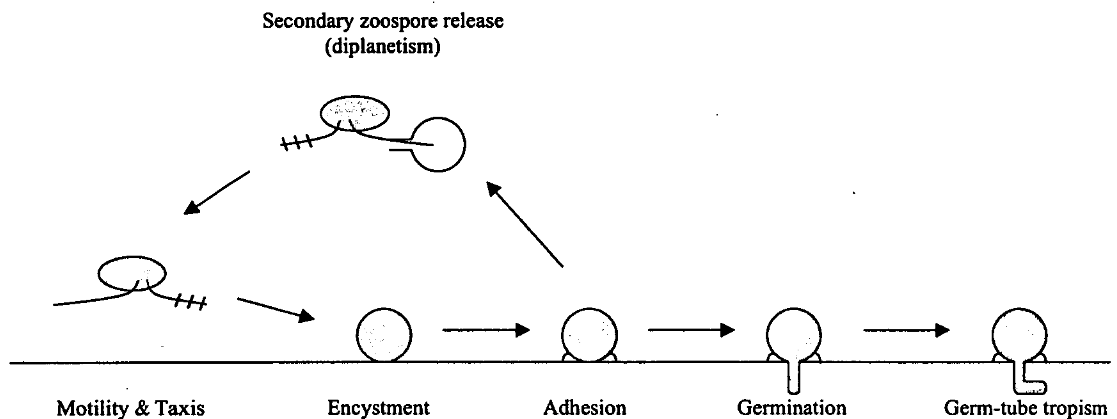
The third option for a stressed mycelial mat is to produce oospores. Oospore production occurs in darkness, low nutrition but in the presence of sterols and at the optimal temperature. For an oospore to germinate blue light, particular enzymes and cations, a temperature change and the elapse of a time-period are required. A germinating oospore in a balanced nutritional state produces both germ tubes and sporangia (Elliott, 1983).

1.6. The infection sequence of zoosporic fungi.

The infection sequence of the zoosporic fungi in irrigation systems based on NFT is essentially as follows (Figure 1.2). Zoosporangia form from a mycelial network. Once the

cytoplasm of the sporangia has differentiated into zoospores, the zoospores are liberated and locate a host by taxis. At a suitable location on a host the zoospore will encyst and proceed to germinate. Zoospore germination can either be direct or indirect (diplanetism). Diplanetism is the release of a secondary zoospore, whereas direct germination is the production of a germ tube which, facilitated by tropism, will infect the host.

Figure 1.2. The homing sequence of a zoosporic fungus in an irrigation system based on NFT.



(Adapted from Deacon and Donaldson, 1993)

1.6.1. Vegetative growth.

Growth by *Phytophthora* and *Pythium* is typically fungal, that is, filamentous growth is achieved by tip growth coupled with branching. The nutrients that drive both vegetative growth and reproduction are derived from absorbing simple nutrients directly across the cell wall and membrane. Complex polymers are degraded extracellularly by enzymes prior to absorption (Erwin and Ribierio, 1996).

Apical growth involves the controlled expansion of the cell wall and plasmalemma (Heath, 1995). This polarised cell growth only occurs when the expansion of the cell surface is focused at one point (Gow, 1994). The model proposed by Robertson (1958 and 1959), one of the first workers to suggest a feasible explanation of tip growth, 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 in 1993. This new model assumes a continuous secretion at the apex of an expansible mixture of wall polymers, that is continuously removed ventrically towards the base of the extension zone, where a rigid

complex arises by interactions between and among the constituent wall polymer chains. As new wall material is added it becomes progressively more cross-linked and able to resist turgor pressure.

Robertson's (1958 and 1959) suggestion that tip growth is driven by turgor pressure is negated by recent advances in knowledge. In *Achyla bisexualis* it was found that at low turgor pressure hyphal growth was maintained by softening the apical wall (Money and Harold, 1992). Later work by these workers found that the Oomycete *Saprolegnia ferax* could generate tip-growing hyphae in the absence of measurable turgor (Money and Harold, 1993). It appears that there is an interplay between turgor pressure and wall biogenesis, which ultimately is controlled by the cytoplasm and the cytoskeleton (Harold, 1997).

1.6.2. Sporangiogenesis.

In the sporangia of *Ph. cinnamomi* structural integrity was found by Hyde and Hardham (1992) to be maintained through extensive microtubule arrays using the nuclei as nodes of microtubule attachment.

Sporangia formation in Oomycete fungi requires high moisture levels, either in the form of high relative humidity (circa. 100%) or a matric potential approaching zero (Ribeiro, 1983). Pfender *et al.* (1977) working with *Ph. megasperma* in soil found maximum sporangiogenesis in flooded soils with a matric potential of -0.28 MPa. Sidebottom and Shew (1984) found that for *Ph. parasitica* the optimum matric potential for sporangial production was between -4 and -20 kPa. Similar results were seen in their follow-up work (Sidebottom & Shaw, 1985). In 1982, Bernhardt and Grogan reported that both *Ph. capsici* and *Ph. parasitica* failed to form sporangia in saturated soils unless incubated for two days at a high matric potential (-20 to -30 kPa). However, a degree of water salinity has been found to enhance sporangial production in *Ph. parasitica* (Blaker & MacDonald, 1982).

Low nutrient levels also encourage sporangial formation; for example, a glucose concentration greater than 2.8 mM is inhibitory (Ribeiro, 1983). The nitrogen source also has a major influence on sporangiogenesis. Singh (1973) found, of the nitrogen sources tested, that L-α-alanine was the greatest promoter of sporangiogenesis of *Phytophthora palmivora*, while other L-amino acids and ammonium sulphate caused complete suppression of sporangial formation. However, with respect to L-aspartic acid and L-glutamic acid there are

conflicting reports (see Ribeiro, 1983). Some workers found that these amino acids inhibited sporangial formation, while others observed a stimulatory effect. However, these differences could be due to different concentrations being used.

Halsall and Forrester (1977) demonstrated that certain cations (Ca^{2+}, Fe^{3+}, Mg^{2+} and K^{+}) influenced sporangia numbers in four *Phytophthora* species. For example, *Ph. cinnamomi* produced maximum sporangia numbers in solutions when cation concentrations were 0.71 mM for Ca^{2+}, 0.16 mM for Mg^{2+}, 0.62 mM for K^{+} and 0.11 mM for Fe^{3+}. Halsall (1977) found that the presence of Cu^{2+} and Mo^{2+} suppressed sporangial numbers while elevated levels of Zn^{2+} enhanced sporangial formation.

One of the distinctive characteristics of the Pythiaceae is the inability to synthesise sterols *de novo*, although they are essential for asexual and sexual reproduction (Webster, 1980). Low concentrations of exogenous sterols increase vegetative growth, stimulate asexual reproduction, influence zoospore formation and play a role in the formation of sexual reproductive structures (Ribeiro, 1983). Vegetative growth of *Ph. lateralis* is unaffected by β-sitosterol concentration up to 200 $\mu g.ml^{-1}$, but sporangial formation is highly dependent on β-sitosterol concentration; a concentration of 10 $\mu g.ml^{-1}$ was found to be optimal for sporangiogenesis. Hendrix (1964) also found that various sterols at low concentrations induced asexual and sexual reproduction in species of both *Pythium* and *Phytophthora*; in the case of *Py. aphanidermatum* sterol supplements were associated with the production of large zoosporangia.

Limitation of atmospheric oxygen significantly reduces sporangial formation (Ribeiro, 1983). Mitchell & Zentmyer (1971) also found that either decreasing oxygen or increasing carbon dioxide concentration reduced sporangial formation. However, the sporangia under investigation went on to produce oospores - suggesting that the structures observed were, in fact, not sporangia.

For *Phytophthora lateralis* Englander & Roth (1980) noted that illumination of cultures produced four times as many sporangia as in cultures that were not illuminated. Hendrix (1967) found that *Ph. palmivora* and *Ph. capsici* respond to light in a similar manner as *Ph. lateralis*. Similarly, Hendrix (1967) observed that sporangia of *Ph. capsici* did not liberate zoospores in a dark regime and that sporangial morphology was affected by the absence of light. In addition, Harrish (1965) found that light enhanced the formation of sporangia of

many species of *Phytophthora* with the exception of *Ph. syringae,* where light was found to inhibit sporangial formation. There are a few species of *Phytophthora* where light has no apparent effect on sporangiogenesis. Brasier (1969) observed that two isolates of *Ph. palmivora* responded positively to light while a third isolate failed to respond.

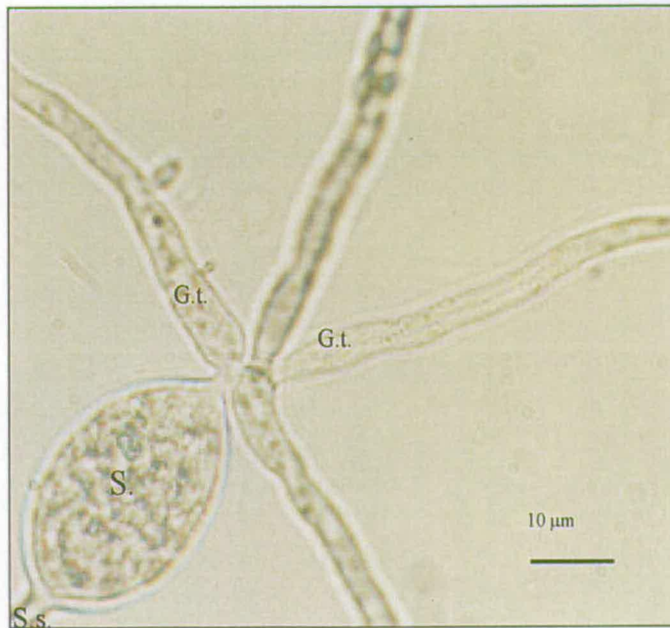
Other factors which affect sporangia formation include colony size (Davison & Tay, 1986), aeration (Mitchell & Zentmyer, 1971; Davison & Tay, 1986), the presence of microorganisms (Marx & Bryan, 1969; Ayres & Zentmyer, 1971), age of mycelium (Ayres & Zentmyer, 1971), UV (Ribeiro *et al.*, 1976) and culture medium (Blaker & MacDonald, 1983; Sidebottom & Shew, 1984; Sidebottom & Shew, 1985). The suggestion that the presence of microorganisms affects sporangia formation can be explained by their ability to remove sporangia-suppressing nutrients by catabolism, or by the production of metabolites which stimulate sporangial formation.

1.6.2.1. Germination of sporangia.

The contents of a sporangium can either germinate directly by producing germ tubes (direct germination) (Figure 1.3) or by the cytoplasm differentiating and cleaving into zoospores which subsequently are liberated into the surrounding liquid medium (indirect germination) (Figure 1.4).

Clerk (1972) identified several factors that promote direct germination of sporangia of *Phytophthora palmivora*. These factors were: a temperature similar to or greater than for optimal mycelial growth (22 °C); 1% peptone or yeast extract; cocoa pod extract and exudate; 10 mM $CaCl_2$; 1-10 mM $MgSO_4$; 0.5% fructose, galactose, glucose, lactose, maltose or sucrose; 100 ppm arginine, aspartic acid, glutamic acid, glycine, leucine, tryptophan or thiamine. In contrast, zoospore formation was inhibited by cocoa pod extract, peptone and yeast extract, several amino acids, carbohydrates and inorganic salts. Ribeiro (1983) has confirmed that these factors are conducive to direct germination for other species of *Phytophthora*. In addition to these factors, Pfender *et al.* (1977) report that the matric potential of soil also governs whether sporangia of *Ph. megasperma* germinate directly or release zoospores. MacDonald & Duniway (1978) also found that high matric potential ($\psi <$ 5 mbar) inhibited zoospore release, but solute potential had little influence of zoospore release.

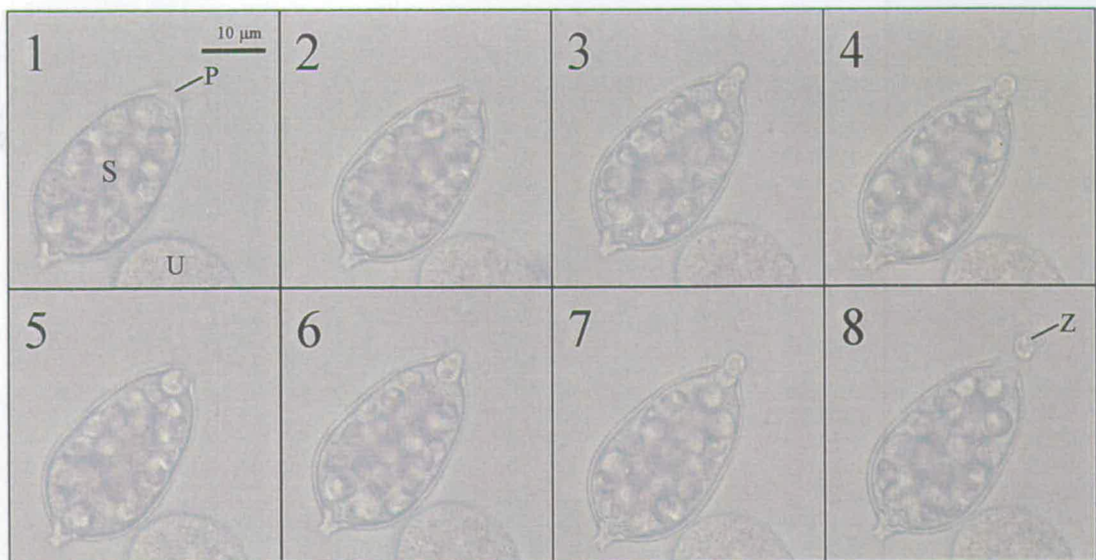
Figure 1.3. Direct germination of a sporangium of *Phytophthora palmivora*.



Four germ tubes (G.t.) originating from a single sporangium (S). The sporangium is attached to a stalk (S.s.), which can be seen in the bottom-left of the photomicrograph.

Photomicrograph supplied by D. Grayson.

Figure 1.4. Indirect germination (zoospore release) of a sporangium of *Phytophthora palmivora*.



Over the series of photomicrographs (1-8) the papillium (P) of a sporangium with differentiated cytoplasm (S) can be seen to dissolute and a zoospore (Z) released. Proximal to this sporangium is a sporangium with undifferentiated cytoplasm (U).

Photomicrographs supplied by D. Grayson.

In the nutrient solution of an irrigation system based on NFT, the matric potential is low and cations are present (Appendices 8 to 13). The concentrations of amino acids and other organic compounds are dependent on the age of the crop, whether the system is run-to-waste or recirculated, and presence of sterilisation equipment. In a recirculating system, the electrical conductivity (solute potential) is kept constant, but organic compounds will accumulate.

Zoospores are formed through the processes of sporangial cytoplasmic differentiation and cleavage. Discharge of zoospores from sporangia of *Phytophthora* is driven by high relative turgor pressure in the sporangium, which can only occur in a hypotonic medium. When the limit of elasticity for the sporangium wall (papillium) is exceeded then it ruptures allowing the ejection of zoospores into the environment (Gisi, 1983). In *Pythium*, zoospores are released from a vesicle that forms out of a sporangium.

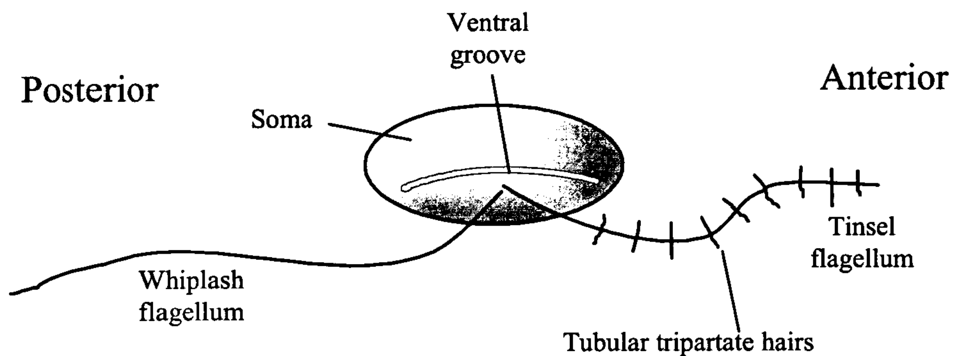
Although the apparent cause of zoospore release is high turgor pressure there are other factors which have been correlated with zoospore release. In *Pythium* spp. Shipton (1987) discovered that a range of cations, including calcium, regulates zoospore release. Transmission electron micrographs suggest that both calcium and potassium can cause as much as an eight fold thickening of the sporangial vesicle wall. This elevated wall stability undermines the mechanism of zoospore release. Personal observations of *Py. aphanidermatum* revealed that the vesicle inflates rapidly (~ 2s) from the sporangium. It is hard to conceive that a fully formed wall, as suggested by Shipton (1987), binds the vesicle. However, some gelatinous material probably surrounds the vesicle. The thickness of the vesicle 'wall', as observed by Shipton (1987) was 35-115 nm (in the absence of cations), but 170-970 nm in the presence of cations. A typical phospholipid bilayer is about 10 nm thick (Gennis, 1989), therefore it can be assumed that the vesicle is more than membrane-bound cytoplasm.

In earlier work Shipton (1985) noted that young (one-day old) colonies had greater releases of zoospores than older colonies. Hendrix (1967) found in addition to sterols being a requirement for sporangia production that sterol deficiency inhibited differentiation of cytoplasm into zoospores.

1.6.3. Motile zoospores.

Zoospores of Oomycetes are biflagellate with the point of insertion for both flagella in the deep groove on the zoospore ventral surface (Figure 1.5). The anterior 'tinsel' flagellum generates 90% of the swimming thrust (Carlile, 1983); its appearance is due to mastigonemes (tubular hairs) on the flagella which act like oars of a boat (Holwill, 1982). The posterior flagellum is longer than the anterior and lacks mastigonemes. The action of the posterior flagellum has given rise to the name 'whiplash flagellum'; this action is responsible for zoospore turning (Carlile, 1983).

Figure 1.5. Simplified diagram of a typical zoospore of the Oomycota.



Adapted from Warburton, 1997.

During swimming acyl glycerides and free fatty acids provide the major energy source for zoospores of *Ph. palmivora* (Bimpong, 1975). These lipid reserves are large enough to supply the energy required for swimming up to several days in the absence of exogenous nutrients (Hickman & Ho, 1966). However, zoospores of *Ph. drechsleri* have been reported to utilise exogenous sugars, albeit at a low rate compared to during cyst germination (Barach *et al.* 1965). More recent work by Penington *et al.* (1989) found that zoospores and cysts of *Ph. palmivora* were impermeable to amino acids, glucose and inorganic phosphate. Uptake of these substances was observed only after the emergence of germ tubes. The consumption of carbohydrates and proteins during motility is negligible; however, during cyst germination usage of these nutrients increases (Bimpong, 1975). During motility the regulation of the tricarboxylic acid cycle is modified as indicated by NAD-isocitrate and malate dehydrogenase concentration increasing slightly, while NADP-isocitrate and succinate dehydrogenase concentration decreases slightly.

Extended zoospore motility is also reported by Bimpong and Clerk (1970) for *Ph. palmivora* zoospores in distilled water at 17 °C. They found that the motility time was reduced in dense zoospore populations, in the presence of calcium chloride, low pH, various salts solutions, amino acids and sugars. They, along with Benjamin and Newhook (1982), found that motility is curtailed through frequent contact with surfaces, although the magnitude of this effect is species specific. Most of these factors (Ca^{2+}, amino acids, and sugars) are present, at varying concentrations, in the nutrient solution of an irrigation system.

The default swimming movement of Oomycete zoospores *in vitro* is a helical path with the soma rotating about the long axis (Carlile, 1983). A 'random walk' was believed to be an inherent property of biflagellated zoospores (for example, Allen & Newhook, 1973); however, Warburton (1993) found this only in *Phytophthora* spp., whereas *Pythium* spp. did not make random turns in the absence of obstacles.

Donaldson and Deacon (1993a) found that zoospore swimming behaviour of *Py. aphanidermatum* was modified through calcium-modulating drugs to cause five distinct swimming modes. The implication of this work is that calcium plays a major regulatory role in zoospore swimming. Other group II cations such as magnesium have also been cited as influencing swimming of flagellate cells (Tamm, 1989; Bloodgood, 1991).

Deacon and Donaldson (1993) have reviewed the homing sequence of zoospores. It is evident that many factors are involved during each phase of the homing sequence. Zoospore chemoattractants include certain amino acids and sugars, with various cations being essential for processes that facilitate the zoosporic homing sequence; for example, calcium is required for motility and encystment.

Taxis of zoospores of *Phytophthora* spp. was reviewed by Carlile (1983), updated and extended to include zoospores of *Pythium* spp. by Deacon and Donaldson (1993). Swimming zoospores of Oomycete fungi exhibit various taxes including geotaxis, electrotaxis, rheotaxis and chemotaxis. The chemotactic nature of *Pythium aphanidermatum* is well studied - for example, by Royle & Hickman (1964) and Donaldson & Deacon (1993c).

The *in vitro* studies on the effects of electric currents on zoospore motility are extensive (Troutman & Wills, 1964; Ho & Hickman, 1967; Katsura & Miyata, 1971; Khew & Zentmyer, 1974; Miller *et al.*, 1988; Morris *et al.*, 1992). *In vivo* this phenomenon may cause

zoospores to accumulate at root tips, which are known to generate electric fields (Salisbury & Ross, 1991). The relevance of these *in vitro* findings is contentious. Hickman (1970) argues they are of little or no significance, whereas Gow *et al.* (1992) believe electrotaxis may contribute to host location. Deacon and Donaldson (1993) suggest that electrotaxis works only in conjunction with chemotaxis. The observations of Hawes *et al.* (1987) who found that *Ph. dissotocum* zoospores are attracted to single root cap cells in suspension supports the view that electrotaxis is non-essential in host location.

Morris and Gow (1993) suggest the possible mechanism of electrotaxis to be a combination of modulated turning frequency and electrophoresis. They observed that electrical fields of physiological magnitude increased the turning frequency of zoospores of *Ph. palmivora* three-fold and that the charge on the posterior flagellum relative to the charge on the anterior flagellum dictates whether the zoospore is attracted to the anode or cathode. In *Py. aphanidermatum* the posterior flagellum has a relative negative charge and was orientated to the cathode in an applied electric field while *Py. palmivora* with a relative positive charge on the posterior flagellum was generally orientated to the anode.

Various taxes may influence the swimming direction of zoospores in relation to the surface of the medium. For *Phytophthora* spp. Carlile (1983) suggests that negative geotaxis is responsible for keeping zoospores near plant rootlets, as observed by Cameron and Carlile (1977). This increases proximity of zoospores to the host and improves the chance of infection.

Chemotaxis of zoospores of *Phytophthora* and *Pythium* species have been extensively researched *in vitro*. Donaldson and Deacon (1993c) working with three species of *Pythium* reported significant positive chemotaxis to five out of the 19 L-amino acids tested, but not to any of the equivalent D-amino acids. Chemotactic response to sugars was varied, with D-mannose being the strongest attractant tested. Ethanol is also a chemoattractant for zoospores of some fungi (Allen & Newhook, 1973; 1974; Cameron & Carlile, 1978). In terms of infection, ethanol might serve as a signal that a plant is compromised through anaerobic conditions. In the development of a dipstick immunoassay of *Ph. cinnamomi* that exploits zoospore taxis, Cahill and Hardham (1994) found that in addition to amino acids, sugars and alcohols, various phenolics, isovaleraldehyde, abscisic acid (a phytohormone) and pectin are all chemoattractants. Allen and Harvey (1974), working with *Ph. cinnamomi*, and Cameron and Carlile (1980), working with *Ph. palmivora*, found negative chemotaxis to low

molecular weight cations. Both groups concluded that effectiveness of the cation as a repellent was proportional to its ionic conductivity (position in the Hofmeister lyotropic series[1]).

Host specific taxis of Oomycete zoospores cannot only operate at the species level (for example, Goldberg *et al.*, 1989) but zoospores of some species can also differentiate between different plant cultivars (Chi & Sabo, 1978). Plants when wounded release a spectrum of compounds including amino acids and sugars. Some of these compounds are general attractants, while others may be fungus-specific non-attractants which interfere with chemotaxis (Donaldson & Deacon, 1993b).

1.6.4. Zoospore encystment.

Anatomical changes that occur during zoospore encystment have been reported for species of both *Pythium* (Grove, 1970; Lunny & Bland, 1976; Grove & Bracker, 1978) and *Phytophthora* (Reichle, 1969; Hemmes & Hohl, 1971; Bimpong & Hickman, 1975; Hardham *et al.*, 1991). During encystment the naked zoospore soma becomes spherical in shape. In *Blastocladiella emersonii* (a zoospore producing Chytridimycete) a glycoprotein cyst coat is exuded through the zoospore membrane and beneath this a microfibrillar cyst wall forms (Truesdell & Cantino, 1971). During encystment the flagella are either shed or retracted (Ho & Hickman, 1967; Reichle, 1969; Grove & Bracker, 1978), and cytoplasmic organelles are reorganised, and occupy positions different from those found in the motile zoospore. Certain organelles, such as the water expulsion vacuole, though present in the naked zoospore, are absent in the walled cyst.

1.6.4.1. Factors involved in inducing zoospore encystment.

The process of zoospore encystment is closely coupled with site-selection. The chemical nature of a substrate can induce zoospore encystment. Zoospores of *Py. coloratum* were observed by Shishkoff (1989) to encyst on hypodermal cells of onion (*Alluim cepa* L.), and it was found that more zoospores encyst (i) next to killed plant cells than living cells, (ii) next to short cells of the hypodermis than could be statistically expected by chance and (iii) on

---

[1] Hofmeister lyotropic series: $K^+ > Na^+ > Cs^+ > Li^+ > Mg^{2+} > Ca^{2+} > Ba^{2+}$

non-fluorescent long cells than fluorescent long cells - autofluorescence is caused by suberination of the lamella. Thus *Py. coloratum* showed site selection.

Chemotaxis to root exudates is well established (for example, Zentmyer, 1961). Fucosyl residues present in root surface slime of plant roots have been implicated in the binding of cysts of *Py. aphanidermatum* (Hinch & Clarke, 1980; Longman & Collow, 1987; Estrada-Garcia *et al.*, 1990b). Root surface slime is rich in uronic acids and uronate-rich compounds (such as poly-D-galacturonic acid, pectin and alginate) which have been shown to cause encystment of zoospores (Irving & Grant, 1984; Grant *et al.*, 1985; Hardham & Suzaki, 1986; Zhang *et al*; 1990; Jones *et al*; 1991; Donaldson and Deacon, 1993b). Zoospores of the pathogenic *Py. marinum* readily encyst on thalli of *Porphyra* spp. (red algal host), but have not been observed to encyst on green or brown algae which are not hosts (Kerwin *et al.*, 1992). *Py. graminicola* also discriminates between hosts (graminaceous) and non-hosts (non-graminaceous), albeit not to the same degree as *Py. marinum* (Mitchell and Deacon, 1987). Other host surface factors implicated in the encystment of fungal zoospores include chitin and cellulose (Mitchell & Deacon, 1986). Hydrostatic pressure (Held, 1972), temperature, pH, dilution and mechanical agitation (Ho & Hickman, 1967) are some of the known physical treatments which induce zoospore encystment. Chemical factors that are ubiquitous in irrigation solutions and induce zoospore encystment are ions (Bryt *et al.*, 1982), amino acids (Donaldson & Deacon, 1993c), lectins (Hardham & Suzaki, 1986; Longman & Callow, 1987) and phosphatidic acid (a phospholipid) (Zhang *et al.*, 1992). Cysts of *Phytophthora* are able to adhere to a wide range of solid, non-biological surfaces including glass, polystyrene plastic, cellophane, and to a lesser extent Teflon and Parafilm (Bartnicki-Garcia & Sing, 1987).

1.6.4.2. The mechanics of zoospore encystment.

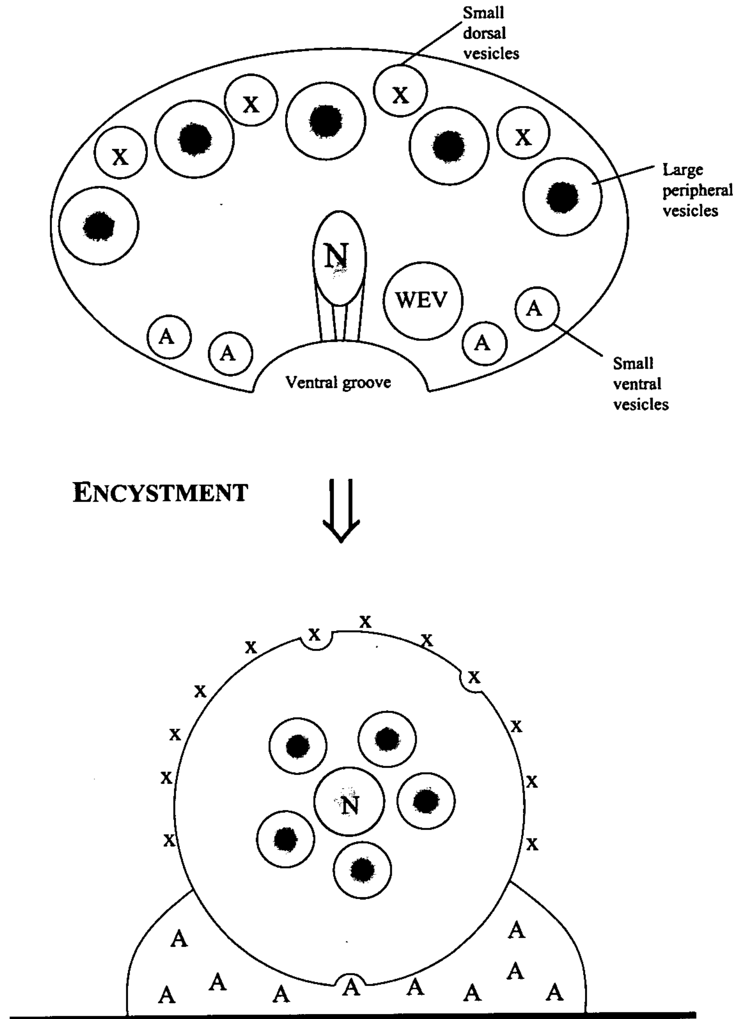
Within zoospores during encystment the pre-formed peripheral vesicles fuse with the plasma membrane and deposit a coat on the spore surface that is principally glycoprotein (Sing & Bartnicki-Garcia, 1975a, b). Calcium has been implicated in triggering the fusion of peripheral vesicles to the plasma membrane and the exocytosis of coat material (Hemmes & Pinto da Silva, 1980). From underneath the cell coat a cell wall develops over the following several minutes (Sing & Bartnicki-Garcia, 1975a).

Using monoclonal antibodies and lectins, other types of vesicles, and their fate, have been identified in encysting zoospores of *Ph. cinnamomi* (Hardham *et al.*, 1986; Gubler & Hardham, 1988). Small peripheral dorsal vesicles exocytose the glycoprotein that forms the cell coat, whereas small peripheral ventral vesicles exocytose an adhesive material, which mediates attachment to the adjacent surface. This adhesive material (high molecular weight proteins and glycoproteins) covers approximately one third of the cell surface and binds non-specifically to a large range of substrates (Gubler *et al.*, 1989). A third category of vesicles, the large peripheral vesicles, do not exocytose their contents, but move away from the plasma membrane and disperse throughout the cytoplasm (Gubler & Hardham, 1988). These large vesicles are believed to contain proteins for later use by the cyst (Gubler *et al.*, 1990; Estrada-Garcia, 1990a), although Grant *et al.* (1986) had previously supposed that the contents of these vesicles formed the cyst wall. Also during encystment the nucleus becomes spherical and relocates to the centre of the cyst, and the water expulsion vesicle disappears approximately 4 min after the onset of encystment (Mitchell & Hardham, 1999). Hardham (1995), reviewing the immunocytochemical studies on these vesicles, concludes that the smaller dorsal and ventral vesicles contain secretory material used for cyst adhesion, whereas the large peripheral vesicles are nutrient storage structures. Figure 1.6 shows schematically the changes that occur during encystment.

Grant *et al.* (1986) hypothesised that calcium had a central role in the encystment of zoospores. During zoospore motility endogenous calcium concentration is relatively high whereas sodium concentration is relatively low. This cation status was related to an active water expulsion vacuole. In hypo-osmotic conditions an active water expulsion vacuole is essential for the wall-less zoospore to avoid rupturing.

Early in the encystment sequence Grant *et al.* (1986) postulate that endogenous calcium level is reduced through expulsion of calcium to the environment. This is followed by sodium uptake, which coincides with reduced water expulsion vacuole activity. Some of the exogenous calcium binds to the zoospore surface. Calcium gated monovalent ion translocators are believed to regulate sodium uptake, and consequently influence water expulsion vacuole activity. Reduced water expulsion vacuole activity in hypo-osmotic conditions causes the zoospore turgor pressure to increase and the cell to swell, causing rupturing of peripheral vesicles - this has been recently confirmed by Mitchell and Hardham (1999).

Figure 1.6. Schematic diagram of the ultrastructural changes that occur in a zoospore during encystment.



The small ventral vesicles (A) discharge their adhesive contents at the ventral groove. The small dorsal vesicles (X) discharge their glycoprotein contents around the rest of the spore to from a cyst coat. The large peripheral vesicles and nucleus (N) assume a central location within the cyst, and the water expulsion vesicle (WEV) disappears.

Adapted from Warburton (1997).

In the presence of EGTA (a calcium chelator) adhesion is suppressed in newly encysted zoospores. In older cysts, which have lost adhesive properties, the addition of Ca^{2+} restores adhesion (Donaldson & Deacon, 1992). Deacon and Donaldson (1993), in a review, analyse the *in vitro* data of four papers (Irving *et al.,* 1984; Mitchell & Deacon, 1986; Iser *et al.*

1989; Donaldson & Deacon, 1992) and proposed a model of autoregulation of infection from zoospores with Ca^{2+} holding a central role. Their model is:

> Glycoprotein adhesive is fixed to surfaces by released Ca^{2+}. Adhesion prevents dispersion of released Ca^{2+}, enabling reabsorbtion and triggering of germination via a control centre, the fixed germination point. Otherwise, high (7 mM) Ca^{2+} is needed (e.g. suspended cysts). Orientation of encystment ensures that the Ca^{2+} signal is received by the control centre, causing autonomous germination after zoospore docking. Specific amino acids (host derived) can synergize Ca^{2+} uptake from low external levels, leading to germination of non-adhered or 'disorientated' cysts.

1.6.5. Diplanetism.

Diplanetism is the release of a secondary zoospore from a walled cyst. Cerenius & Söderhäll (1985) believe repeated zoospore emergence (diplanetism) in the genus *Aphanomyces* is an adaptation to parasitism. In the absence of nutrients (SDW), the majority of adhered cysts of *Py. aphanidermatum* will undergo diplanetism within 16h (Jones *et al.*, 1991), but in the presence of nutrients (bacteriological peptone) virtually all cysts will produce a germ-tube, and therefore are unable to undergo diplanetism. Von Broembsen and Deacon (1996) suggest that diplanetism requires no specific trigger, and is therefore the default mechanism in an adhered cyst. External calcium alone suppresses cyst diplanetism (von Broembsen & Deacon, 1996 & 1997). In contrast, calcium has a central role in the direct germination of cysts for species of both *Pythium* and *Phytophthora*. Donaldson and Deacon (1992) confirmed this hypothesis by chelating free ionic calcium and finding that direct germination was suppressed

1.6.6. Cyst germination.

Observation of encysted zoospores invariably germinating from the side of the cyst nearest to an attractant has given rise to the supposition that zoospores have a predetermined point of germination, and that during encystment the point of germination is orientated towards the attractant (e.g. Royle & Hickman, 1964; Allen & Newhook, 1973).

Mitchell and Deacon (1986) working with two *Pythium* species (*Py. aphanidermatum* and *Py. graminicola*) confirmed the above observations. When pre-encysted zoospores were placed near an attractant there was no preferential germination towards the attractant.

However, the germ-tubes often changed direction and grew towards the attractant. That is, germ tubes exhibited positive chemotropism; chemotropism was not found in vegetative hyphae.

Jones *et al.* (1991) found cysts germinated rapidly and with almost 100% efficiency on roots, in contrast to the maximum of approximately 70% germination *in vitro* found by Donaldson and Deacon (1992). This suggests exogenous factors promote cyst germination. However, zoospores and cysts are impermeable to amino acids, glucose and inorganic phosphate (Pennington *et al.*, 1989). But after germination, that is when a germ tube is present, the up-take of these low molecular weight molecules occurs. Therefore, it is expected that the exogenous triggers only bind to the cyst surface.

The inability of the cyst to uptake amino acids as exogenous triggers coupled with the central role of calcium in germination precludes the uptake of calcium by symport, but amino acids might act through receptors on the cyst membrane to open Ca^{2+} channels. Ca^{2+}-channel-linked glutamate receptors are widely reported in other organisms (e.g. Gilbertson *et al.*, 1991; Hollmann *et al.*, 1991) and might be expected to occur in Oomycete zoospores. However, Ca^{2+} channels linked to aspartate, asparagine or alanine receptors have not been reported (Warburton, 1997). A receptor-mediated role of amino acids would be consistent with the species-specific effects, as found for alanine by Donaldson and Deacon (1992).

During germination of cysts the large peripheral vesicle contents (glycoprotein) are degraded. This observation led Gubler and Hardham (1990) to suggest that these glycoprotein deposits are endogenous nitrogen stores for germ-tube formation. Energy for germ-tube formation is liberated from acyl glycerides and free fatty acids by the glyoxylate cycle and indirectly through the citric acid cycle, although both these cycles are more active during zoospore motility (Bimpong, 1975).

In parallel to energy liberation and glycoprotein degradation the amount of mRNA present is elevated; prior to germination zoospores contain a low level of translational mRNA (Penington *et al.*, 1989). The implication of this is that germ tube synthesis requires *de novo* protein synthesis; this is not a universal phenomenon in fungal spore germination (van Etten *et al.*, 1983). In addition to mRNA translation the nuclei of cysts undergo multiplication as the germ tube extends (Hooley *et al.*, 1982).

Exogenous nutrition is required to a far greater extent by germinating cysts than by motile zoospores. Barach *et al.* (1965) working with *Ph. drechsleri* suggested that exogenous nutrition is of little significance to motile zoospores; and that the availability of external nutrition (e.g. root exudates) enhances germ tube elongation. The uronic acids present in pectins and other complex uronates of root slime are inducers of germination (Grant *et al.*, 1985). Donaldson and Deacon (1993c) found for *Pythium* and *Phytophthora* species that amino acids and simple sugars also enhance germ tube elongation. The study of Byrt *et al.* (1982) concludes with stating that the presence of simple sugars or amino acids is a prerequisite for germ tube elongation. For germination oxygen was believed not to be required, but oxygen concentration has been positively correlated with germ tube growth (Davison & Tay, 1986).

1.6.7. Infection.

Infection of glasshouse crops is poorly researched. Literature on the diseases caused by the Pythiaceae predominantly covers which factors suppress or enhance disease. One of the best documented diseases caused by the Pythiaceae is black shank of tobacco (*Nicotiana tabacum*); this is due to the high value of tobacco and the potential destructiveness of black shank (MacKenzie *et al.*, 1983). This disease system is particularly relevant to infection of tomatoes (*Lycopersicon esculentum*) by *Ph. parasitica* because the pathogen is the same (species) and the hosts are in the same putative clade of the Solanaceae (Olmstead & Sweere, 1994).

1.6.7.1. Factors affecting infection.

The development level of tobacco plant root systems has influence on the infection by the pathogen (English & Mitchell, 1988 & 1989). Root tips possessing a meristem are more susceptible than older, larger roots. The soil matric potential has a large effect on infection of susceptible tobacco seedlings (Shew, 1982 & 1983). Although the major propagule of infection in soil is unknown, Shew (1982 & 1983) suggests that under higher matric potentials (close to saturation) the conditions are conducive to zoospore production and hence elevate infection. In addition to soil matric potential, Sidebottom and Shew (1985) correlated soil texture, soil drainage class, fumigation and nutrient status (calcium, magnesium, zinc and phosphorous) with black shank incidence. It is therefore suggested that disease expression is influenced by a combination of microbial, phytological, physical and

chemical factors. Kannwischer and Mitchell (1981) found that 300 zoospores per tobacco plant were the minimum number of zoospores required to achieve infection of a plant. However, the number of zoospores required to cause infection in other environments would be different.

1.6.7.2. Penetration of host.

De Zoeten *et al.* (1982) visualised infection of tobacco callus tissue by *Ph. parasitica.* var. *nicotianae* (although this varietal epithet is no longer valid - Hall, 1993) by transmission and scanning electron microscopy. When incubated with callus material it was observed that zoospores encyst and germinate on the material within three hours of introduction. The germ tubes often penetrated callus cells some distance from the place of germination. By 24 hours the host cells were still turgid in the susceptible callus yet collapsed in the 'resistant' callus because of a hypersensitive reaction. In this collapsed 'resistant' tissue, penetration by the fungus at 48 hours was to a depth of 5 to 8 layers of cells, whereas in the susceptible turgid tissue penetration was greater than 50 layers.

Benhamou and Côté (1992) using more advanced electron microscopical techniques showed that within 24 hours the epidermis of tobacco roots is colonised. The entire cortex is colonised (but not the xylem vessels) by the time the pathogen has reached the paratracheal parenchyma at 96 hours. The pathogen produces a battery of extracellular enzymes, particularly pectinases that diffuse extracellularly and break down the middle lamella. Cellulases are also present, but are of less significance. This is possibly because the pathogen cell wall contains cellulose (Bartnicki-Garcia & Wang, 1983). The host plant responds to infection by i) filling intercellular spaces with fibrillar pectin material, ii) plugging of sieve pores and plasmodesmata with amorphous material and iii) occluding non-invaded xylem vessels by using a coating material of undetermined chemical nature.

1.6.7.3. Host reaction to infection.

In stems and leaves challenged with *Ph. parasitica* the *de novo* synthesis of endo-1,3-β-glucosidases and endochitinases is activated (Meins & Ahl, 1989). This response is non-specific to the pathogen. The effect of a chitinase on an Oomycete pathogen is questionable in light of its cell wall biochemistry, that is, Oomycetes generally lack chitin in their cell walls (Bartnicki-Garcia & Wang, 1983). In callus material the production of phytoalexins

was found when infected with *Ph. parasitica*, although the stimulus for production is not a primary response to infection, but due to cell stress (Budde & Helgeson, 1981).

The hypersensitive reaction of the host plant to pathogens, involving rapid cell death and subsequent necrosis constitutes the primary mechanism of resistance to infection (Goodman & Novacky, 1994). *Phytophthora* spp. produce elicitins that cause non-host plants to display a hypersensitive reaction. For example, *Ph. cryptogea* secretes cryptogein (a haloprotein elicitin). Cryptogein is stored within the mycelium in the mature form; that is, a signal peptide of the preprotein is removed cotranslationally with no post-translational modifications, and can only be synthesised while the mycelium has a nitrogen source (Tercelaforgue *et al.*, 1992). Panabieres *et al.* (1995) have characterised a cluster of four genes in the *Ph. cryptogea* genome, of which two encode for cryptogeins, and the other two for other elicitins. These proteinaceous elicitins induce hypersensitive-like necroses in non-host tobacco plants.

Cryptogeins are lethal to tobacco cell cultures at low (100 nM) concentrations, with the sub-lethal concentrations causing the production of ethylene and phytoalexins (Blein *et al.*, 1991). Ethylene biogenesis has been implicated in activation of genes responsible for the plant defence response (Kim & Hwang, 2000). Increased extracellular pH and electrical conductivity were also observed in tobacco cells incubated with cryptogein. This activity has been correlated with decreased intracellular pH and depolarization of the plasma membrane, although membrane integrity is maintained. These observations are consistent with cryptogein targeting plasmalemma ATPase (Blein *et al.*, 1991), and other hypersensitive reactions (for example, see Goodman & Novacky, 1994). The invasion of tomato roots by *Pythium* group F (a minor ubiquitous pathogen in soil-less cultures) also causes massive induction of host responses (Rey *et al.*, 1998).

Ph. infestans also produces extracellular protein elicitors of similar molecular weight to cryptogein. A particular elicitor of *Ph. infestans* is INF1. Like cryptogein, it induces a hypersensitive-like response in non-host tobacco (Kamoun *et al.* 1998). However, in two major hosts of *Ph. infestans*, potato and tomato, a hypersensitive-like response is not induced by *Ph. infestans* (Kamoun *et al.*, 1997). During the early stages of infection of potato by *Ph. infestans* the production of INF1 does not occur, although other genes are translated in response to initial plant infection (Pieterse *et al.*, 1994). The *inf1* gene found to be expressed

at a late stage of infection has been correlated with extended leaf necrosis, saprophytic growth and profuse sporulation (Kamoun *et al.*, 1997).

The late expression of this elicitor may be to evade plant defences, such as the hypersensitive reaction. Although elicitor production is essential for completion of infection they are not known to have catalytic ability, but they can bind to other molecules such as carbohydrates, proteins and lipids (Templeton *et al.*, 1994). In liquid culture elicitins are the most abundant secretory proteins, but their function is still unknown. However, Yu (1995) suggests their function may be similar to cerato-ulmin toxin of *Ceratocystis ulmi* - that is, to increase the non-polarity or hydrophobicity of the microbial cell wall, and in the production of aerial hyphae, fruiting bodies, conidia and appressoria. The evidence presented by Kamoun *et al.* (1997) of maximum *infl* expression concurrent to sporulation supports Yu's (1995) hypothesis.

Tobacco plants are highly sensitive to elicitins of *Phytophthora* spp. In the review by Templeton *et al.* (1994), it is stated that *Ph. nicotinanae* var. *nicotinae* (=*Ph. parasitica*) does not produce elicitins. However, there is substantial evidence that the black shank pathogen does synthesis elicitins (for example, parasiticein - Capasso, 1999).

1.7. Glasshouse irrigation systems.

This project was partially in collaboration with Horticultural Research International (HRI), Stockbridge House (Yorkshire), where the modern, well-equipped greenhouse unit comprises approximately 50 individual compartments ranging in size from 15 m^2 to 900 m^2. The glasshouses include a 16 section multifactorial unit, which was ideal for detailed replicated studies of the greenhouse environment. A computer individually controls the heating, ventilation, carbon dioxide, irrigation and nutrients supplied to each greenhouse. Readings were taken every few minutes to ensure that a constant computer record of all environmental factors was logged.

Most of the British industry involved in growing fruit and vegetables under glass uses the nutrient film technique. Irrigation rates are generally 30% greater than required to overcome variances in the system, resulting in run-off to the environment. In the run-off phosphates and nitrates are present. It is inevitable that for environmental reasons legislation will be passed to stop the present practice of run-to-waste (see Section 1.10). The main option would

be to recirculate the irrigation water. Received wisdom suggests that in these systems disease inoculum build-up would be rapid. However, in trials at Stockbridge it was found that although there is occasional build up of *Pythium* and *Phytophthora* disease inoculum, it disappears rapidly. It would appear that in closed irrigation systems natural control of *Pythium* and *Phytophthora* spp. operates.

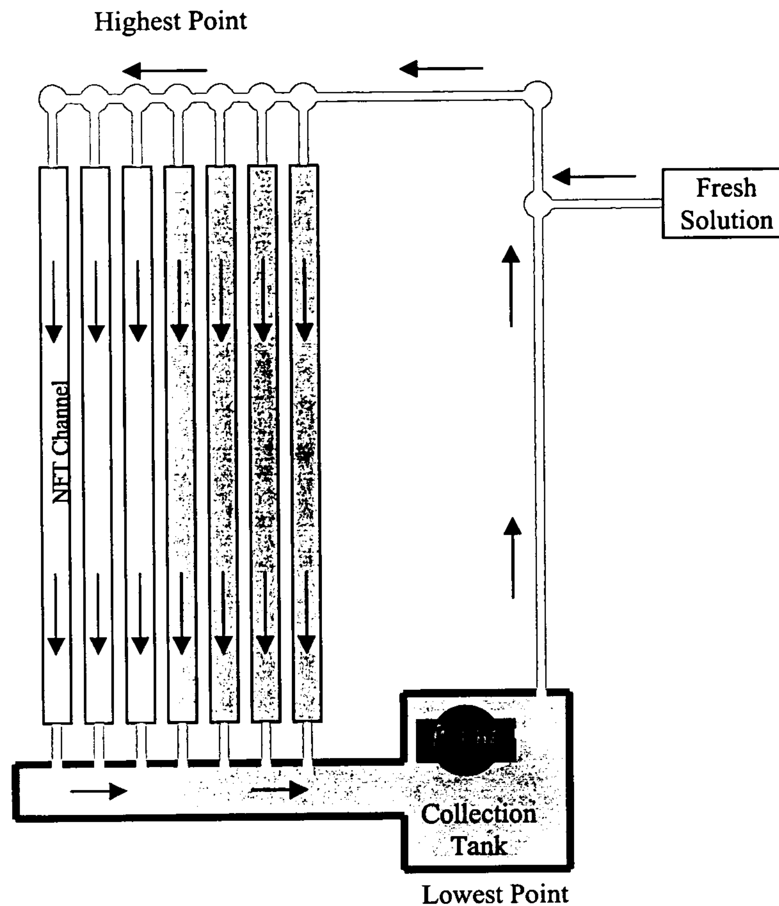
1.7.1. Nutrient film technique.

Nutrient film technique (NFT) has been described as the World's first method of crop production without a solid rooting medium (Cooper, 1979). In NFT, solid rooting medium, such as soil or peat, is replaced with a shallow stream of nutrient solution. This arrangement permits the root mat to develop above the solution in moist air, which forms a film on the aerial roots. This confers the advantages of having available to the roots at all times nutrients, water and air (oxygen). In conventional solid rooting medium systems abundant water has the detrimental effect of anaerobiosis or if water is lacking then water stress can occur. The availability of nutrients in solid rooting media can be limited by slow rates of diffusion and high affinity with the rooting medium. In NFT systems nutrients are proximal and available to roots because of a rapidly flowing medium.

1.7.1.1. Construction of a recirculating NFT installation.

A NFT installation is laid out with the channels discharging directly into a collection trench. The collection trench is dug along the lowest of the four sides of the site. At the lowest corner of the site, which coincides with an end of the collection trench, is located the nutrient circulating pump. Small bore tubing (one per channel) delivers the nutrient solution to the head of each channel from the recirculation pump. Very little filtration is required in NFT systems because the collection trench/tanks act as sedimentation tanks. Fresh solution is added to the recirculated solution to maintain the volume of liquid in the system and the concentration of nutrients. A schematic representation of a recirculating NFT installation can be seen in Figure 1.7.

Figure 1.7. Schematic representation of a recirculating NFT installation.



Nutrient solution is delivered to the top of each NFT channel, with excess solution draining into a collection trench and tank, which is at the lowest point in the glasshouse. The excess nutrient solution is recirculated and topped-up with fresh nutrient solution as necessary.

A suitable nutrient film is achieved when the gradient down the water channel is uniform and not subject to local depressions, the water flow is not rapid so deep flow builds up, the channel width is adequate so the root mass does not dam the nutrient solution, and the channel base is flat and not curved. The depth of the nutrient solution is typically only a few millimetres, so that most of the root mat develops in the NFT channel and roots are above the surface of the nutrient solution.

1.7.1.2. Operation of a NFT installation.

The calculation of water consumption by crops in a NFT system is complicated by the interaction of many factors, including plant size, growth stage, flux of solar radiation and solution temperature. Water consumption of over 1.5 l per day per plant is not uncommon (Cooper, 1979).

The optimal pH of the nutrient solution is between pH 6.0 and 6.5. Suitable acids for lowering pH are phosphoric acid and nitric acid. In systems where calcium is present in high concentrations nitric acid is recommended because the addition of phosphoric acid to a solution with free calcium causes calcium to be precipitated out as calcium phosphate. The nutrient solution should have an electrical conductivity greater than 2.0 mΩ, but less than 3.0 mΩ with most crops showing optimal growth in nutrient solutions with an electrical conductivity of 2.5 mΩ.

The theoretically ideal concentrations for elements in nutrient solution for NFT cropping are: nitrogen as nitrate 14.3 mM; phosphorus 1.9 mM; potassium 7.7 mM; calcium 4.3 mM; magnesium 2.1 mM; iron 214 μM; manganese 36 μM; boron 27μM; copper 2 μM; molybdenum 2 μM; and zinc 2 μM. Nitrogen is supplied as nitrate ions and not ammonium ions because ammonium is damaging to young tomato plants (Cooper, 1979).

The ratio of potassium to nitrogen uptake by tomatoes varies throughout the season. The ratio of potassium to nitrogen in the nutrient solution affects both fruit quality and yield. If the ratio is too low then fruit quality is affected whereas a ratio that is too high is detrimental to yield. However, it has been found that a wide tolerance to the potassium to nitrogen ratio exists between 26:1 and 1:10 with the effect on both yield and fruit quality minimal as long as the amount of either potassium and nitrogen was not too high to be toxic or too low to be deficient (Cooper, 1979).. For three main nutrients (nitrogen, phosphorus and potassium) the concentrations at which deficiency becomes evident is 715 μM nitrogen; 160 μM for phosphorus; and 510 μM for potassium. There is little information available on toxicity and deficiency of nutrients in NFT systems. However, The tolerances are believed to be large because of the nature of the NFT. That is, the lack of solid rooting medium and continuous flow of solution does not permit localised build up of nutrient salts around roots.

Plants require little sodium and very little chloride, consequently in recirculating irrigation systems sodium chloride build-up occurs. For tomatoes a water supply with less than 1.3 mM sodium should be used to avoid salt build-up (Cooper, 1979). However, in Barbados the water has a high calcium content (10 mM) and has been successfully used for NFT tomato and cucumber production.

1.8. Microbiology of irrigation systems.

Hydroponic systems at the beginning of the growing season are relatively free of disease causing organisms. However as the growing season progresses disease causing organisms build up in the irrigation water. By 1994 four viral, three bacterial and 21 fungal pathogens had been identified as causal agents of root disease in hydroponically grown crops with 13 of the fungal pathogens listed being able to produce zoospores (Table 1.1).

Although Stanghellini and Rasmussen (1994) describe hydroponic systems as lacking microbiological buffering, Berkelmann and Wokanka (1993) carried out quantitative and qualitative examination of bacterial flora in circulating nutrient solutions of hydroponic systems where tomatoes were grown in rockwool. Twenty hours after planting the tomatoes the population of aerobic heterotrophic bacteria was between 10^5 and 10^6 cfu.ml^{-1}. In a control system lacking tomato plants the bacterial population was between 500 and 900 cfu.ml^{-1}. Over the 12 week experimental period there were no significant changes in bacterial population densities. By a random process 160 bacterial strains were isolated from the experiment and subsequently identified (Table 1.2)

Table 1.1. Infectious agents isolated from roots of hydroponically grown crops.

Pathogen	Proof of pathogen spread by recirculating of infested nutrient solution.
Bacterial	
Clavibacter michiganese	inconsistent
Erwinia carotovora	no
Pseudomonas solanacearum	yes
Viral	
Lettuce Big Vein Virus (transmitted by *Olpidium brassicae)*	yes
Melon Necrotic Spot Virus (transmitted by *Olpidium radicale*)	yes
Tomato Mosaic Virus	yes
Cucumber Green Mottle Mosaic Virus	yes
Fungal (non-zoosporic)	
Colletotrichum coccodes	inconsistent
Fusarium oxysporum f.sp. *lycopersici*	inconsistent
F. oxysporum f.sp. *radicis-lycopersici*	inconsistent
F. oxysporum f.sp. *cucumerinum*	no
Pythium ultimum	inconsistent
Verticillium dahliae	no
Verticillium tricorpus	no
Thielaviopsis basicola	not tested
Fungal (zoosporic)	
Phytophthora cryptogea	yes
Phytophthora nicotianae (=*Ph. parasitica*)	yes
Plasmopara lactucae-radicis	yes
Pythium aphanidermatum	yes
Pythium debaryanum	yes
Pythium dissotocum	yes
Pythium intermedium	yes
Pythium irregulare	not tested
Pythium myriotyum	yes
Pythium sylvatcum	not tested
Olpidium brassicae	yes
Olpidium radicale	yes
Spongospora subterranea	no

Taken from Stanghellini & Rasmussen (1994).

Table 1.2. Bacterial flora of a hydroponic system.

Genera	Proportion of isolates
Pseudomonas spp.	40%
(*Ps. facilis*)	(29%)
Agrobacterium spp.	13%
Xanthomonas spp.	9%
Comamonas spp.	8%
Azospirillum spp.	4%
Enterobacter spp.	3%
Flavobacterium spp.	< 2%
Alcaligenes spp.	< 2%
Rhodococcus spp.	< 2%
Yersinia spp.	< 2%
Cytophaga spp.	< 2%
Aureobacterium spp.	< 2%
Other*	18%

* isolates that could not be identified with certainty or could not be identified.

Adapted from Berkelmann and Wokanka (1993)

1.8.1. Susceptibility of nutrient solutions to root pathogens.

Stanghellini and Rasmussen (1994) list several routes of pathogen introduction into greenhouse irrigation systems. In general, root-infecting pathogens are seldom airborne. However, *Fusarium oxysporum* f.sp. *radicis-lycopersici,* the most destructive of the non-zoospore producing pathogens of hydroponic systems, is airborne.

From the list of pathogens (Table 1.1) only *Clavibacter michiganese* and melon necrotic spot virus are seed-borne. However, melon necrotic spot virus is transmitted by zoospores of *Olpidium radicale.* Soil is not intentionally present in hydroponic systems. However, soil is constantly being introduced into irrigation system through shoes on personnel. In some greenhouses, washed river sand is used as ground cover or in pathways between production areas. However, this sand can be infected with plant pathogens including *Pythium* species. Peat and peat-mixtures are also major sources of disease inoculum for hydroponic systems. Species of *Fusarium* and *Pythium* along with *Olpidium brassicae* and *Thielaviopsis basicola* have been isolated from peat. Reservoir and surface water, such as rivers and streams, which can be used in NFT systems, are naturally infested with many potential plant pathogens. River water has been found to be a source of *Pythium dissotocum.* Fungus gnats (*Bradysia inpatiens*) and shore flies (*Scatella stagnalis*) are common insect pests in glasshouses. Both

insects have been shown to be capable of aerial transmission of *Pythium aphanidermatum* and other phytopathogens.

Stanghellini and Rasmussen (1994) describe the environmental factors that influence disease in hydroponic systems. In field conditions it is temperature and soil moisture that have the greatest influence on disease occurrence. However, in all hydroponic systems the zoosporic fungal pathogens are favoured because of the abundance of water. Therefore, temperature is the most important environmental factor governing disease onset and prevalence in hydroponic systems. For example, *Pythium aphanidermatum* is most destructive at nutrient temperatures above 25 °C, whereas below 20 °C it is of little concern. Similarly, *Plasmopara lactucae-radicus* is also favoured by warm temperatures, but inhibited below 18 °C. In contrast, *Phytophthora cryptogea* is most destructive at 15 °C and causes little or no damage at 25 °C.

Disease spread in irrigation systems is more rapid than in soil based systems for several reasons. The substrate in irrigation systems (nutrient solution) lacks the microbial diversity that is found in soils, and consequently does not have the same degree of biological buffering (Stanghellini and Rasmussen, 1994). That is, microorganisms in natural soils limit pathogens by antagonism, nutrient competition and fungistasis.

The lack of microbiological buffering in hydroponic systems permits a small amount of disease inoculum to lead to substantial infection of disease and subsequent crop loss. For example, Menzies *et al.* (1996) found that two million zoospores of *Pythium aphanidermatum* per hundred litres of nutrient solution (20 zoospores per ml) caused all the cucumber plants in the experiment to die 28 days post-inoculation.

Staunton (1978), perceiving that many growers fear that if disease enters a NFT system it may spread quickly through the nutrient system, set-up a demonstration to show that these fears were mainly unfounded. This demonstration consisted of five tomato diseases (fusarium wilt[2], verticillium wilt[3], didymella[4], brown root-rot[5] and bacterial canker[6]) being

---

[2] Causal agent: *Fusarium oxysporum* f.sp. *lycopersici* (Valenzuelaureta *et al.*, 1996).
[3] Causal agent: *Verticillium dahliae* (Bender & Shoemaker, 1984).
[4] Causal agent: *Didymella lycopersici* (Fagg & Fletcher, 1987).
[5] Causal agent: *Pyrenochaeta lycopersici* (Hockey & Jeves, 1984).
[6] Causal agent: *Corynebacterium michiganensis* subsp. *michiganensis* (Chang *et al.*, 1992).

introduced into separate NFT systems and the spread of disease was observed over a four-month period.

Overall this demonstration was convincing, that is little disease was observed. However, both fusarium wilt and verticillium wilt pose little threat in NFT systems because commercial varieties of tomato are resistant to these diseases. Staunton (1978) failed to include zoospore-producing pathogens in his demonstration. In the year of that demonstration, Evans (1977) found that many tomato crops growing poorly in NFT had phytophthora rot, with *Ph. cryptogea* being the most frequent causal agent, and less often *Ph. parasitica*.

In addition to *Phytophthora* spp. being responsible for poor root growth in NFT systems, Evans (1977 & 1979) found *Colletotrichum coccodes* to be rotting the tomato cortex with characteristic sclerotia being present. *Colletotrichum atramentarium* was also associated with the rotting of tomato roots. In addition to these pathogens *Pythium* spp. were also isolated. However *Pythium* spp. are more prevalent in cucumbers than tomatoes grown in NFT systems; for example, *Pythium ultimum* causes wilting in cucumbers to an extent that is of commercial significance.

Moulin *et al.* (1994) assessed the relative pathogenicity of 39 isolates of *Pythium* spp. from four species (*Py. aphanidermatum, Py. irregulare, Py. sylvaticum* and *Py. ultimum*) on cucumbers grown in rockwool, hydroponically (without a solid substrate) and in sand-peat mix. All four *Pythium* species were found to be pathogenic to cucumbers grown in the sand-peat mix, but only *Py. aphanidermatum* was pathogenic to cucumbers grown in rockwool and hydroponically. However, pathogenicity was greater in rockwool than in the hydroponic system.

Van Voorst *et al.* (1987) grew tomatoes in a greenhouse by NFT and inoculated the plants with *Ph. nicotinae* (=*Ph. parasitica*) either by direct inoculation or by adding fungal spores to a container within the recirculating nutrient solution. In the case of direct inoculation a polycyclic epidemic seemed to establish. However, the addition of fungal spores seemed to cause a monocyclic epidemic. In both cases inoculum freely circulated throughout the NFT system, and was present in the nutrient solution at least 6 days post-inoculation. It is this freedom for inoculum to circulate freely and rapidly throughout the NFT system that is concerning to growers.

1.9. Disease control.

The opinion that hydroponic systems are not overtly susceptible to pathogens (for example, Staunton, 1978) is contrary to the evidence suggesting that hydroponic systems are innately susceptible to root pathogens. With the present legislative trend (Section 1.10) fuelled by environmental concerns it is apparent that recirculation technology is likely to be adopted. Consequently methods for controlling disease will have to be found.

Below (Sections 1.91 to 1.9.4) the control options are grouped into four categories: plant breeding, fungicides, biological control, and natural control. The fungicide control category is simply the chemical control methods that predominate in contemporary agriculture and horticulture. The third, biological control, is the use of microorganisms to control disease. Waecher-Kristensen *et al.* (1993) identify three kinds of microbial control that are aimed at improving the microbiological stability of hydroponic cultures. These are plant growth promoting rhizobacteria, microbial antagonists to phytopathogens and bacteria that remove unwanted organic compounds, such as phenolic acids, from the nutrient solution. The fourth ambiguous category (natural control) includes physical treatments such as filtration and pasteurisation. This final group also includes non-toxic chemical control such as the supplement of silicates to control root diseases.

1.9.1. Plant breeding.

Breeding for resistance to *Ph. infestans* in tomato began over 50 years ago (Richards & Barratt, 1946). A dominant resistance gene specific to *Ph. infestans* race 0 was identified in *Lycopersicon pimpinellifolium* and transferred to *L. esculentum* (Bonde & Murphy, 1952), but this resistance was overcome by a new race of the pathogen (Conover & Walter, 1953). Subsequent breeding has focused on selecting partial resistance to the pathogen (Moreau *et al.*, 1998a).

1.9.2. Chemical control.

Chemical fungicides are usually the main component of any disease control program in agriculture or horticulture. However, these are mainly used to control foliar diseases. Soil

pathogens are difficult to control with fungicides because of the problems of introducing the fungicide into the soil and achieving contact between the pathogen and fungicide. Some fungicides once in the soil leach away and have environmental implications, while microbiological processes break down other fungicides. Some fungicides are systemic and can be redistributed by the plant from above ground portions to the roots. Examples of systemic fungicides used in drenches in nurseries and horticulture are benomyl[7] and metalaxyly[8].

However, no fungicides are currently registered for use against root diseases in hydroponic systems in the United States or Canada for food or horticultural crops (Paulitz, 1997). The reason for non-registration is phytotoxicity by fungicides in hydroponic systems. In soil systems the soil provides a buffer, which may bind the fungicide and reduce uptake by plants. In addition to this, microbial breakdown of fungicides occurs in soil, but may be less efficient in hydroponic systems, therefore leading to a build-up of fungicide in the nutrient solution. Also in greenhouse systems where crops are harvested daily the normal pre-harvest fungicide free period cannot be achieved. Rapid acquired fungicide resistance by fungi is also a reason for non-registration. Phenylamide (metalaxyl) fungicides are specifically active against the Peronosporales (including *Pythium* and *Phytophthora* species). However, strains of these organisms have been resistant to metalaxyl fungicides for at least a decade (Staub, 1991), and Zoquier and Young (1985) found *in vitro* sensitivity of the fungicide metalaxyl by *Ph. infestans* during vegetative growth, although the release of zoospores was relatively insensitive.

The chemical control of diseases caused by zoosporic fungi of the Oomycetes presents several problems. The unique biochemical properties of the Oomycetes, namely substitution of cellulose for chitin in the cell wall and the absence of *de novo* sterol synthesis render many traditional fungicides useless. The Oomycetes are diploid and thus can exhibit high phenotypic plasticity through recombination at sexual reproduction (oospore production). The problems of chemical control of the Oomycetes and the reasons underlying these problems are shown in Table 1.3.

---

[7] Also known as Benlate or Methyl 1-(butycarbamoyl)-2-(benzimidozolecarbamate)
[8] Also known as Ridomil, Subdue or *N*-(2,6-Dimethylphenyl)-*N*-(methoxyacetyl)-alanine methyl ester.

Table 1.3. The problems of chemical control of the Oomycetes and the reasons that underlie these problems.

Problem	Reason
Insensitive to polyoxin antibiotics.	Cell walls contain cellulose and not chitin.
Insensitive to potent sterol synthesis inhibitory fungicides.	Sterols are not synthesised *de novo*.
Insensitive to systemic benzimidazole, oxathiin and hydroxypyrimids fungicides	Structural proteins and enzymes active in energy production are structurally different from other fungi.
Some biochemical pathways are not typical. Therefore Oomycetes are tolerant to some fungicides.	Utilisation of enzymes with distinctly different properties to other fungi, including some that are active in RNA synthesis
Oomycetes are highly versatile and adaptable.	Oomycetes are diploid

Summarised from Bruin & Edgington (1983) pp 193-194.

The commercial use of fungicides to control disease in recirculating irrigation systems is not promising. Commercially it is not wise to register a fungicide for use in recirculating irrigation systems because of the high selection pressure on the pathogen to develop fungicide tolerance.

1.9.3. Biological control.

Pythium spp. have poor viability in soil relative to other root-colonising organisms, and often can only act as primary colonisers (Kommedahl & Windels, 1979). However, in hydroponic systems where the initial level of microbiological life is low, and there is efficient dissemination of zoospores in the nutrient solution, then there are opportunities for diseases caused by *Pythium* to become established. A control option for *Pythium* spp. in hydroponic systems would be the introduction of competitive root colonising microorganisms. Rankin & Paulitz (1994) isolated five rhizo-bacterial species from cucumber roots of which two had several favourable indices of control; for example, one bacterial isolate indirectly caused the plant to produce 88% more fruit than the *Pythium aphanidermatum* inoculated control plants. Other aspects of cucumber production that are affected positively through the suppression of *Pythium* spp. infection by rhizo-bacteria are fruit weight, shoot dry weight and fruit quality.

Zhou and Paulitz (1993) showed that *Pseudomonas corrugata* and *Ps. fluorescens* interfered with the chemotactic properties of cucumber root exudate. Generally, the presence of these pseudomonads significantly reduced attraction of zoospores of *Py. aphanidermatum* to cucumber roots, zoospore encystment at the root and germination of cysts on the roots. Presumably the mechanism for these phenomena was the catabolism by pseudomonads of chemoattractants and chemicals which induce zoospore encystment and germination. However, direct antibiosis of zoospores by chemicals like pyronitrin is another possible mechanism of control.

Cassinelli *et al.* (1993) collected 253 bacterial strains from the rhizosphere of cultivated and non-cultivated plants grown in *Pythium ultimum* suppressive soils of different northern regions of Italy. In a preliminary *in vitro* screen 18 strains inhibited *Py. ultimum.* Nine of the strains belonged to the species *Pseudomonas fluorescens* and *Ps. putida.* The *in vitro* mechanism of inhibition by fluorescent pseudomonads was by siderophore production, whereas for non-fluorescent pseudomonads the production of antibiotic-like substances was found to be responsible for inhibition of *Py. ultimum.* Buysens *et al.* (1996) suggested that it is not competition for iron (mediated by siderophore chelation) that is responsible for inhibition of *Pythium* by various pseudomonads, but certain siderophores (for example, pyoverdin or pyochelin) directly antagonise *Pythium* spp. *Pseudomonas* spp. have also been implicated in suppressing infection of cucumber roots by *Py. aphanidermatum* by inducing systemic resistance in the host (Chen *et al.*, 1998).

Arbuscular mycorrhiza on the apices of tomato roots protect the apices from pathogenic infection by *Ph. parasitica* (Fusconi *et al.*, 1999). However, these observations were made in soil systems, and the relevance in soil-less systems is unknown.

1.9.4. Natural control.

'Natural' control of zoospore-producing fungi may be achieved by the use of 'soft' chemicals, for example, calcium supplements to irrigation water to control diseases caused by *Pythium* and *Phytophthora* (von Broembsen and Deacon, 1997).

Non-toxic control options have been investigated in small scale recirculating irrigation systems. Stanghellini *et al.* (1996a) demonstrated that supplementing irrigation water with non-ionic surfactants caused the elimination of zoospores and 100% control of the spread of

Ph. capsici from a point source; in the absence of the supplement all pepper plants died within two weeks. Stanghellini *et al.* (1996b) also found that spread of *Py. aphanidermatum* was completely controlled with the supplement of a non-ionic surfactant.

Garibaldi and Gullino (1993) advocate that maintaining contaminant free systems is the method for keeping hydroponic systems disease free. However, the authors recognise that "the large number of plants per m^2 might create conditions favouring the multiplication of pathogens" and that "pathogens contaminating these soil or soil-mix starter blocks multiply quickly and then spread rapidly into the nutrient system". It would seem that contamination is inevitable in hydroponic systems and consequently prevention of pathogen introduction alone cannot be the sole strategy of disease control.

An option for control of zoospores investigated by Goldberg *et al.* (1992) is the filtration of irrigation water prior to recirculation. They found that the use of a 7 μm filter with a 20 μm pre-filter removed all zoospores from the system. However, the commercial viability of such a control technique is questionable because the filters were only operated for three cycles of irrigation water.

Chérif and Bélanger (1992) supplemented recirculating nutrient solutions with potassium silicate and found that *Pythium ultimum* was suppressed. The supplements significantly reduced plant mortality, root decay and yield losses, and increased root dry weight and number of fruit. Results from the controls suggested that potassium silicate acted as a disease suppression agent rather than a fertiliser. However, the phenomenon by which potassium silicate confers disease suppression is not understood, but induced systemic resistance is a possibility (Fawe *et al.*, 1998). The presence of potassium silicate did not reduce the viability of the pathogen, because throughout the trial it could be reisolated from the nutrient solution.

Purified milled chitosan from crab shell suppresses *Py. aphanidermatum* zoospores from causing root-rot of cucumbers (Ghaouth *et al.*, 1994). The suppression of infection was associated with the triggering of several host defence responses, including the induction of structural barriers in root tissues and the stimulation of the non-specific antifungal hydrolases (chitinase and β-1,3-glucanase). Chitosan also induced mechanical defensive structures - papillae were impregnated with electron-opaque substances. Chitosan has some fungicidal activity; for example, Ghaouth *et al.* (1994) demonstrated that chitosan inhibited

biomass gain of the pathogen. Microscopic examination of hyphae grown in the presence of chitosan revealed alteration of the plasma membrane and modifications to the cell wall.

Calcium has been shown to be involved at many stages of the homing sequence (Figure 1.2). This led von Broembsen & Deacon (1997) to supplement irrigation water with calcium; this caused a significant level of disease control. This is a promising finding because calcium acts at several points of the zoospore homing sequence, and is therefore broad acting.

The role of saponins has been suggested to be, in part, antifungal (Osbourn, 1996). Deacon and Mitchell (1985) demonstrated that intact oat roots, which are known to synthesis saponins, can both attract and cause lysis of zoospores from a range of fungi; this is probably why oats are less susceptible to zoosporic pathogens than other cereals (Hampton & Bucholtz, 1962; Kilpatrick, 1968).

1.10. Legislation.

Financial and environmental constraints have caused the UK industry to consider the utilisation of recirculation technology to ensure that the nutrient solution is retained and re-used, but not lost to the environment. However, the perceived threat from root pathogens disseminated in recirculating solution currently prevents the adoption of recirculation technology in the UK (McPherson *et al.*, 1995). In Sweden, pending environmental legislation is expected to impose restrictions on the release of nutrient solution from greenhouses. Such legislation would force recirculation technology and other non run-to-waste technologies to be adopted by the Swedish industry (Waecher-Kristensen, *et al.*, 1993)

1.11. Summary

The Pythiaceae although morphologically similar to the eumycota have different cell wall biochemistry and require an exogenous supply of β-hydroxyl sterols for reproduction. Taxonomically, the Pythiaceae are phylogenetically closer to diatoms and brown algae than Basiodomycetes or Ascomycetes.

Mycelial growth is typically fungal, with hyphae absorbing simple nutrients across the plasmamembrane, while complex polymers are degraded to simple molecules by extracellular enzymes prior to absorption. Primarily, nutrient stress and high levels of

moisture induce sporangial production. Sporangia will germinate directly in a warm nutrient-rich environment, whereas zoospores are released into cool wet nutrient-poor environments as found in irrigation systems based on NFT.

The naked, motile zoospore relies on endogenous nutrient supplies for energy. The normal helical path is modified by various stimuli (nutrients, electric fields, gravity etc.). At a suitable location, typically one proximal to a host, the zoospore encysts. Simple nutrients, cations and various plant-specific polymers induce encystment. Similar factors prompt the cyst to germinate, but in the absence of nutrients the cyst is likely to undergo diplanetism. The germ-tube will locate a suitable infection point principally by chemotropism. Various enzymes, particularly pectinases, facilitate penetration of the host.

The biology of the Pythiaceae enables them to be suitable pathogens in irrigation systems based on NFT. In NFT irrigation systems the abundance of moving water and the lack of microbial competition facilitates the production and dissemination of the major pathogen propagule - the zoospore. Environmental and economic pressures are conducive to the adoption of recirculating technology, which would possibly increase disease pressure.

Traditional methods for controlling fungal diseases, that is the use of fungicides, are not appropriate for the control of the pathogenic species of the Pythiaceae in irrigation solutions. Biological control, particularly the use of pseudomonads, is an option. The supplements of natural chemicals (for example, calcium, silicates and saponins) are also potential control options.

1.12. Aims of the project.

Judged on the need of industry for non-fungicidal control of the Pythiaceae in irrigation systems based on NFT, this project assayed the effects of several potential control methods on two major pathogens in the Pythiaceae. The pathogens were *Py. aphanidermatum* and *Ph. parasitica*. The potential control methods are:

- Cationic supplements to the irrigation solution.
- Natural suppression in recirculating irrigation systems.
- The supplement of natural toxins (β-escin, gramicidin S and ethanol).
- The synergism or antagonism between these potential control methods.

1.13. Major findings of this project.

The major findings of this project were:

- Overall, potassium is superior to calcium at suppressing the *in vitro* infection related behaviour of both *Py. aphanidermatum* and *Ph. parasitica*.
- It is possible to suppress infection-related behaviour of both fungi by altering the potassium to calcium ratio, while maintaining constant nitrate concentration and electrical conductivity.
- A calcium concentration of approximately 5 mM during sporangiogenesis markedly increases the subsequent release of zoospores into water.
- The cationic supplement made during sporangiogenesis affects the behaviour of the zoospores that are subsequently released.
- When nitrate is the counter-ion the effect of the cation is more distinct than when the counter-ion is chloride.
- There are chemicals present in commercial irrigation solutions and oat extracts that extend the motile period of zoospores.
- There are chemicals present in commercial irrigation solutions that antagonise the effectiveness of cationic supplements, but not the effectiveness of ethanol.
- Ethanol at very low concentrations suppresses sporangiogenesis and zoospore release.
- The toxicity of β-escin is antagonised by Ca^{2+} and K^{+}, but not Mg^{2+} and Na^{+}.
- The presence of a cyst wall reduces the toxicity of β-escin, but not of gramicidin S.

- The toxicity of gramicidin S is antagonised by divalent cations, but not monovalent cations.
- Overall *Ph. parasitica* was less susceptible to the various treatments than was *Py. aphanidermatum.*

Chapter 2.

General materials and methods.

2.1. Media

All media were sterilised by autoclaving at 121 °C (18 psi) for 15 minutes.

2.1.1 Agars.

Clarified V8 agar (CV8A): 330 ml of centrifuged (20 min. at 1000 g) V8 juice (Campbells Soups Co.) diluted 1:5 (v/v) with distilled water; 1.5 % (w/v) Bacto Difco agar added prior to being autoclaved.

V8 agar (V8A): 100 ml V8 juice (Campbels Soups Co.), 20g Oxoid No. 3 agar and 900 ml of distilled water.

Potato dextrose agar (PDA): Oxoid potato dextrose agar (39g) was added to distilled water to give a final volume of 1 litre.

Nutrient Agar (NA): Oxoid nutrient agar (28g) was added to distilled water to give a final volume of 1 litre.

Blood Agar (BA): NA plus 6% sterile fresh horse blood. Plates were supplied by Mr. M. Dye, Institute of Cell, Animal and Population Biology, the University of Edinburgh.

2.1.2. Broths

Yeast Extract Broth (1% v/v): 10 g of Oxoid Yeast extract in 1 L of DW.
Neutralised Bacteriological Peptone Broth (1% v/v): 10 g of Oxoid Neutralised Bacteriological Peptone in 1 L of DW.
Bacteriological Peptone Broth (1% v/v): 10 g of Oxoid Bacteriological Peptone in 1 L of DW.
Peptone P Broth (1% v/v): 10 g of Oxoid Peptone P in 1 L of DW.
Gelatine Broth (1% v/v): 10 g of Oxoid Gelatine in 1 L of DW.
Potato Extract Broth (1% v/v): 10 g of Difco Potato Extract (dehydrated) in 1 L of DW.
Malt Extract Broth (1% v/v): 10 g of Sigma Malt Extract (from starch digestion) in 1 L of DW.
Nutrient Broth (1% v/v): 10 g of Difco Nutrient Broth in 1 L of DW.

Clarified V8 Broth (CV8B): As for CV8A, but without agar.

Concentrated CV8B: As for CV8B, but undiluted with DW.

2.1.3. Mineral salts solution (MSS).

The mineral salts solution comprised: 3.08 g $Ca(NO_3)_2 \cdot 4H_2O$; 1.49 g $MgSO_4 \cdot 7H_2O$; 0.51 g KNO_3 in 1 l of distilled water. After being autoclaved 1 ml of chelated iron (II) solution was added. This comprised 0.65 g Ethylenediaminetetraacetic acid (EDTA), 0.38 g KOH and 1.25 g $FeSO_4 \cdot 4H_2O$ dissolved sequentially in 50 ml of distilled water; this solution was filter sterilised through a sterile 0.22 μm Millipore membrane prior to being added to the salt solution.

2.1.4. Mineral nutrient solution.

The mineral nutrient solution comprised: 10 g Na_2HPO_4; 3g KH_2PO_4; 1 g K_2SO_4; 1 g NaCl; 200 mg $MgSO_4 \cdot 7H_2O$; 1 mg $CaCl_2 \cdot 2H_2O$; 100 ng $FeSO_4 \cdot 7H_2O$ in 1 l of distilled water. Mineral nutrient solution was made up from stock solutions.

2.1.5. Preparation of crude oat extracts.

In a tray (15 x 25 x 6 cm) with the base covered to a depth of 2 cm with washed sterile sand (approximate grain diameter of 500 μm), oat seeds were liberally sprinkled (approximately 2 seeds per cm^2) over the sand and then covered with a 1 cm layer of sand. The tray was incubated on the bench-top (ambient temperature was approximately 19 °C) and watered as required. When seedlings were 6 to 8 cm tall the sand was washed away with tap water.

For each seedling the terminal 5 mm of each root was removed with a sharp razor blade. For the remaining portion of each seedling the leaves were removed, again with a sharp razor blade. This left the remains of the seed attached to the majority of the root system. Therefore, each seedling was divided into three parts: 1) terminal root portions, 2) leaves, and 3) seed attached to the roots.

2.1.5.1. Terminal root tip extract.

To each 1 g of terminal root tips 20 ml of DW was added, then homogenised (Ultra Turrax at 24000 rpm for 1 min). The homogenate was then diluted one-to-one (v/v) with DW and clarified by centrifugation (1400 g for 5 min). The supernatant was boiled for 10 min and then frozen (-11 °C) until required.

2.1.5.2. Leaf extract.

To each 15 g of leaves 100 ml of DW was added, then homogenised in a mortar with a pestle (2 g of clean sand was added to aid this process). The homogenate was diluted one-to-one (v/v) with DW and then clarified by centrifugation (1400 g for 5 min), boiled for 10 min, filtered through Whatman No. 1 paper to remove the green precipitate, and the filtrate was then frozen (-11 °C) until required.

2.1.5.3. Seed and root complex extract.

To 58 g of oat seeds attached to roots 125 ml of DW was added, then homogenised in a mortar with a pestle (2g of clean sand was added to aid this process). The homogenate was then made up to 500 ml with DW, blended in a kitchen blender (for 1 min), boiled for 15 min, clarified by centrifugation (1400 g for 5 min.), and then frozen (-11 °C) until required.

2.2 Origin, maintenance and preservation of strains.

2.2.1 *Phytophthora parasitica*.

The isolate of *Phytophthora parasitica* Dastur (=*Ph. nicotianae* van Breda de Haan) originated from diseased *Cantharanthus roseus* (L.) G. Don (bedding 'vinca') in a commercial glasshouse in Oklahoma, USA. The fungus was maintained on clarified V8 agar. *Ph. parasitica* was preserved in McCartney bottles on PDA squares (1 cm × 1 cm) in SDW at room temperature.

2.2.2. *Pythium aphanidermatum.*

The isolate of *Pythium aphanidermatum* (Edson) Fitz. (CBS 634.70) was originally isolated from *Lycopersicon esculentum* and was maintained on V8 agar. *Py. aphanidermatum* was preserved in McCartney bottles on PDA squares (1 cm × 1 cm) in SDW at room temperature.

2.2.3. *Brevibacillus brevis.*

Both strains of *B. brevis*, the wild type and a gramicidin-S-deficient mutant (E-1), were supplied by Dr. B. Seddon, the Department of Agriculture, the University of Aberdeen, Scotland. Both strains were maintained on NA.

2.3. Standard techniques.

2.3.1. Production of zoospores.

2.3.1.1. *Ph. parasitica.*

The mycelium of a mature plate of *Ph. parasitica* (7-14 days old) (Section 2.2.1) was covered with a layer of CV8B (12 ml) and aerial hyphae dislodged by a sterile glass spreader. The inoculated broth was dispensed in 3 ml aliquots into small (5 cm diameter) Petri dishes (Sterilin, UK). After 48h incubation in the dark at 23 °C mats were washed with three aliquots of 3ml of MSS per plate and then returned to a second incubator (20 °C with constant light) for 24h then washed once more with MSS. This was followed by the final incubation of 48 to 72h at 20 °C with constant illumination. For zoospore release the MSS was removed with three washes of SDW (3 ml) before a pre-chilled (11 °C) release solution (4 ml) was added. This was followed by a cold shock of 30 min (11 °C). Synchronous zoospore release occurred over 2h in a 23 °C incubator in the dark.

2.3.1.2. *Py. aphanidermatum.*

The method for zoospore production was essentially as for *Ph. parasitica* except that an intact mycelial mat of *Py. aphanidermatum* was transferred to a 250 ml medicine bottle containing 100

ml of CV8B, shaken vigorously to produce inoculated broth which was subsequently decanted into Petri dishes. If mycelial growth in the broth was good the first wash with MSS was at 24h, otherwise the first wash was at 48h. The time between the second MSS wash and zoospore release was consistently 48h.

2.3.1.3. Bulk zoospore production.

For bulk production of zoospores for motility and germination experiments larger (standard 9 cm diameter) Petri dishes were employed which would contain 10 ml of inoculated broth. The volume of wash solution was increased proportionally to 10 ml and the volume of release solution increased to 13 ml. All other factors were identical to the standard protocol for zoospore production (Sections 2.3.1.1 and 2.3.1.2).

2.3.2. Mechanical induction of zoospore encystment.

Motile zoospores were induced to encyst synchronously by transferring aliquots of the spore suspension to 1.5ml Eppendorf tubes and holding them against a vortex mixer for 70 seconds. Immediately after vortexing the spores were added to the required treatments. The exception was in the experiments of Chapter 6, where a distinction between immature and mature cysts was made. Immature cysts are cysts that were immediately used in experiments after vortexing, whereas cysts that were added 10 minutes post-vortexing were classed as mature. Immature cysts were presumed to have cell walls that were still forming, whereas mature cysts were assumed to have fully formed cell walls (Hardham *et al.*, 1991).

2.3.3. Effects of treatments on mycelial growth.

To each pre-weighed small Petri dish was added 1 ml of CV8B inoculated with mycelial fragments and 3 ml of supplemented CV8B. The supplement depended on the particular experiment. Dishes were swirled around to mix the two broths and cause them to cover the entire base of the dish. The Petri dishes were then sealed with Parafilm and incubated in the dark at 23 °C for 48 hours. After the incubation period, the mats were washed with three 3 ml aliquots of distilled water and then placed in an incubator (23 °C) without lids for 72h to dehydrate the mycelial mats before re-weighing to establish the gain in biomass.

2.3.4. Effects of treatments on zoosporangiogenesis.

The standard protocol for zoospore production (Section 2.3.1) was followed with the MSS being supplemented with the various treatments. However, when assaying the effect of irrigation solutions on sporangiogenesis (Chapter 4), the MSS was substituted with various irrigation solutions. For assays on the effect of either $Ca(NO_3)_2$ or KNO_3 on sporangiogenesis the concentration of the salt was simply adjusted. In the assays on the effect of $CaCl_2$ on sporangiogenesis, $Ca(NO_3)_2$ was substituted with various concentrations of $CaCl_2$. For the effect of the concentration of $Mg(NO_3)_2$ on sporangiogenesis the concentration of $Ca(NO_3)_2$ was reduced from 13mM to 7mM and the 6 mM $MgSO_4$ was replaced with 6mM $CaSO_4$ to maintain constant SO_4^- and Ca^{2+} concentrations across the series of experiments.

Because *Py. aphanidermatum* sporangia are morphologically highly variable and difficult to differentiate from hyphae, enumeration of sporangial production and viability was by an indirect measurement; namely the number of zoospores subsequently liberated into SDW. This indirect measurement indicated the cumulative effect on sporangial production and ability to liberate zoospores, which is a useful epidemiological index.

It was observed that *Ph. parasitica* released zoospores prior to the cold-shock treatment, so before washing off the MSS, an aliquot (500 μl) of MSS was removed and fixed (100 μl 16% (v/v) formaldehyde and 12% (v/v) glutaldehyde) and zoospore density was later enumerated by haemacytometer counts. Direct video recordings of sporangial density and the fraction of sporangia discharged were also made. Three random microscope fields of view for each plate were video recorded with a Panasonic F15 video (CCD) camera mounted on a Leitz Wetzlar research microscope (4× objective) attached to a Panasonic AG6720 SVHS video recorder and a Panasonic BT-M1420PY colour video monitor. A 1.25× magnification lens in the objective turret increased the effective magnification.

The number of sporangia and percent of empty sporangia were enumerated from the video recordings. Frequently part of the field of view was obscured or out of focus. To compensate for this, the screen of the monitor was divided into 20 equal sized squares and sporangia were only

counted in the squares that were in focus. Then the number of sporangia per square was determined and multiplied by 20 to give sporangia per screen. After washing the mats free of MSS with SDW and liberation of zoospores into SDW, a further aliquot of zoospores was removed for enumeration and the mats were videoed for a second time to determine the post-shock density of sporangia and the percentage of sporangia that had discharged their contents.

2.3.5. Effects of treatments on zoospore release.

The zoospore release solution (SDW) was supplemented with various test treatments. After a 2 hour release period the fungal mat was removed, the dish contents swirled and 875 μl of the zoospore suspension was transferred by pipette to an Eppendorf tube containing 125μl of 20% (v/v) formaldehyde and 15% (v/v) gluteraldehyde aqueous solution to fix the spores. Enumeration of zoospores was achieved by loading a heamocytometer slide with a sample (circa. 100 μl) of the zoospore suspension and counting while illuminated with dark field optics with a UNILUX-12 bifocal microscope at a final magnification of 100× (10× objective and 10× eyepiece).

2.3.6. Effects of treatments on zoospore motility.

Test solutions were added to either 1 ml of zoospore suspension of *Py. aphanidermatum* or 2 ml of zoospores of *Ph. parasitica* in a small Petri dish. The final volume of solution in all cases was 4 ml. After 2 hours incubation at 23°C in the dark three random fields of view from the base of each Petri dish were recorded as before (Section 2.3.1), but with a 10× objective.

Once recordings of zoospores had been made, the videotape was replayed frame by frame and zoospore motility was assessed by marking directly on the monitor screen the location of all zoospores in sequential frames. For those zoospore (cysts) that were stationary the proportion that had germinated was recorded.

2.3.7. Effects of treatments on cyst germination.

In Eppendorf tubes, 800 μl of test solution was added to 200 μl of vortex-encysted spores. The contents of the tubes were mixed by 2 s of vortexing and then incubated at 23 °C in the dark for 2 hours. Then the cysts were examined microscopically and the proportion that had germinated was determined. A cyst was deemed to have germinated when the germ tube length was greater than the cyst diameter.

2.4. Culturing of *Brevibacillus brevis* in various liquid media.

To 500 ml of broth (in 1 l conical flask) a loop-full of *B. brevis* was inoculated. The type of broth was dependent on the particular experiment. However, incubation was always at 30 °C on a rotary shaker (112 rpm). When required, the broth was clarified by centrifugation (1800 g for 20 minutes). Adjustments from this protocol, when made, are indicated in the text.

2.5. Source of reagents.

All reagents were obtained from Sigma (Sigma-Aldrich Company Ltd., Fancy Road, Poole, Dorset, BH12 4QH, UK) and were ACS reagent grade or equivalent.

2.6. Statistics.

All statistics were performed using Microsoft Excel (version '97). All data expressed as a percentage were arcsine transformed prior to data analysis.

Chapter 3.

The effects of cations on the zoosporic infection sequence.

3.1. Introduction.

The effects of cations, particularly Ca^{2+}, on aspects of zoospore biology and cyst germination are well established (Donaldson & Deacon, 1992; Donaldson & Deacon, 1993a; Bloodgood, 1991; Tamm, 1989; Grant *et al.*, 1986; Warburton & Deacon, 1998). Calcium is one of the principal cations added to irrigation systems and is known to curtail zoospore motility and promote the germination of encysted zoospores. Both these phenomena in an irrigation system theoretically suppress inoculum dispersal and re-dispersal through diplanetism. This has recently been demonstrated in small-scale trials by von Broembsen and Deacon (1997).

The major cations present in irrigation systems are Ca^{2+}, Mg^{2+} and K^{+}. Calcium and potassium are usually added as nitrate salts, whereas magnesium is added as a sulphate salt. Virtually all research on the effects of these cations on zoospore biology has been with chloride salts. The accumulation of chloride in irrigation systems can lead to phytotoxicity. Consequently any prescribed cationic supplement would not be in the form of chloride salts. The addition of nitrate salts or possibly sulphate salts would be agronomically acceptable, but the higher demand for nitrogen by plants, suggests that nitrate salts are the more likely candidates.

The work presented in this chapter assessed the effect on several stages of the zoosporic infection sequence of *Py. aphanidermatum* and *Ph. parasitica in vitro* of various supplements of Ca^{2+}, Mg^{2+} and K^{+}. Nitrates were used because of the applied focus of this work, and to contrast with previous findings where chlorides were used. The infection by zoosporic fungi is polycyclic. Consequently all stages of the infection sequence can be present in an irrigation system at one time. Therefore, an understanding of the cumulative effect of cationic supplements on the infection sequence is required. The cumulative effect of cation supplements on zoosporic fungi is also assessed in this chapter.

3.2. Effects of supplementing CV8 broth with cations on mycelial growth.

Curtailment of mycelial growth in irrigation solutions would reduce the resource base for the production of the principal infection unit, the zoospore. The vegetative growth of *Py. aphanidermatum* and *Ph. parasitica* was assessed by biomass production in cation-supplemented CV8 broth (see Section 2.3.3) (Figures 3.1 to 3.8). *Py. aphanidermatum* grown in

unsupplemented broth formed a complete mat - the hyphal fragments grew and interlaced to form a single mat. In contrast, *Ph. parasitica* produced many small, dense, distinct colonies, with each colony presumably formed from a small viable mycelial fragment.

Supplements up to 80 mM KNO_3 or $CaCl_2$ to CV8B had no significant ($P \geq 0.093$) effect on biomass gain of *Py. aphanidermatum* when compared to the unsupplemented broth (Figures 3.1 and 3.3). Supplements of $Mg(NO_3)_2$ and $Ca(NO_3)_2$ (Figures 3.2 and 3.4) were both negatively correlated with biomass gain in the range of 0 to 80 mM (Table 3.1). Suppression of mycelial growth was greater when the broth was supplemented with calcium than with magnesium nitrate.

Table 3.1. Correlation statistics and the equations for lines of best fit for the effects of $Ca(NO_3)_2$ and $Mg(NO_3)_2$ concentration on mycelial growth of *Py. aphanidermatum*.

Salt	Line of best fit	*R*	*F*	*df*
$Ca(NO_3)_2$	y = 13.86 - 0.17x	-0.92	1.38×10^{-10}	24
$Mg(NO_3)_2$	y = 11.00 - 0.11x	-0.85	1.07×10^{-7}	23

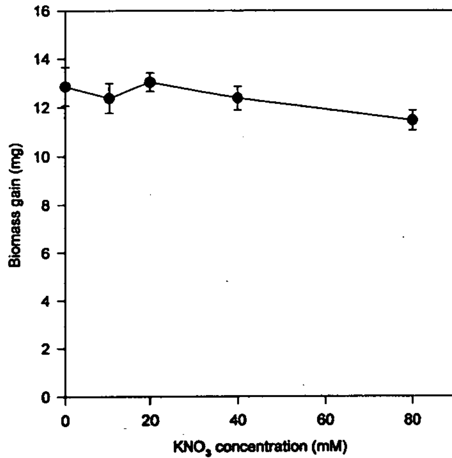
Supplements up to 80 mM of KNO_3 or $CaCl_2$ to CV8B had no significant ($P \geq 0.182$) effect on biomass gain of *Ph. parasitica* when compared to the unsupplemented control broth (Figures 3.5 and 3.7). However, 40 mM $Ca(NO_3)_2$ very significantly (t-test, P = 0.005) increased biomass gain of *Ph. parasitica* compared to the unsupplemented control (Figure 3.8). The other three supplements of $Ca(NO_3)_2$ (10, 20 and 80 mM) were not significantly different ($P < 0.05$) from the control. Supplements of $Mg(NO_3)_2$ of 20 mM or more significantly suppressed biomass gain of *Ph. parasitica* (ANOVA, $P = 0.011$, $\sigma_d = 1.9$ mg) compared to the unsupplemented control (Figure 3.6).

Thus, these experiments indicated that biomass production by both of the fungi was unaffected by KNO_3 (up to 80 mM) and was progressively suppressed by increasing the concentration of $Mg(NO_3)_2$ supplement. Only *Py. aphanidermatum* was adversely affected by $Ca(NO_3)_2$ supplements. In contrast biomass production by *Ph. parasitica* was unaffected by these calcium supplements up to 80 mM.

A 330 ml can of V8 typically contains 1.49 g of potassium, 106 mg of calcium and 2.3 mg of iron (Anon., 1997). If partitioning of salts is assumed to be equal between the supernatant and pellet, then the background concentrations of cations in CV8B are 1.6 mM Ca^{2+}, 23 mM K^{+} and 25 μM Fe^{n+}.

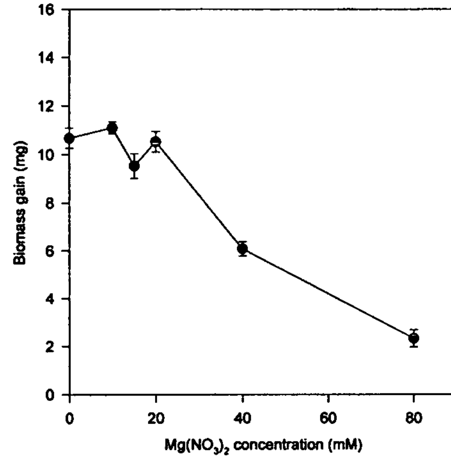
Figures 3.1 - 3.4. Effect of salt concentration on biomass gain* of *Pythium aphanidermatum* during a 48h incubation at 23 °C in the dark.

Figure 3.1 Effect of KNO_3.



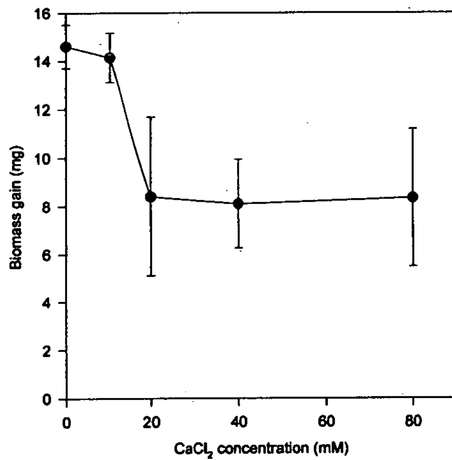
n = 5.

Figure 3.2 Effect of $Mg(NO_3)_2$.



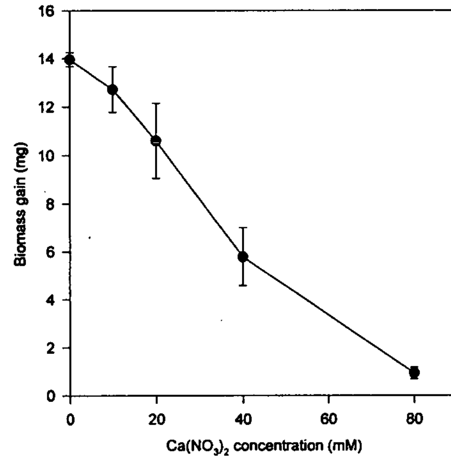
n = 4.

Figure 3.3 Effect of $CaCl_2$.



n = 5.

Figure 3.4 Effect of $Ca(NO_3)_2$.

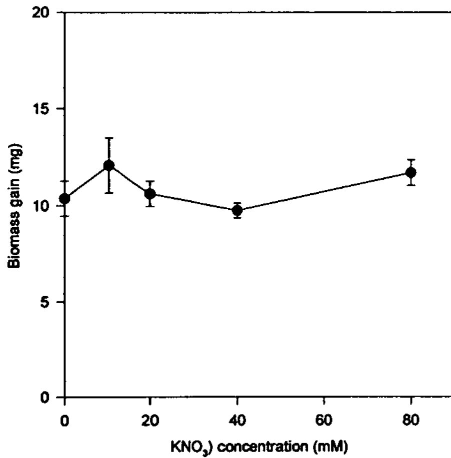


n = 5.

* Means ± s.e.m. for n replicates, assessed by arithmetic dry mass gain of pre-weighed Petri-dishes.

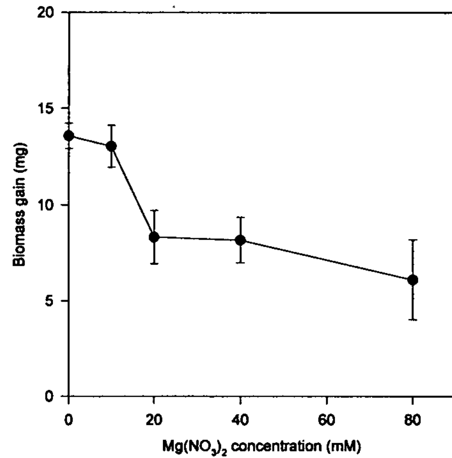
Figures 3.5 - 3.8. Effect of salt concentration on biomass gain* of *Phytophthora parasitica* during a 48h incubation at 23 °C in the dark.

Figure 3.5 Effect of KNO_3.



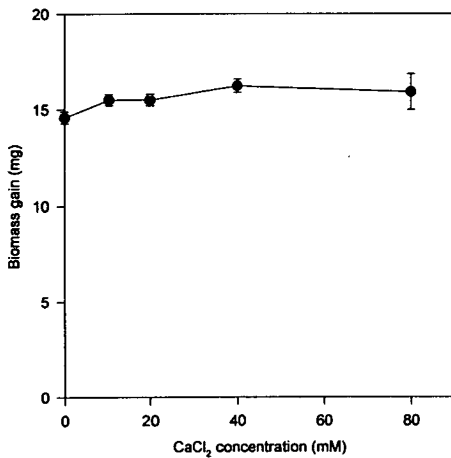
n = 3.

Figure 3.6 Effect of $Mg(NO_3)_2$.



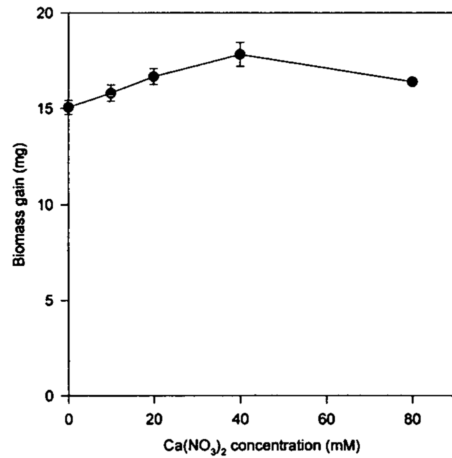
n = 3.

Figure 3.7 Effect of $CaCl_2$.



n = 5

Figure 3.8 Effect of $Ca(NO_3)_2$.



n = 5

* Means ± s.e.m. for n replicates, assessed by arithmetic dry mass gain of pre-weighed Petri-dishes.

3.3. Zoosporangiogenesis and subsequent zoospore liberation in cation supplemented mineral salt solutions.

3.3.1. Effect on *Pythium aphanidermatum*.

Py. aphanidermatum under nutrient-stress produces morphologically highly variable sporangia that are generally inflated, lobe forming structures (Plaats-Niterink, 1981). Because they are complexes of swollen hyphal branches, visualisation and enumeration of these sporangia was difficult. Therefore, an indirect measurement of zoosporangiogenesis that had epidemiological relevance was chosen. The number of zoospores subsequently liberated into SDW was used as a measurement of the cumulative effect of the Mineral Salts Solution (MSS) on sporangial production and the ability to liberate zoospores.

Supplements of Ca^{2+}, Mg^{2+} or K^{+} to MSS (as in Section 2.3.4) did not significantly ($P > 0.11$) affect subsequent zoospore release from *Py. aphanidermatum* sporangia into SDW (Table 3.2). In the light of other findings reported in this chapter, the effect of a 5 mM $Ca(NO_3)_2$ supplement to MSS on subsequent zoospore release was compared to calcium-free MSS. It was found that the 5 mM Ca^{2+} test supplement caused a four-fold increase in the number of zoospores released compared to the Ca^{2+} free control. This was highly significant ($P = 3.32 \times 10^{-3}$) (Table 3.3).

Table 3.2. Effect of various supplements to Mineral Salt Solution on zoosporangiogenesis, assessed indirectly by measurement of subsequent zoospore release (zoospores.μl^{-1})* from sporangia-bearing mycelia of *Py. aphanidermatum*.

Supplement concentration	Supplement salt			
	$Ca(NO_3)_2$	$Mg(NO_3)_2$	KNO_3	$CaCl_2$
0 mM	488 ± 56	463 ± 136	348 ± 39	220 ± 23
10 mM	505 ± 76	517 ± 97	227 ± 37	342 ± 102
20 mM	423 ± 38	325 ± 32	413 ± 114	255 ± 93
30 mM	428 ± 30	252 ± 70	277 ± 134	365 ± 66
40 mM	109 ± 16	300 ± 18	355 ± 90	335 ± 47
P (ANOVA)	1.05×10^{-1}	1.92×10^{-1}	6.52×10^{-1}	5.83×10^{-1}
σ_d	143	262	129	102

* Mean ± s.e.m for (3 replicates dishes), based on two haemocytometer counts per replicate.

Table 3.3. Effect of Mineral Salt Solution supplemented with 5 mM $Ca(NO_3)_2$ on zoosporangiogenesis, assessed indirectly by measurement of subsequent zoospore release (zoospores.μl^{-1})* from sporangia-bearing mycelia of *Py. aphanidermatum*.

Treatment	Zoospore release (zoospores.μl^{-1})
Control (no added Ca^{2+})	138 ± 48
Test (5 mM Ca^{2+})	563 ± 43
P (t-test)	3.32×10^{-3}

* Mean ± s.e.m (for 3 replicates dishes), based on two haemocytometer counts per replicate.

3.3.2. Effect on *Phytophthora parasitica*.

3.3.2.1. Effect on sporangial density.

The sporangial morphology of *Ph. parasitica* was more consistent than for *Py. aphanidermatum*. Sporangia of *Ph. parasitica* are distinct terminal, ovoid structures that are easy to enumerate *in vitro*. Although sporangial morphology is regular the distribution within the hyphal mat was observed to be highly variable and clustered; this was consistent with previous observations (Waterhouse, 1956).

Supplementation of MSS with any of the three nitrate salts (up to 40 mM) had no significant ($P > 0.05$) effect on sporangial density (Figures 3.9, 3.10 and 3.12). However, $CaCl_2$ concentrations up to 15 mM noticeably suppressed sporangial density, whereas between 15 mM and 40 mM the trend was reversed. There was significant (t-tests, $P \leq 0.025$) suppression of sporangial density by 15 mM $CaCl_2$ compared to the Ca^{2+}-free control and the 40 mM supplement.

In another experiment (Figure 3.13), higher concentrations of KNO_3 were used. MSS supplemented with up to 168 mM KNO_3 did not significantly (ANOVA, $P > 0.05$) affect sporangial density, although 0.5 M KNO_3 suppressed sporangiogenesis (as assessed by sporangial density) to 5% of the K^+-free control.

Figures 3.9 - 3.12. Effect of salt type and salt concentration in MSS on the sporangial density* of mats of *Phytophthora parasitica*.

Figure 3.9 Effect of KNO_3.

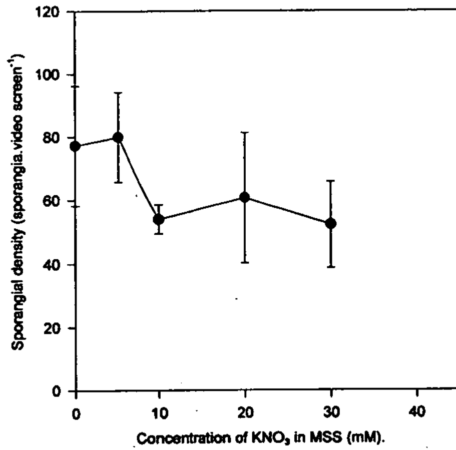


Figure 3.10 Effect of $Mg(NO_3)_2$.

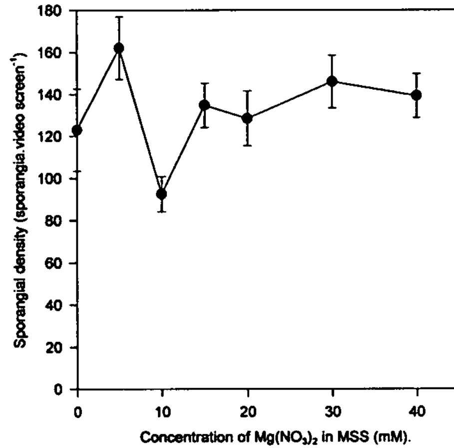


Figure 3.11 Effect of $CaCl_2$.

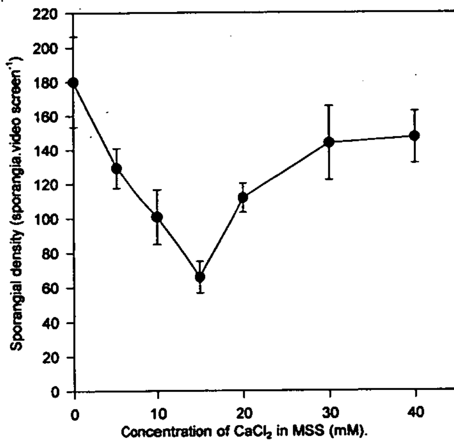
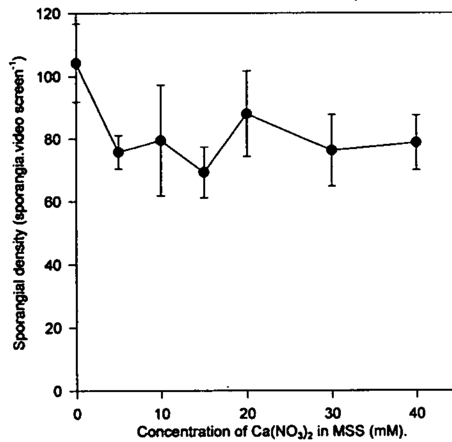
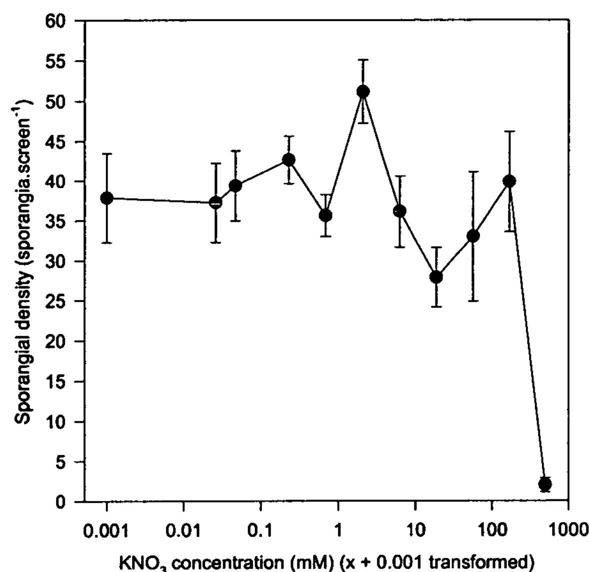


Figure 3.12 Effect of $Ca(NO_3)_2$.



* Means ± s.e.m. for 3 replicates, based on 6 random fields of view (3 fields prior to cold-shock and 3 fields after zoospore release) per replicate.

Figure 3.13. Effect of KNO_3 concentration in MSS on the sporangial density* of mats of *Phytophthora parasitica.*



* Means ± s.e.m. for 4 replicates, based on 5 random fields of view per replicate.

3.3.2.2. Effect on zoospore release.

It was observed that *Ph. parasitica* released zoospores prior to washing with the SDW release solution. That is, zoospores were released into the modified MSS prior to the cold shock. This pre-shock zoospore release into MSS was enumerated (as in Section 2.3.4) (Figures 3.14 to 3.17). The concentrations of $Mg(NO_3)_2$ and KNO_3 supplement were negatively correlated with zoospore release, whereas the concentration of $CaCl_2$ supplement was positively correlated with zoospore release (Table 3.4). The peak in zoospore release in the 5 mM $Ca(NO_3)_2$ supplement was found to be significantly different from all other supplements (ANOVA $P = 0.001$, $\sigma_d = 3.3$ zoospores.μl^{-1}). All other supplements of $Ca(NO_3)_2$ (excluding the 5 mM) were not significantly different from each other ($P > 0.05$). The number of zoospores released into 5 mM Ca^{2+} MSS was at least fifteen times greater than the number released into the Ca^{2+}-free control, and three times greater than into the 10 mM Ca^{2+} treatment.

Table 3.4. Correlation statistics and the equations for lines of best fit for the effects of KNO_3, $Mg(NO_3)_2$ and $CaCl_2$ concentration on zoospore release into MSS from sporangia of *Ph. parasitica*.

Salt	Line of best fit	*R*	*F*	*df*
KNO_3	y = 3.6 - 0.07x	-0.49	4.13×10^{-2}	17
$Mg(NO_3)_2$	y = 6.9 - 0.18x	-0.75	1.06×10^{-4}	20
$CaCl_2$	y = 0.9 + 0.09x	0.73	1.33×10^{-4}	20

All KNO_3 supplements to MSS had no significant ($P > 0.05$) effect on post-shock zoospore release (Figure 3.14) - that is, the number of zoospores released into SDW after the modified MSS had been removed. The number of zoospores released post-shock into the 5 mM $CaCl_2$ supplement was significantly greater ($P < 0 .05$) than for all other $CaCl_2$ supplements (Figure 3.16). Again, a peak in zoospore numbers was observed in the 5 mM $Ca(NO_3)_2$ supplemented MSS treatment. There was a very highly significant (y = 12.7 - 0.26x, $F = 6.61 \times 10^{-4}$, $R = 0.77$, $df = 14$) negative correlation between $Ca(NO_3)_2$ supplements from 10 mM to 40 mM and zoospore release. Zoospore release into all $Mg(NO_3)_2$ supplements except for the 10 mM supplement was not significantly different from each other ($P > 0.05$). The peak in zoospore numbers in 10 mM $Mg(NO_3)_2$ MSS was significantly ($P = 0.037$) greater than in the Mg^{2+} free control.

Figures 3.14 - 3.17. Effect of salt type and salt concentration in MSS on zoospore release* from mats of *Phytophthora parasitica* .

Figure 3.14 Effect of KNO_3.

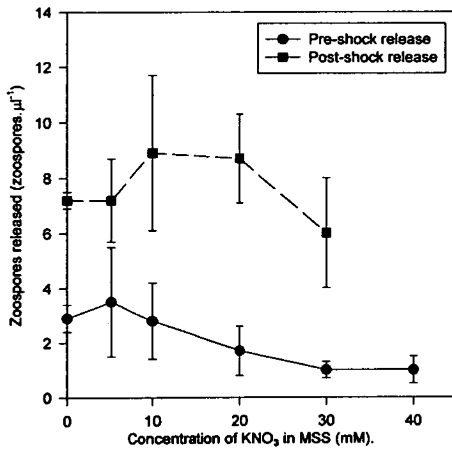


Figure 3.15 Effect of $Mg(NO_3)_2$.

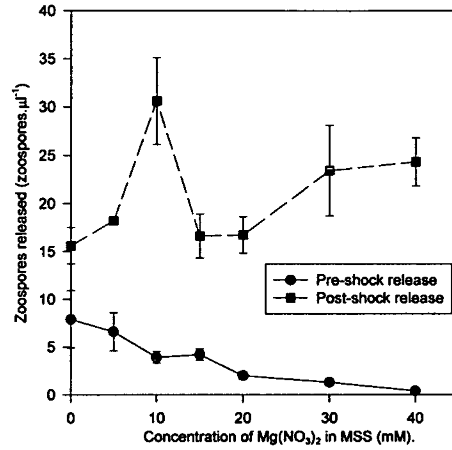


Figure 3.16 Effect of $CaCl_2$.

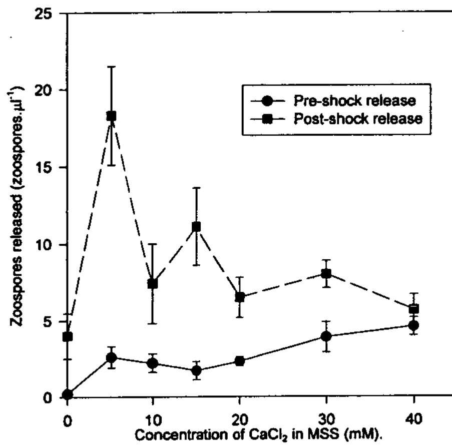
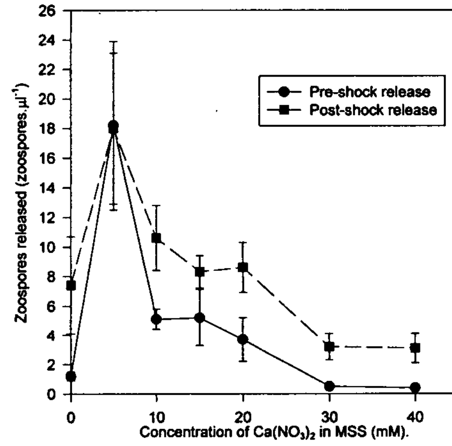


Figure 3.17 Effect of $Ca(NO_3)_2$.



* Means ± s.e.m. for 3 replicates, based on 2 haemocytometer counts per replicate.

3.3.2.3. Effects of treatments on sporangial discharge.

In addition to the indirect measurement of sporangiogenesis (zoospore release) two direct measurements of the effect of salt and salt concentration on zoosporangiogenesis were made. The first measurement was sporangial density (Section 3.3.2.1). The second was microscopic examination of sporangia to determine if they had released their contents. The concentrations of $CaCl_2$ and KNO_3 supplement were both negatively correlated with the fraction of sporangia that had discharged their contents prior to the removal of MSS (Figures 3.18 and 3.20 and Table 3.5). The relationship between pre-shock sporangial discharge and $Mg(NO_3)_2$ concentration was exponential ($y = 12.4 \cdot 10^{-0.0279x} - 1$) (Figure 3.19). The fitted data and the original data were very highly significantly correlated ($F = 4.98 \times 10^{-6}$, $R = 0.82$). In the $Ca(NO_3)_2$ free MSS and MSS supplemented with 20 mM or greater concentrations of $Ca(NO_3)_2$, few (< 1%) sporangia had discharged their contents. However, a peak (~13%) in sporangial discharge was observed when $Ca(NO_3)_2$ supplements were between 5 and 15 mM.

Table 3.5. Correlation statistics and the equations for lines of best fit for the effect of KNO_3 and $CaCl_2$ concentration on pre-cold-shock sporangial discharge of *Ph. parasitica*.

Salt	Line of best fit	*R*	*F*	*df*
KNO_3	y = 6.3 - 0.2x	-0.62	6.19×10^{-3}	17
$CaCl_2$	y = 11.3 - 0.2x	-0.66	9.97×10^{-4}	20

Post-cold-shock sporangial discharge was assessed when the MSS had been removed and replaced with SDW. Significant differences were not observed ($P = 0.928$) in the fraction of sporangia that had discharged their contents after being bathed in MSS supplemented with various concentrations of KNO_3 (Figure 3.18). The relationship between post-shock sporangial discharge and $Mg(NO_3)_2$ concentration was again exponential ($y = 23.9 \cdot 10^{-0.0175x}$) (Figure 3.19). The correlation between observed and fitted data was very highly significant ($F = 1.56 \times 10^{-6}$, $R = 0.84$, $df = 20$). The various $Ca(NO_3)_2$ supplements did not significantly ($P > 0.05$) affect sporangial discharge (Figure 3.21). Although a peak (28% of sporangia with discharged

contents) was distinctly observed in the 5 mM supplement, in all other $Ca(NO_3)_2$ supplements the fraction of discharged sporangia was between 5% and 14%. The lack of significance can be attributed to high variation in the results for supplements with 20 and 30mM $Ca(NO_3)_2$. There was also a peak in sporangial discharge in the 5 mM $CaCl_2$ supplement (Figure 3.20). After this peak, all subsequent supplements further suppressed sporangial discharge - that is, there was negative correlation between sporangial discharge and $CaCl_2$ concentration between 5 and 40 mM ($y = 29.2 - 0.5x$, $F = 4.67 \times 10^{-3}$, $R = 0.81$, $df = 16$).

Figures 3.18 - 3.21. Effect of salt type and salt concentration in MSS on sporangial discharge* in mats of *Phytophthora parasitica* .

Figure 3.18 Effect of KNO_3.

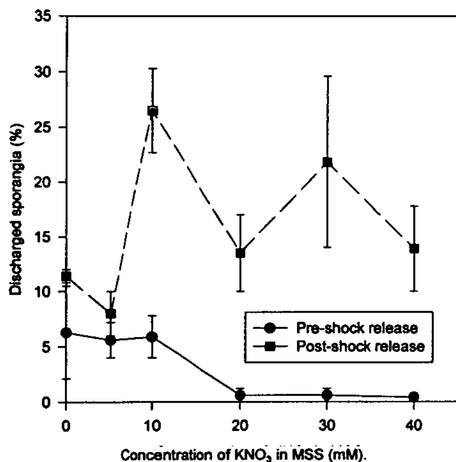


Figure 3.19 Effect of $Mg(NO_3)_2$.

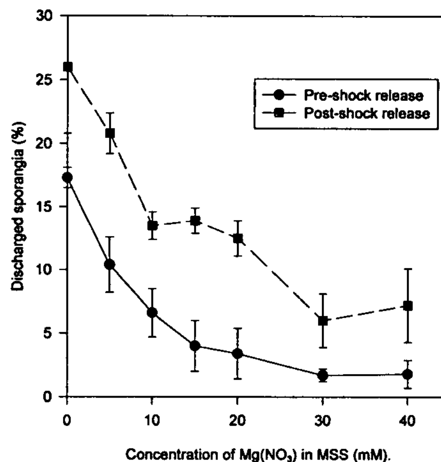


Figure 3.20 Effect of $CaCl_2$.

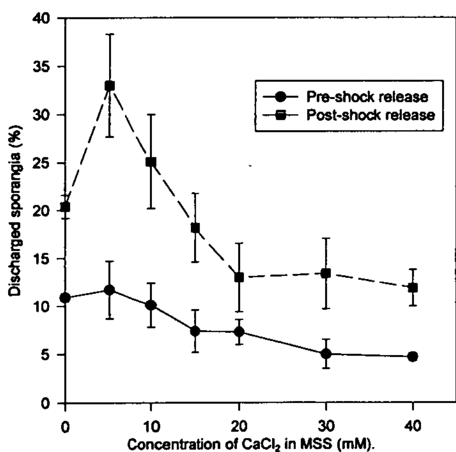
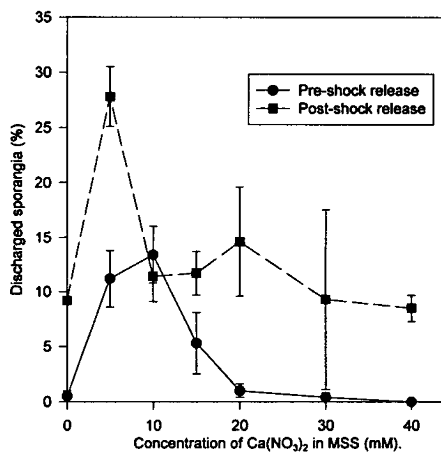


Figure 3.21 Effect of $Ca(NO_3)_2$.



* Means ± s.e.m. for 3 replicates, assessed before the cold shock and after zoospore release, based on scoring 3 fields of view per replicate.

3.4. Zoospore release into various salt solutions.

Mycelia of both *Py. aphanidermatum* and *Ph. parasitica* when bathed in MSS (13 mM Ca^{2+}, 6 mM Mg^{2+}, 5 mM K^{+}, 90 µM Fe^{2+}) at 20 °C in light produced sporangia. After *Pythium* has been subjected to cold shock the sporangia formed vesicles containing undifferentiated cytoplasm. After the cytoplasm has differentiated into zoospores the vesicle ruptured and zoospores were released. In contrast, in *Phytophthora* the vesicular stage is absent and cytoplasmic differentiation occurs within the sporangia. To establish the effect of the various salts at several concentrations on the release of zoospores from pre-formed sporangia of *Phytophthora* and *Pythium* the release solution was supplemented with one of the four salts ($Ca(NO_3)_2$, $CaCl_2$, $Mg(NO_3)_2$ and KNO_3) at test concentrations of 0, 5, 15, 20, 30 or 40 mM.

Only $CaCl_2$ did not significantly ($P > 0.05$) suppress zoospore release from pre-formed sporangia of *Py. aphanidermatum*, but the lack of significance was due to the high variation in zoospore release from sporangia bathed in 10 mM $CaCl_2$ (Figure 3.22). The overall trend was that increasing $CaCl_2$ concentration suppressed zoospore release. It was consistently noted that zoospore release from sporangia was highly variable. The three nitrate salts all significantly suppressed zoospore release from pre-formed sporangia ($P < 0.05$) (Figures 3.22, 3.23 and 3.24). A concentration of 40 mM of any tested salt reduced zoospore release to between 10 and 20% of the salt-free control (SDW).

The effects of salts on suppressing zoospore release from pre-formed sporangia of *Ph. parasitica* were less noticeable. *Ph. parasitica* released fewer zoospores than *Py. aphanidermatum*. Typically *Ph. parasitica* released approximately 100 zoospores.μl^{-1} into SDW, whereas *Py. aphanidermatum* releases approximately 500 zoospores.μl^{-1}. Zoospore release from pre-formed sporangia of *Ph. parasitica* was negatively correlated with calcium concentration irrespective of the counter ion (Cl^- or NO_3^-) (Figures 3.28 and 3.29 and Table 3.6). The fitted lines were very similar. Overall, increasing the KNO_3 or $Mg(NO_3)_2$ concentration reduced the number of zoospores released (Figures 3.26 and 3.27). The most effective salt at suppressing zoospore release from sporangia of *Ph. parasitica* was KNO_3, with 40 mM of this salt reducing zoospore release to 26% of the control value. In contrast, the calcium salts were the least effective, with 40 mM reducing zoospore release to about half that of the control.

Table 3.6. Correlation statistics and the equations for lines of best fit for the effect of calcium salts on zoospore release from pre-formed sporangia of *Ph. parasitica*.

Salt	Line of best fit	*R*	*F*	*df*
$CaCl_2$	y = 99 - 1.3x	-0.57	8.2×10^{-3}	20
$Ca(NO_3)_2$	y= 101 - 1.2x	-0.52	1.60×10^{-2}	20

Figures 3.22 - 3.25. Effect of salt type and salt concentration in release solution on zoospore release* from pre-formed sporangia of *Pythium aphanidermatum.*

Figure 3.22 Effect of KNO_3.

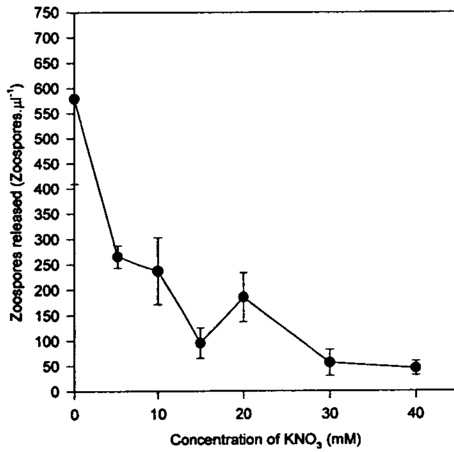


Figure 3.23 Effect of $Mg(NO_3)_2$.

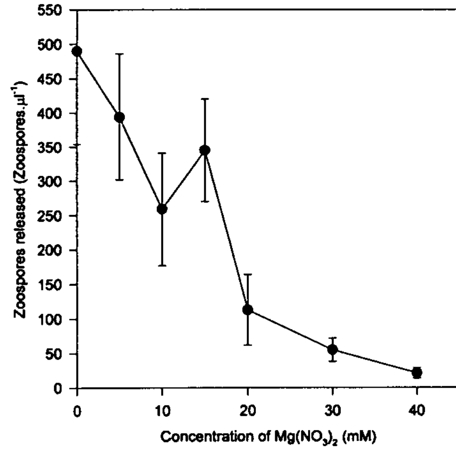


Figure 3.24 Effect of $CaCl_2$.

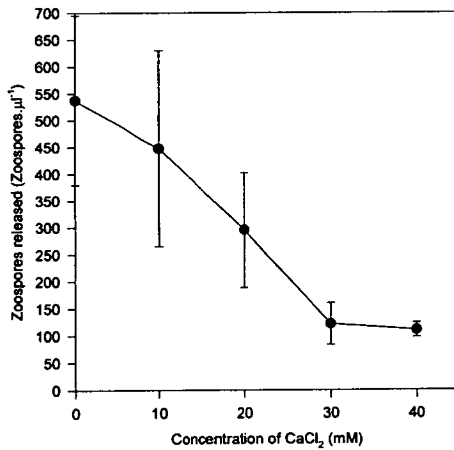
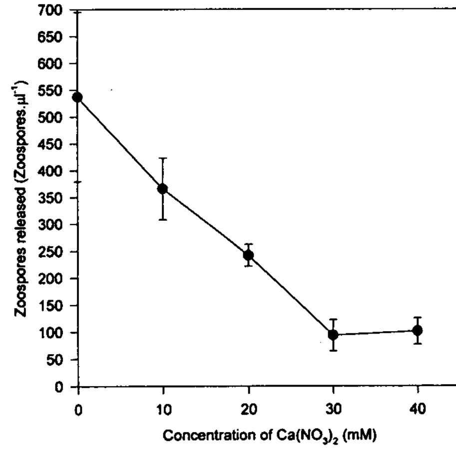


Figure 3.25 Effect of $Ca(NO_3)_2$.



* Means ± s.e.m. for 3 replicates, based on 2 haemocytometer counts per replicate.

Figures 3.26 - 3.29. Effect of salt type and salt concentration in release solution on zoospore release* from pre-formed sporangia of *Phytophthora parasitica.*

Figure 3.26 Effect of KNO_3.

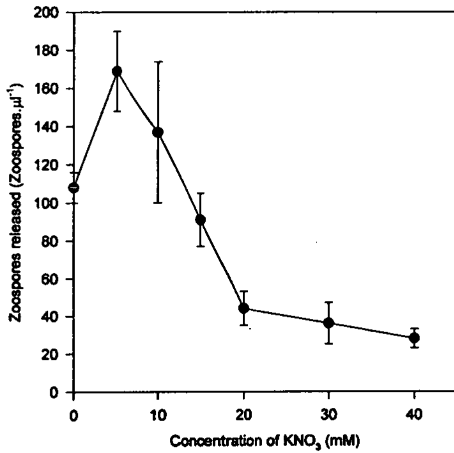


Figure 3.27 Effect of $Mg(NO_3)_2$.

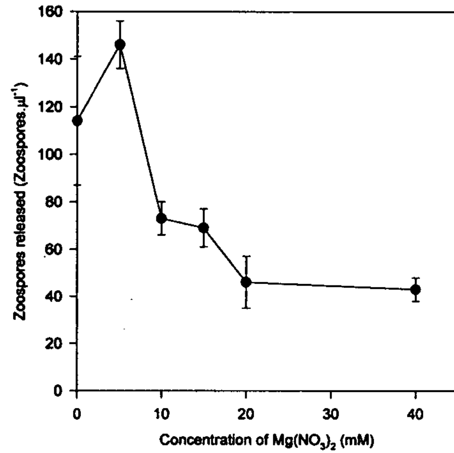


Figure 3.28 Effect of $CaCl_2$.

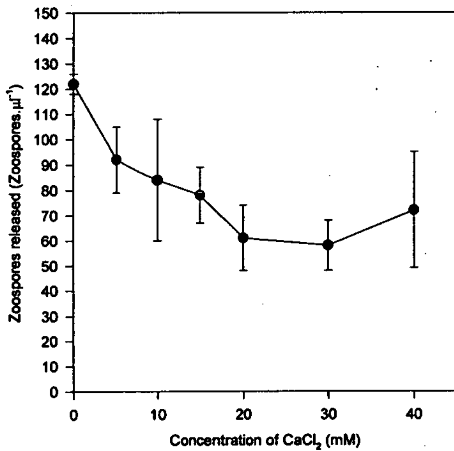
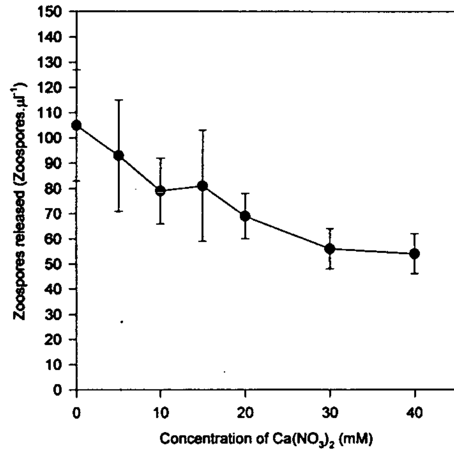


Figure 3.29 Effect of $Ca(NO_3)_2$.



* Means ± s.e.m. for 3 replicates, based on 2 haemocytometer counts per replicate.

3.5. Motility of zoospores and subsequent cyst germination in various salt solutions.

Zoospores released into SDW were transferred to Petri dishes supplemented with various salts at various concentrations (see Section 2.3.6). After 2h the base of the Petri dish was examined microscopically to determine the fraction of zoospores that had retained motility and the fraction of cysts that had germinated.

Increasing salt concentration caused increased suppression of zoospore motility by inducing encystment of zoospores of *Py. aphanidermatum* (Figures 3.30 to 3.33). A 5 mM KNO_3 or $Mg(NO_3)_2$ supplement significantly (t-tests, $P = 1.5 \times 10^{-4}$ and $P = 0.031$, respectively) reduced zoospore motility compared to the SDW control. Although 5 mM $CaCl_2$ did significantly (t-test, $P = 0.029$) reduce zoospore motility compared to the control, the same concentration of $Ca(NO_3)_2$ did not significantly (t-test, $P = 0.093$) reduce zoospore motility. A concentration of 15 mM of either KNO_3 or $Ca(NO_3)_2$ caused all observed zoospores to encyst within the 2h incubation period. To achieve complete suppression of zoospore motility with $CaCl_2$ required a concentration of 20 mM.

After 2h swimming in the SDW controls approximately 40% of zoospores of *Py. aphanidermatum* were still motile. The comparable value for *Ph. parasitica* was 10% after a one hour incubation (Figures 3.34 to 3.37). The effectiveness of the tested salts to suppress zoospore motility was less with *Ph. parasitica* than with *Py. aphanidermatum*. To significantly reduce zoospore motility required either 10 mM KNO_3 (t-test, $P = 3.4 \times 10^{-4}$), 20 mM $Ca(NO_3)_2$ (t-test, $P = 0.018$), 20 mM $Mg(NO_3)_2$ (t-test, $P = 5.6 \times 10^{-3}$) or 15 mM $CaCl_2$ (t-test, $P = 0.021$). To completely suppress zoospore motility required either 40 mM $CaCl_2$, 40 KNO_3 or 30 mM $Ca(NO_3)_2$. Concentrations of $Mg(NO_3)_2$ up to 40 mM did not completely suppress motility of zoospores of *Ph. parasitica*.

Once zoospores had encysted they had the potential to germinate. In SDW controls approximately 20% of all cysts of *Py. aphanidermatum* had germinated during the 2h incubation (Figures 3.38 to 3.41). Overall, $Mg(NO_3)_2$, $Ca(NO_3)_2$ and $CaCl_2$ supplements increased the proportion of cysts that had germinated. The highest mean levels of germination were 79% in

40mM $Ca(NO_3)_2$, 71% in 30 mM $Mg(NO_3)_2$ and 26% in 40 mM $CaCl_2$. Low concentrations of KNO_3 (5 and 10 mM) noticeably increased the proportion of cysts that had germinated, although higher concentrations (15 mM and above) did not significantly ($P > 0.05$) affect the proportion of cysts germinating compared to the SDW control.

KNO_3 concentrations up to 40 mM had no noticeable affect on the germination of cysts of *Ph. parasitica* (Figure 3.42). Similarly, increasing concentrations of $Mg(NO_3)_2$ had no significant ($P > 0.05$) effect on cyst germination, but the proportions of cysts germinating in the $Mg(NO_3)_2$ supplements were all lower than in the SDW control (Figure 3.43). The highest level of germination observed in the $Mg(NO_3)_2$ supplements was 16%, whereas in the control 31% of cysts had germinated; this difference was significant (t-test, $P = 0.021$). Increasing Ca^{2+} concentration increased cyst germination (Figures 3.44 and 3.45). $CaCl_2$ was more effective at promoting cyst germination than $Ca(NO_3)_2$, with 51% of cysts germinating in 40 mM $CaCl_2$, but only 33% germinating in the 40 mM $Ca(NO_3)_2$ treatment.

Figures 3.30 - 3.33. Effects of salt type and salt concentration on motility* of zoospores of *Pythium aphanidermatum.*

Figure 3.30 Effect of KNO_3.

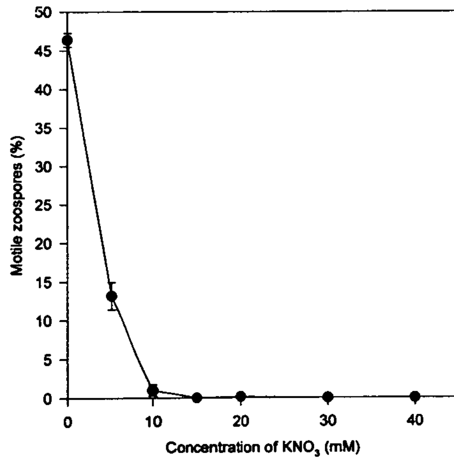


Figure 3.31 Effect of $Mg(NO_3)_2$.

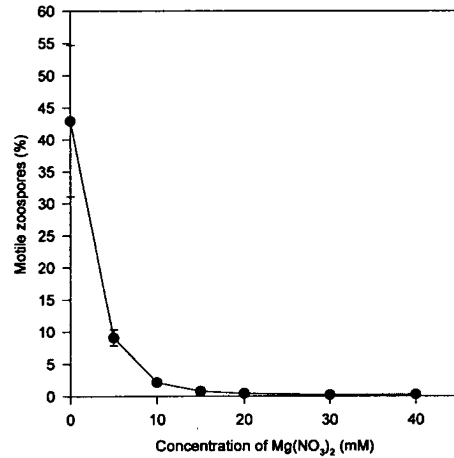


Figure 3.32 Effect of $CaCl_2$.

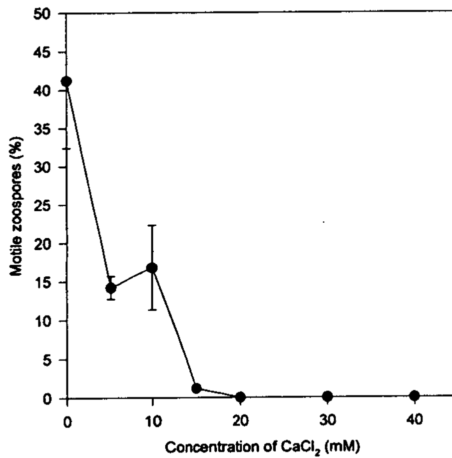
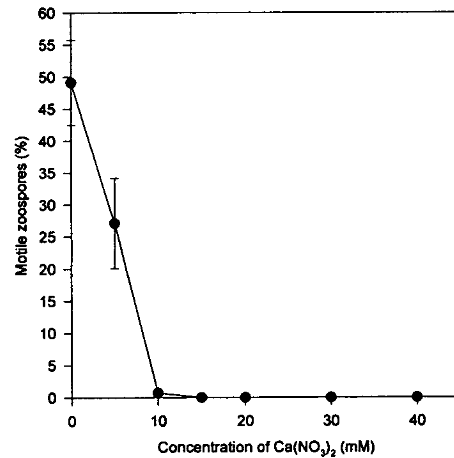


Figure 3.33 Effect of $Ca(NO_3)_2$.



* Means ± s.e.m. for 3 replicates, assessed after 2h, based on scoring 3 fields of view per replicate.

Figures 3.34 - 3.37. Effect of salt type and salt concentration on motility* of zoospores of *Phytophthora parasitica.*

Figure 3.34 Effect of $Ca(NO_3)_2$.

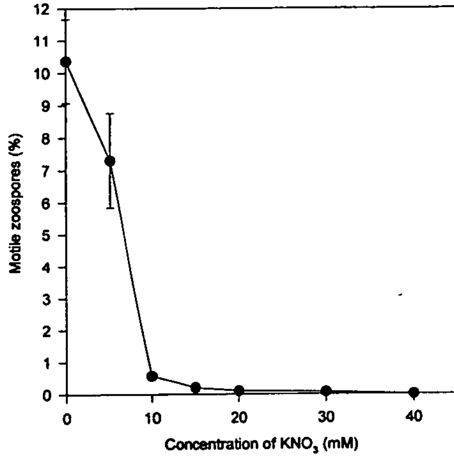


Figure 3.35 Effect of $Mg(NO_3)_2$.

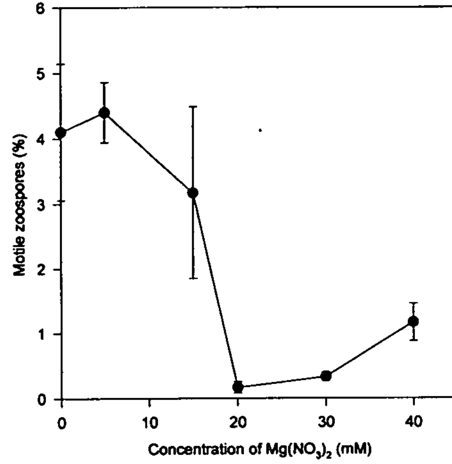


Figure 3.36 Effect of $CaCl_2$.

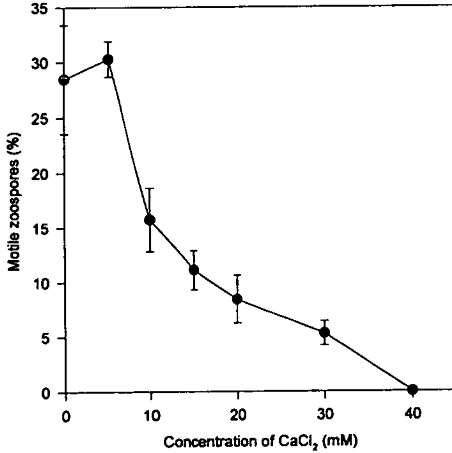
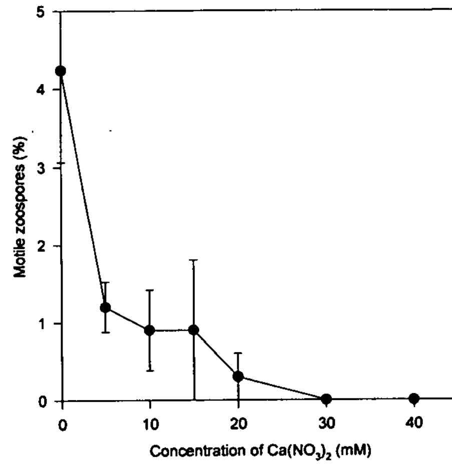


Figure 3.37 Effect of $Ca(NO_3)_2$.



* Means ± s.e.m. for 3 replicates, assessed after 1h, based on scoring 3 fields of view per replicate.

Figures 3.38 - 3.41. Effect of salt type and salt concentration on spontaneous germination* of encysted zoospores of *Pythium aphanidermatum.*

Figure 3.38 Effect of KNO_3.

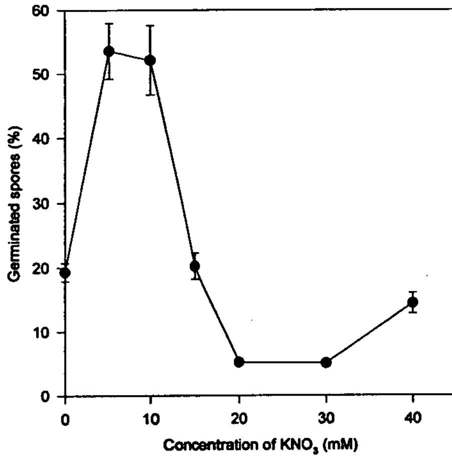


Figure 3.39 Effect of $Mg(NO_3)_2$.

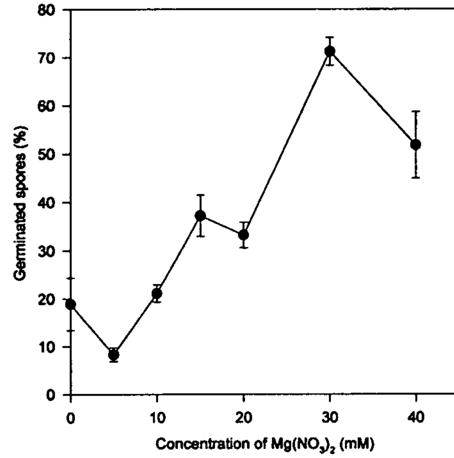


Figure 3.40 Effect of $CaCl_2$.

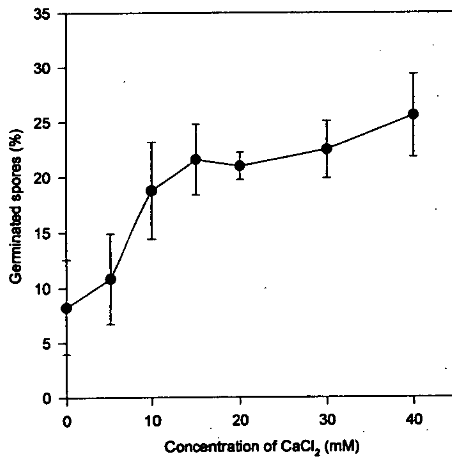
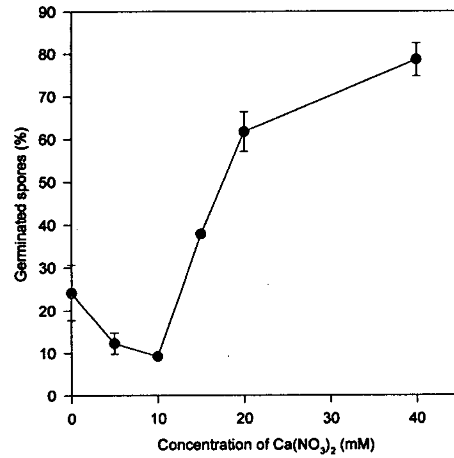


Figure 3.41 Effect of $Ca(NO_3)_2$.



* Means ± s.e.m. for 3 replicates, assessed after 2h, based on scoring 3 fields of view per replicate.

Figures 3.42 - 3.45. Effect of salt type and salt concentration on spontaneous germination* of encysted zoospores of *Phytophthora parasitica.*

Figure 3.42 Effect of KNO_3.

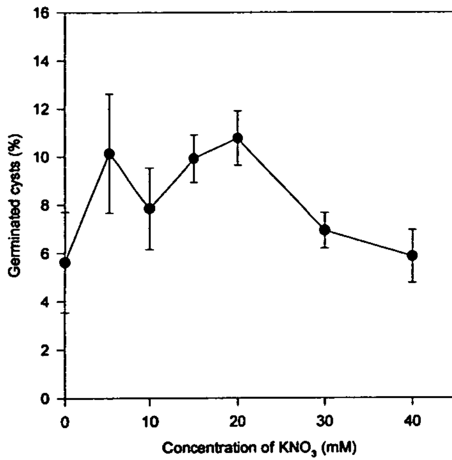


Figure 3.43 Effect of $Mg(NO_3)_2$.

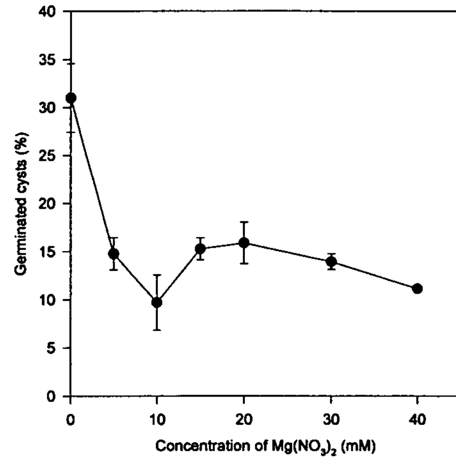


Figure 3.44 Effect of $CaCl_2$.

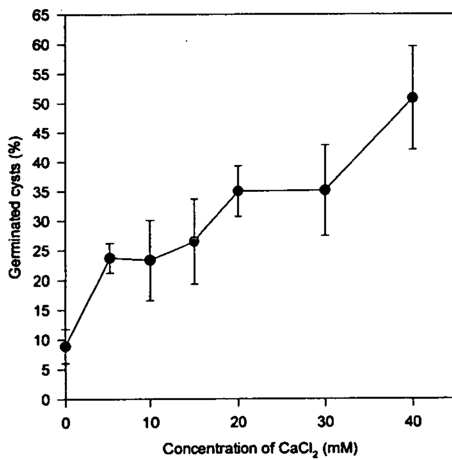
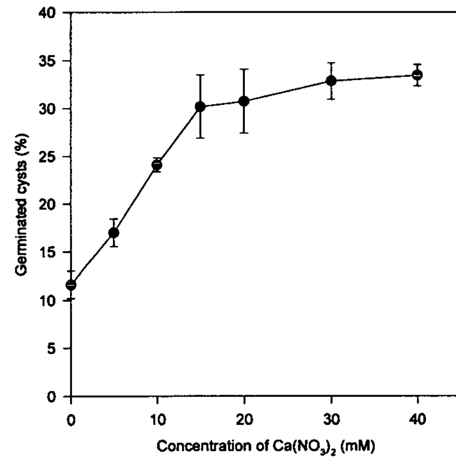


Figure 3.45 Effect of $Ca(NO_3)_2$.



* Means ± s.e.m. for 3 replicates, assessed after 2h, based on scoring 3 fields of view per replicate.

3.6. Germination of vortex-encysted zoospores in various salt solutions.

In the motility assays zoospores naturally encysted and had the potential to germinate. In the following experiments, zoospores were mechanically induced to encyst by vortexing (Section 2.3.2). Without an exogenous supplement, typically 20 to 30% of *Py. aphanidermatum* cysts germinated during the 2h incubation (Figures 3.46 to 3.49). Supplements of KNO_3 up to 40 mM did not significantly effect the proportion of cysts that germinated. For the other three salts, the overall affect was for increasing salt concentration to increase the proportion of cysts that germinated. The maximum level of germination achieved was 50 to 60% in the 30 to 40 mM salt supplements.

The percentage germination of cysts of *Ph. parasitica* in SDW was typically below 10% (Figures 3.50 to 3.52). All supplements of KNO_3 significantly (t-tests, $P < 0.05$) increased cyst germination levels to at least 20%, but the differing concentrations of the supplement had little effect on the proportion of cysts germinating (Figure 3.50). Similarly, all $Mg(NO_3)_2$ supplements significantly (t-tests, $P < 0.05$) increased the proportion of cysts germinating to at least 7.5%, but the differing concentrations of the supplement had little effect on the proportion of cysts germinating (Figure 3.51). Increasing concentrations of $Ca(NO_3)_2$ caused increased levels of cyst germination, with 45% of cysts germinating in 40 mM $Ca(NO_3)_2$ (Figure 3.52).

Figures 3.46 - 3.49. Effects of salt type and salt concentration on germination* of encysted zoospores of *Pythium aphanidermatum*.

Figure 3.46 Effect of KNO_3

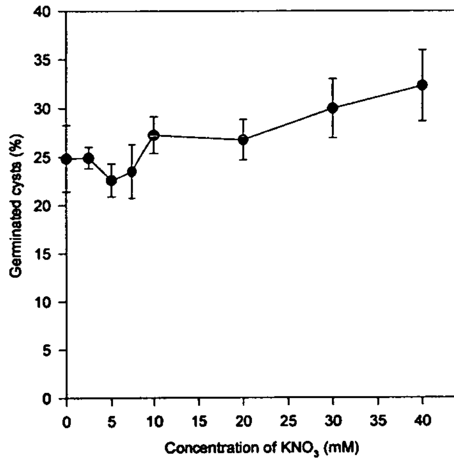


Figure 3.47 Effect of $Mg(NO_3)_2$

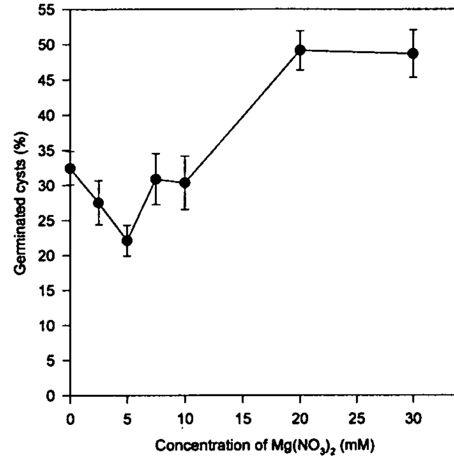


Figure 3.48 Effect of $CaCl_2$

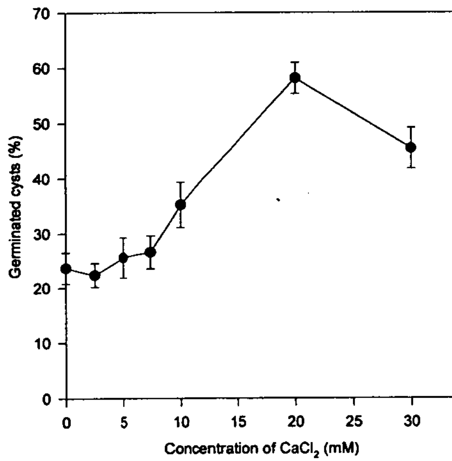
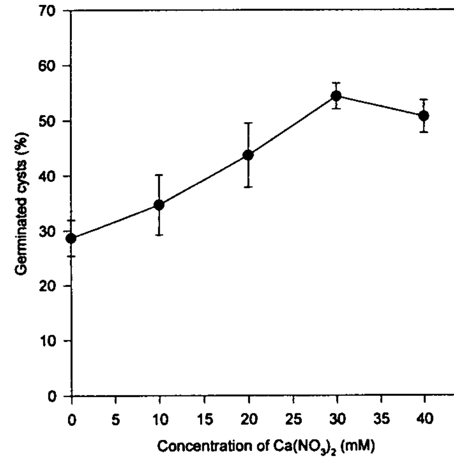


Figure 3.49 Effect of $Ca(NO_3)_2$



* Means ± s.e.m. for 5 replicates, assessed after 2h, based on observations of at least 100 spores per replicate.

Figures 3.50 - 3.52. Effects of salt type and salt concentration on germination* of encysted zoospores of *Phytophthora parasitica.*

Figure 3.50 Effect of KNO_3

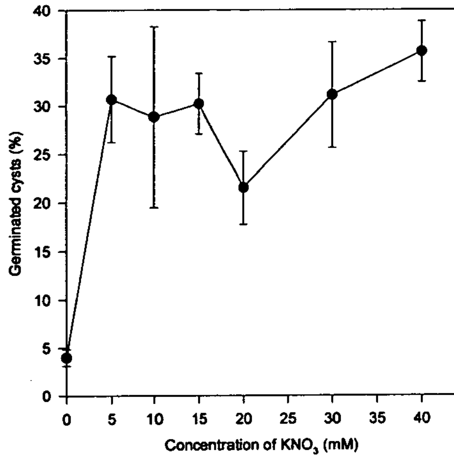


Figure 3.51 Effect of $Mg(NO_3)_2$

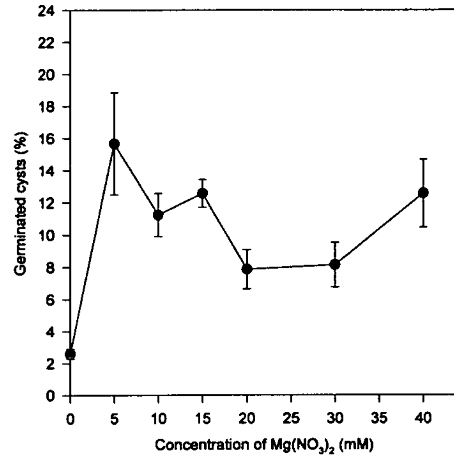
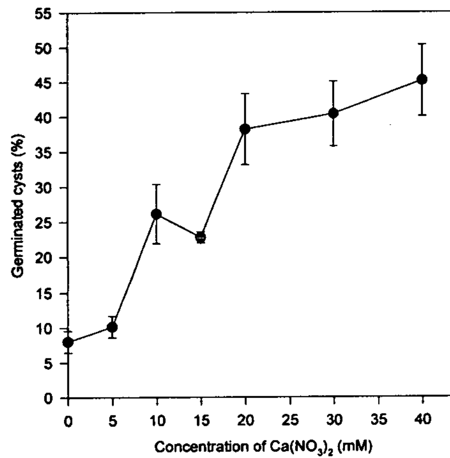


Figure 3.52 Effect of $Ca(NO_3)_2$



* Means ± s.e.m. for 3 replicates, assessed after 2h, based on observations of at least 100 spores per replicate.

3.7. Summary of effects of cations on individual stages of the zoosporic infection sequence.

In the foregoing experiments five stages of the zoosporic infection sequence of two fungi were exposed to three cations at various concentrations. A summary of results is shown in Table 3.7. In some assessments there was consistency in the trends. For example, all three cations (Ca^{2+}, Mg^{2+} and K^{+}) suppressed zoospore release from pre-formed sporangia of both *Ph. parasitica* and *Py. aphanidermatum*. Also, all three cations enhanced germination of vortex-encysted spores of both fungi. In other assessments the effect of the cations was different for the two fungi. Potassium did not affect mycelial growth of either species, whereas calcium clearly suppressed mycelial growth of *Py. aphanidermatum*, but there was evidence to suggest that calcium enhanced mycelial growth of *Ph. parasitica*.

The cations tested did not generally affect sporangial density of *Ph. parasitica* or sporangiogenesis of *Py. aphanidermatum*. However, the effect of cations on the subsequent liberation of zoospores and the proportion of sporangia of *Ph. parasitica* that discharged was dependent on cation species. Magnesium and potassium, in general, suppressed sporangial discharge. At low concentrations calcium caused more zoospores to be released than in the SDW control.

Cation valency was not consistently related to effect on zoospore biology. The divalent cations suppressed mycelial growth of *Py. aphanidermatum*, whereas the monovalent potassium ion did not. However, in several cases, the effects of magnesium and potassium were similar, but different from the effect of calcium. For example, magnesium and potassium had no effect on the pre-shock emptying of sporangia of *Ph. parasitica*, whereas calcium did have a noticeable effect on this.

Germination of cysts was assessed under two different protocols. Under the first protocol, encystment was either spontaneous or salt-induced. The results from these experiments were highly variable, although calcium promoted cyst germination in both fungi. Although high concentrations of potassium had no noticeable effect on germination of cysts of *Ph. parasitica*, a peak in cyst germination in low concentrations of potassium was observed. Under the second

protocol encystment was by mechanical agitation and the effect of the three species of cation on cysts from both fungi was always to increase the proportion of cysts that germinated.

Table 3.7. Summary of effects of cations on individual stages of the zoosporic infection sequence.

Stage	Fungi	Cations	Effect of increasing cation concentration
Mycelial growth	*Py. aphanidermatum*	Ca^{2+} & Mg^{2+}	Suppression
Mycelial growth	Both	K^{+}	No effect
Mycelial growth	*Ph. parasitica*	Ca^{2+}	Possibly enhance
Mycelial growth	*Ph. parasitica*	Mg^{2+}	Suppression
Sporangiogenesis (indirect measurement)	*Py. aphanidermatum*	Ca^{2+}, Mg^{2+} & K^{+}	No effect
Sporangial density	*Ph. parasitica*	Ca^{2+}, Mg^{2+} & K^{+}	No effect
Sporangial discharge (zoospores) - pre shock and post shock	*Ph. parasitica*	Ca^{2+}	Peak at 5 mM
Sporangial discharge (zoospores) - pre shock	*Ph. parasitica*	Mg^{2+} & K^{+}	Suppression
Sporangial discharge (zoospores) - post shock	*Ph. parasitica*	Mg^{2+} & K^{+}	No effect
Empty sporangia - pre shock	*Ph. parasitica*	Ca^{2+}	Peak at 5 mM
Empty sporangia - pre shock	*Ph. parasitica*	Mg^{2+} & K^{+}	Suppression
Empty sporangia - post shock	*Ph. parasitica*	Ca^{2+}	Peak at 5 mM
Empty sporangia - post shock	*Ph. parasitica*	Mg^{2+}	Suppression
Empty sporangia - post shock	*Ph. parasitica*	K^{+}	No effect
Zoospore release	Both	Ca^{2+}, Mg^{2+} & K^{+}	Suppression
Zoospore motility	Both	Ca^{2+}, Mg^{2+} & K^{+}	Suppression
Subsequent germination	*Py. aphanidermatum*	Ca^{2+} & Mg^{2+}	Enhance
Subsequent germination	*Py. aphanidermatum*	K^{+}	Peak at 5-10 mM
Subsequent germination	*Ph. parasitica*	Ca^{2+}	Enhance
Subsequent germination	*Ph. parasitica*	Mg^{2+}	Possible suppression
Subsequent germination	*Ph. parasitica*	K^{+}	No effect
Germination of vortex encysted zoospores.	Both	Ca^{2+}, Mg^{2+} & K^{+}	Enhance

3.8. The compounded effects of cationic supplements on aspects of zoospore biology

A cationic supplement to an irrigation system would target all stages of the infection sequence concurrently: from mycelial growth to infection of a host by a germ tube. To simulate this *in vitro*, the standard protocol was modified so that test salts at the test concentrations was constant in the treatments throughout the experiments. That is, the same concentration of the salt was added to the CV8 broth, the MSS and the release solution. At several stages throughout the experiments, aspects of fungal biology were quantified. These were the number of zoospores released, the proportion of zoospores motile after the incubation period and the proportion of cysts that had germinated during the motility period. In addition to these aspects, a portion of the zoospore suspension was drawn-off after zoospore release, and zoospores were mechanically encysted. After a period of time the proportion of cysts that had germinated after mechanically induced encystment was assessed.

3.8.1. *Pythium aphanidermatum.*

The first aspect of zoospore biology to be assessed was the number of zoospores released into the release solution. The release solution was the test salt at the test concentration. In the KNO_3 and $Mg(NO_3)_2$ free controls the number of zoospores released was approximately 552 and 773 zoospores.μl^{-1}, respectively (Figures 3.53 and 5.54). In the 5 mM KNO_3 treatment the number of zoospores released was reduced very significantly (t-test, $P = 0.0031$) to 150 zoospores.μl^{-1}, that is 27% of the K^+-free control value. The 40 mM $Mg(NO_3)_2$ treatment reduced the number of zoospores released to 1% of the Mg^{2+} free control, but the effectiveness at suppressing zoospore release at lower concentrations was not as noticeable as for KNO_3. Increasing concentrations of $CaCl_2$ reduce the number of zoospores released (Figure 3.55). The 40 mM $CaCl_2$ treatment reduced the number of zoospores released to 5% of the calcium-free control. When the 40 mM calcium supplement was supplied as the nitrate salt, the number of zoospores released was 0.5% of the control (Figure 3.56). However, there was a peak in zoospore release numbers in the 5 mM $Ca(NO_3)_2$ treatment that was 65% greater than in the Ca^{2+} free control.

After the release period, the mycelial mats were removed from the Petri dishes and the number of spores (zoospores and cysts) observed in the bases of the dishes were then scored (Figures 3.57 to 3.60). There was good correlation between the number of zoospores released as assessed by the first method and this method.

The fraction of zoospores observed to be motile after this 2h incubation followed a similar pattern to the release of zoospores (Figures 3.61 to 3.64). In the KNO_3 control 58% of zoospores were motile, but only 5% were motile when 5 mM KNO_3 was present (Figure 3.61). Increasing supplements of KNO_3 had little additional effect on the proportion of zoospores remaining motile. Increasing concentrations of $Mg(NO_3)_2$ caused the proportion of zoospores that remained motile to decrease (Figure 3.62). In the Mg^{2+} free control 86% of spores were observed to be motile, while only 2% were motile in the 40 mM $Mg(NO_3)_2$ treatment. Similarly, increasing concentrations of $CaCl_2$ caused the proportion of zoospores that remained motile to decrease (Figure 3.63). In the $CaCl_2$ free control 95% of spores remained motile during the incubation period, while no zoospore were observed to be motile after being incubated in 40 mM $CaCl_2$. In the $Ca(NO_3)_2$ free control 36% of spores were motile, but only 1.4% were motile in the 20 mM treatment. There was a peak in zoospore motility in the 5 mM treatment with 61% of spores remaining motile during the incubation period.

For the controls of the KNO_3, $Mg(NO_3)_2$ and $CaCl_2$ of (Figures 3.65 to 3.67) the fraction of encysted zoospores that germinated during the 2h incubation was between 17% and 28%.. The lower concentrations of KNO_3 (5 to 15 mM) increased cyst germination levels to at least 65%, whereas in the treatments with 20 mM or greater concentrations of KNO_3, the proportion of cysts germinating was not significantly ($P > 0.11$) different from the K^+-free control (Figure 3.65). Although there was significant (ANOVA, $P = 0.027$) variation in the proportion of cysts germinating in various concentrations of $Mg(NO_3)_2$, there was not an obvious relationship between the proportion of cysts germinating and $Mg(NO_3)_2$ concentration (Figure 3.66). There was not significant (ANOVA, $P = 0.081$) variation in the proportion of cysts germination between the treatments of $CaCl_2$ (Figure 3.67). The effect of increasing $Ca(NO_3)_2$ supplements on cyst germination was complex, with the highest germination levels observed in the control and the lowest in the 5 mM supplement (Figure 3.68). There was also a noticeable peak in the 15

mM treatment which is significantly (t-test, $P = 0.037$) greater than the level in the 10 mM treatment.

The sample of zoospore suspension that was removed prior to the incubation for spontaneous zoospore encystment was vortexed and incubated for 2h. The proportion of cysts that germinated was then enumerated. All supplements of KNO_3 significantly ($P < 0.05$) increased the proportion of cysts that had germinated when compared to the K^+ free control (Figure 3.69), but there was no significant (ANOVA, $P = 0.12$) variation in the proportion of cysts germinating between the different supplements of KNO_3. In the Mg^{2+} free control 17% of cysts had germinated, whereas in the 40 mM $Mg(NO_3)_2$ treatment the percentage germinated had increased to 62% (Figure 3.70). The level of cyst germination in the 40 mM $CaCl_2$ treatment just failed to be not significantly ($P = 0.053$) different from the $CaCl_2$ free control (Figure 3.71). However, the overall trend of increasing $CaCl_2$ supplement was to increase the proportion of cysts that germinated. The effect of increasing $Ca(NO_3)_2$ supplements on cyst germination was again complex, with the highest germination levels observed in the control and the lowest in the 5 mM supplement (Figure 3.72). Also there was a noticeable peak in the 15 mM treatment. The correlation between the proportion of cysts germinating in $Ca(NO_3)_2$ recorded in the two graphs (Figures 3.68 and 3.72) was very highly significant ($R = 0.70$, $F = 8.76 \times 10^{-5}$, n = 25). The data analysed were not arcsine transformed. Transformation of the data would have had negligible affect on the correlation statistics.

Figures 3.53 - 3.56. Effect of salt type and salt concentration on zoospore release* from sporangia of *Pythium aphanidermatum.*

Figure 3.53 Effect of KNO_3

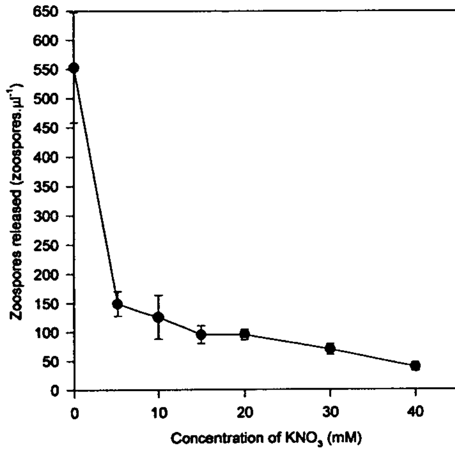


Figure 3.54 Effect of $Mg(NO_3)_2$

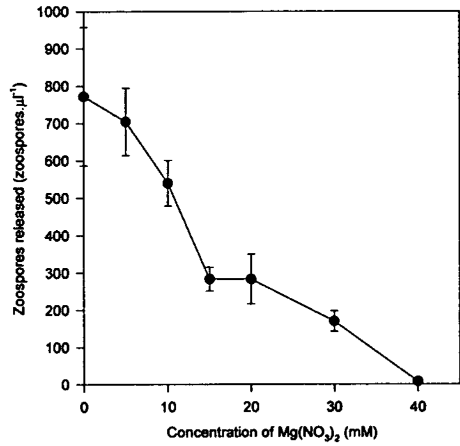


Figure 3.55 Effect of $CaCl_2$

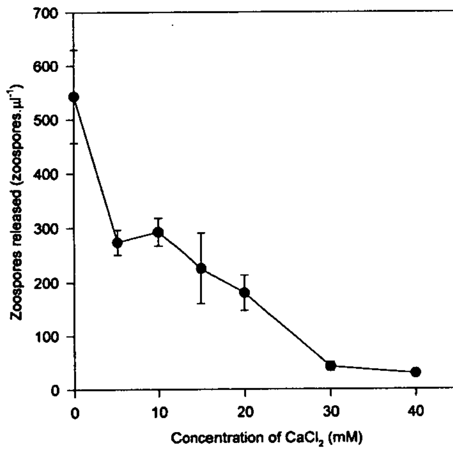
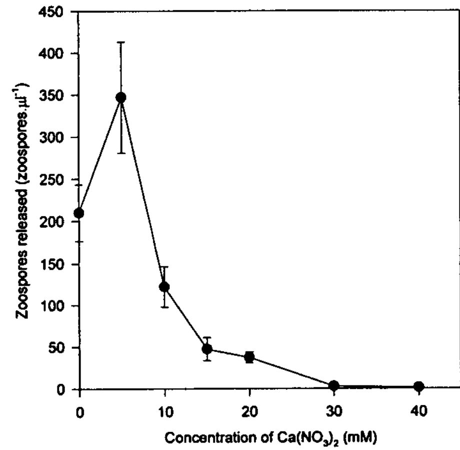


Figure 3.56 Effect of $Ca(NO_3)_2$



* Means ± s.e.m. for 5 replicates, based on 2 haemocytometer counts per replicate.

Figures 3.57 - 3.60. Effect of salt type and salt concentration on the number of spores of *Py. aphanidermatum* observed at the bases of the Petri dishes after 2 h incubation*.

Figure 3.57 Effect of KNO_3

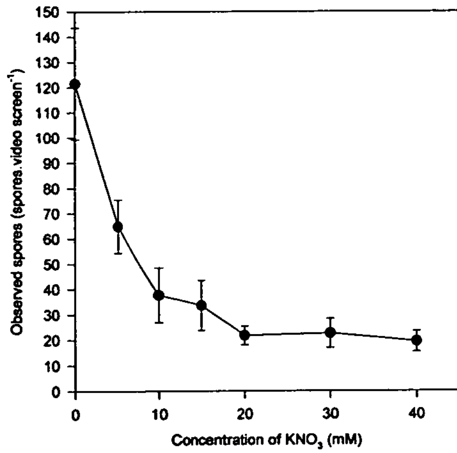


Figure 3.58 Effect of $Mg(NO_3)_2$

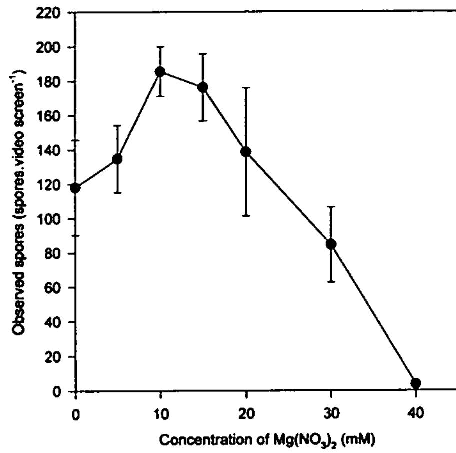


Figure 3.59 Effect of $CaCl_2$

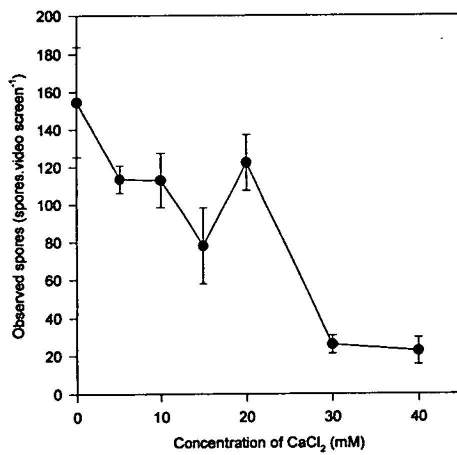
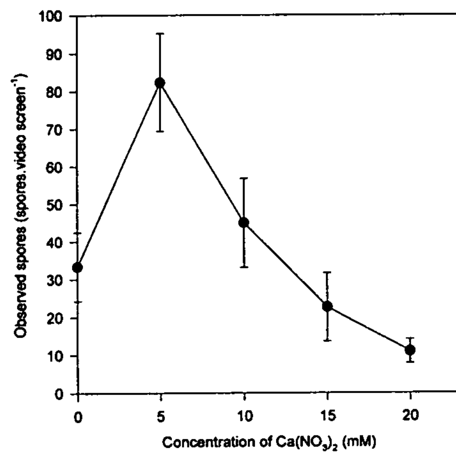


Figure 3.60 Effect of $Ca(NO_3)_2$



* Means ± s.e.m. for 5 replicates, based on 3 fields of view per replicate.

Figures 3.61 - 3.64. Effect of salt type and salt concentration on the proportion of spores of *Py. aphanidermatum* remaining motile after 2 h incubation*.

Figure 3.61 Effect of KNO_3

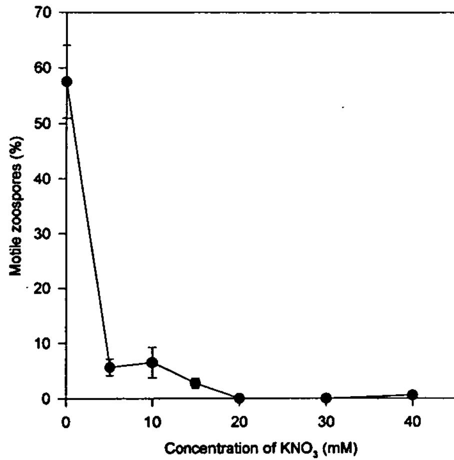


Figure 3.62 Effect of $Mg(NO_3)_2$

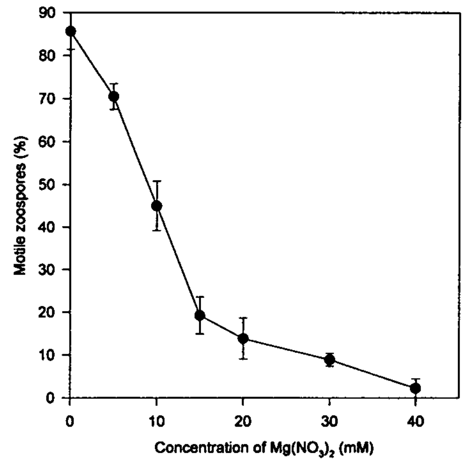


Figure 3.63 Effect of $CaCl_2$

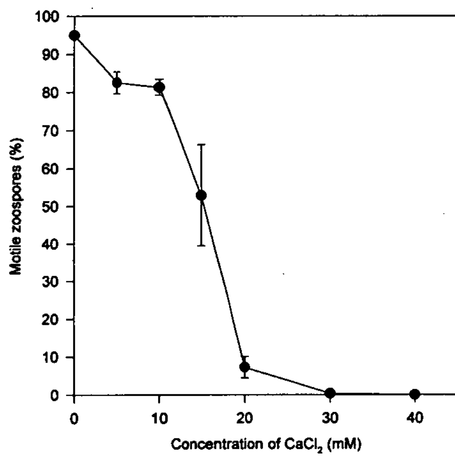
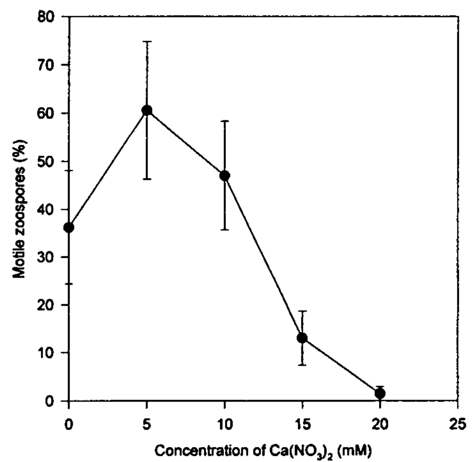


Figure 3.64 Effect of $Ca(NO_3)_2$



* Means ± s.e.m. for 5 replicates, based on 3 fields of view per replicate.

Figures 3.65 - 3.68. Effect of salt type and salt concentration on the proportion of naturally encysted zoospores of *Py. aphanidermatum* that had germinated during 2 h incubation*.

Figure 3.65 Effect of KNO_3

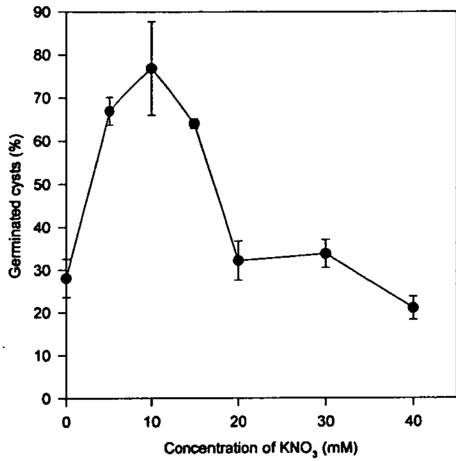


Figure 3.66 Effect of $Mg(NO_3)_2$

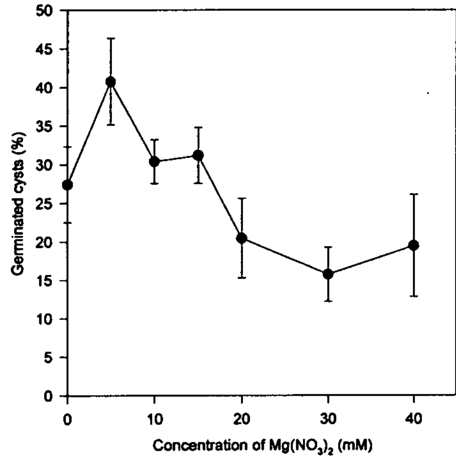


Figure 3.67 Effect of $CaCl_2$

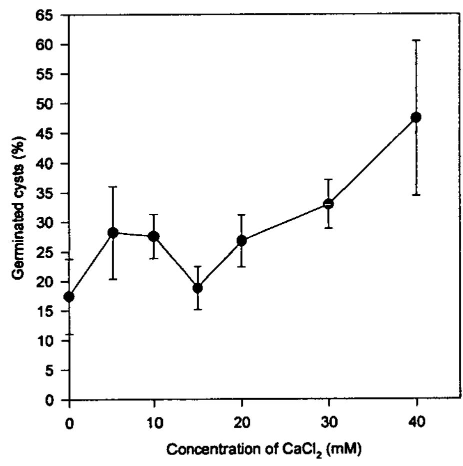
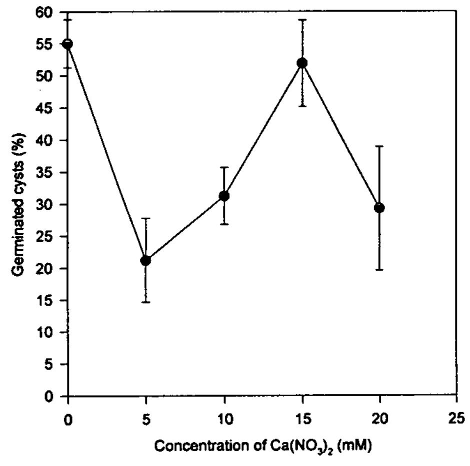


Figure 3.68 Effect of $Ca(NO_3)_2$



* Means ± s.e.m. for 5 replicates, based on 3 fields of view per replicate.

Figures 3.69 - 3.72. Effect of salt type and salt concentration on the proportion of vortex-encysted zoospores of *Py. aphanidermatum* that had germinated during 2 h incubation*.

Figure 3.69 Effect of KNO_3

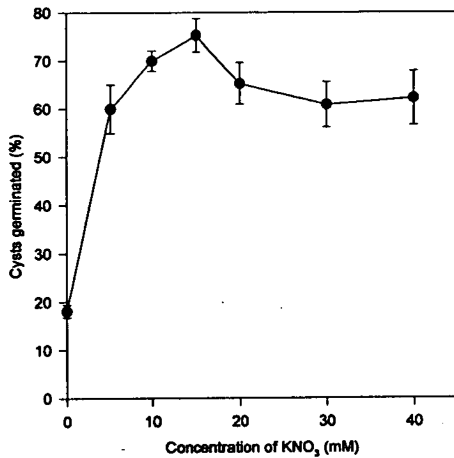


Figure 3.70 Effect of $Mg(NO_3)_2$

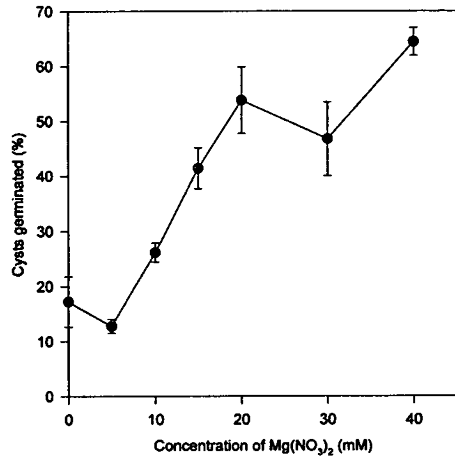


Figure 3.71 Effect of $CaCl_2$

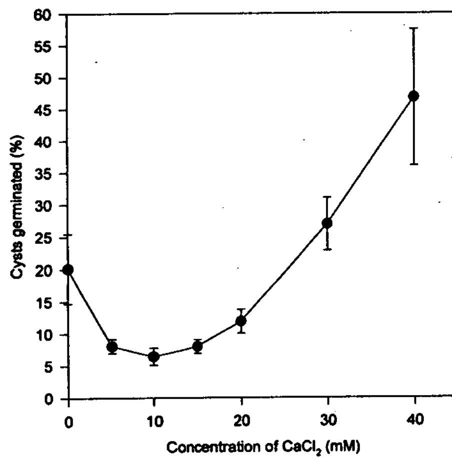
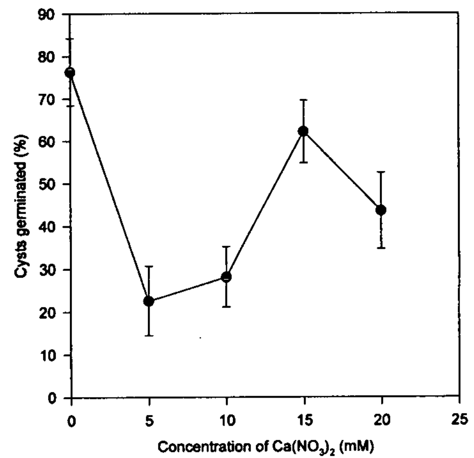


Figure 3.72 Effect of $Ca(NO_3)_2$



* Means ± s.e.m. for 5 replicates, based on a minimum of 100 counts per replicate.

3.8.2. *Phytophthora parasitica.*

Parallel experiments investigating the compounded effects of cationic supplements were performed using *Ph. parasitica*. However, only KNO_3 and $Ca(NO_3)_2$ salts were used, and at fewer concentration levels. Also, two additional aspects of sporangiogenesis were quantified - sporangial density and the proportion of sporangia discharging their contents.

The 5 mM KNO_3 supplement at all stages of the protocol did not significantly suppress pre-shock zoospore release ($P = 0.628$), pre-shock sporangial discharge ($P = 0.495$), post-shock zoospore release ($P = 0.074$), post-shock sporangial discharge ($P = 0.143$), sporangial density ($P = 0.451$), zoospore motility ($P = 0.106$) or the germination of vortex encysted spores ($P = 0.646$) of *Ph. parasitica* (Table 3.8). However, the 5 mM KNO_3 supplement did significantly ($P = 0.039$) suppress germination of cysts that had naturally encysted during the zoospore motility assay. Although the results were not always significant the general trends were consistent with the results from the corresponding experiment with *Py. aphanidermatum*. That is, a 5 mM KNO_3 supplement suppressed both zoospore release and zoospore motility.

In the pre-shock zoospore release assay the 5 mM $Ca(NO_3)_2$ supplement significantly ($P < 0.05$) increased zoospore release in comparison to both the calcium-free control and 20 mM supplement (Table 3.9) - there was no significant ($P > 0.05$) difference between the control and 20 mM supplement. This was not reflected in the degree of pre-cold shock sporangial discharge where the control and 5 mM did not significantly ($P < 0.05$) differ for each other, but sporangial discharge was significantly ($P >0.05$) increased by the 20 mM supplement.

There was no significant ($P > 0.05$) effect on post-shock zoospore release, post-shock sporangial discharge or sporangial density by 5 mM $Ca(NO_3)_2$ when compared to the Ca^{2+}-free control, whereas zoospore release, post-shock sporangial discharge and sporangial density were all significantly (t-tests, $P < 0.05$) greater in the control than in the 20 mM supplement.

The fraction of zoospores motile in the Ca^{2+}-free control was 16%; this rose to 26% in the 5 mM Ca^{2+} treatment. However, in the 20 mM Ca^{2+} supplement, no zoospores were found to be motile. Once the zoospores had encysted they had the potential to germinate. In the control 23% of the encysted spores had germinated. This was not significantly ($P > 0.05$) increased by the 5mM

Ca^{2+} supplement, but in the 20 mM supplement 55% of spores had germinated - this was significantly (multiple range test, $P < 0.05$) greater than in the control and the 5 mM supplement.

Table 3.8. Effects of applications of 5 mM KNO_3 on aspects of biology of *Ph. parasitica*.

Aspect	Units	0 mM K^+	5 mM K^+	*P* (t-test)
Pre-shock zoospore release [a]	zoospores.μl^{-1}	10.3 ± 1.6	9.0 ± 2.0	0.628
Post-shock zoospore release [a]	zoospores.μl^{-1}	22.3 ± 1.9	17.8 ± 0.9	0.074
Vortex encysted zoospore germination [b]	% (and Arcsine)	22.8 ± 5.3 (28.0 ± 3.8)	25.2 ± 2.6 (30.0 ± 1.8)	(0.646)
Pre-shock empty sporangia [c]	% (and Arcsine)	14.4 ± 2.2 (22.1 ± 1.8)	17.6 ± 3.6 (24.5 ± 2.7)	(0.495)
Post-shock empty sporangia [c]	% (and Arcsine)	26.1 ± 3.9 (30.5 ± 2.6)	17.4 ± 3.3 (24.3 ± 2.5)	(0.143)
Sporangia density [d]	sporangia per video screen	26.3 ± 2.7	28.9 ± 1.7	0.451
Motile zoospores [e]	% (and Arcsine)	45.3 ± 3.7 (42.3 ± 2.1)	27.9 ± 8.0 (31.0 ± 5.6)	(0.106)
Subsequent cyst germination [e]	% (and Arcsine)	22.4 ± 2.7 (28.1 ± 1.9)	12.8 ± 2.2 (20.7 ± 2.1)	(0.039)

[a] Means ± s.e.m. for 4 replicates, based on 2 haemocytometer counts per replicate.
[b] Means ± s.e.m. for 4 replicates, based on 100 counts per replicate.
[c] Means ± s.e.m. for 4 replicates, based on 3 fields of view per replicate.
[d] Means ± s.e.m. for 4 replicates, based on 6 fields of view per replicate. Three fields of view observed prior to cold shock and three fields of view observed post cold shock.
[e] Means ± s.e.m. for 4 replicates, based on 3 fields of view per replicate.

Table 3.9. Effects of applications of 5 mM and 20 mM $Ca(NO_3)_2$ on aspects of biology of *Ph. parasitica*.

Aspect (ANOVA)	Units	0 mM Ca^{2+}	5 mM Ca^{2+}	20 mM Ca^{2+}	σ_d	*P*
Pre-shock zoospore release [a]	zoospores.μl^{-1}	2.1 ± 0.6	6.5 ± 0.8	2.3 ± 0.4	0.7	1.64×10^{-3}
Post-shock zoospore release [a]	zoospores.μl^{-1}	9.9 ± 1.0	7.1 ± 0.6	1.1 ± 0.3	1.0	3.97×10^{-5}
Pre-shock empty sporangia [b]	% (and Arcsine)	12.7 ± 1.3 (20.8 ± 1.1)	11.5 ± 0.7 (19.8 ± 0.6)	4.4 ± 1.0 (11.8 ± 1.4)	(1.5)	(4.62×10^{-4})
Post-shock empty sporangia [b]	% (and Arcsine)	15.2 ± 1.3 (22.9 ± 1.1)	19.4 ± 3.3 (25.9 ± 2.3)	7.1 ± 0.5 (15.5 ± 0.5)	(2.1)	(2.48×10^{-3})
Sporangia density [c]	sporangia per video screen	36.4 ± 5.0	28.0 ± 5.3	15.9 ± 1.3	6.1	2.50×10^{-2}
Motile zoospores [d]	% (and Arcsine)	17.9 ± 8.5 (23.6 ± 6.0)	27.5 ± 8.6 (30.3 ± 6.4)	0.0 ± 0.0 (0.0 ± 0.0)	(7.1)	(5.24×10^{-3})
Subsequent cyst germination [d]	% (and Arcsine)	22.6 ± 2.4 (28.3 ± 1.6)	28.8 ± 3.0 (32.4 ± 2.0)	54.4 ±1.9 (47.5 ± 1.1)	(2.3)	(3.19×10^{-5})

[a] Means ± s.e.m. for 4 replicates, based on 2 haemocytometer counts per replicate.
[b] Means ± s.e.m. for 4 replicates, based on 3 fields of view per replicate.
[c] Means ± s.e.m. for 4 replicates, based on 6 fields of view per replicate. Three fields of view observed prior to cold shock and three fields of view observed post cold shock.
[d] Means ± s.e.m. for 4 replicates, based on 3 fields of view per replicate.

3.9. Summary of the effects of cations on various stages of the zoosporic infection sequence when the test supplement was constant throughout the experimental protocol.

Each cation affected the infection sequence differently (see the summary in Table 3.10). Both magnesium and potassium suppressed zoospore release, the number of spores observed on the Petri dish base, and zoospore motility, but enhanced the germination of vortex encysted zoospores. of *Py. aphanidermatum*. In contrast, 5 mM $Ca(NO_3)_2$ increased zoospore release, the proportion of zoospores remaining motile and the number of spores observed on the Petri dish base. The effect of increasing calcium concentration on cyst germinating is complex, but identical patterns were seen in the results from the two assessments of cyst germination (Figures 3.68 and 3.72).

The biology of *Ph. parasitica* in treatments supplemented with 5 mM KNO_3 was generally similar to that in the K^+ free control. Only in the assessment of cyst germination did 5 mM KNO_3 cause a significant difference. Five mM $Ca(NO_3)_2$ caused a peak in pre-shock zoospore release and the proportion of zoospores remaining motile.

When zoospores of *Py. aphanidermatum* had encysted either spontaneously or induced by KNO_3, a peak in subsequent cyst germination was observed (Figure 3.65). This observation was also made in the assessment of individual stages of the infection sequence (Figure 3.34). That is, approximately 10 mM K^+ increased the level of cyst germination, but higher concentrations (circa. 30 mM) of K^+ the level of germination was comparable to the K^+ free control.

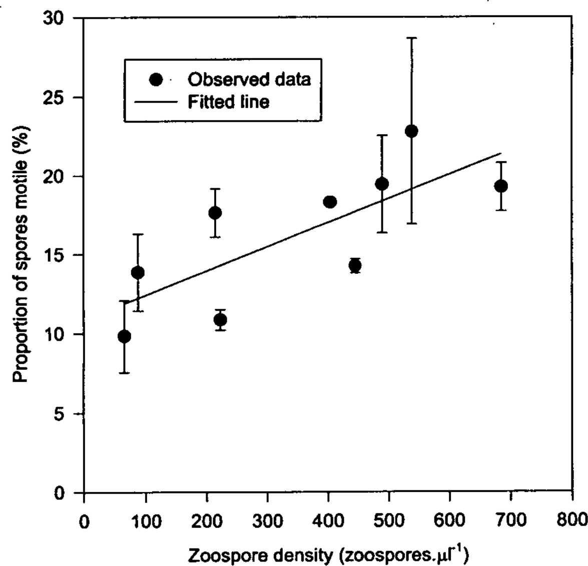
Table 3.10. Summary of effects of cations on stages of the zoosporic infection sequence when the test supplement was constant throughout the experimental protocol

Stage	Fungi	Cations	Effect of increasing cation concentration
Zoospore release	*Py. aphanidermatum*	Mg^{2+} & K^+	Suppression
Zoospore release	*Py. aphanidermatum*	Ca^{2+}	Suppression, but peak at 5 mM
Zoospore release (pre- & post-shock)	*Ph. parasitica*	K^+	No effect
Zoospore release (pre-shock)	*Ph. parasitica*	Ca^{2+}	Peak at 5 mM
Zoospore release (post-shock)	*Ph. parasitica*	Ca^{2+}	Suppression
Empty sporangia (pre-shock)	*Ph. parasitica*	Ca^{2+}	Suppression
Empty sporangia (pre- & post-shock)	*Ph. parasitica*	K^+	No effect
Empty sporangia (post-shock)	*Ph. parasitica*	Ca^{2+}	Suppression
Sporangial density	*Ph. parasitica*	Ca^{2+}	Suppression
Sporangial density	*Ph. parasitica*	K^+	No effect
Spores observed on Petri dish base.	*Py. aphanidermatum*	Mg^{2+} & K^+	Suppression
Spores observed on Petri dish base.	*Py. aphanidermatum*	Ca^{2+}	Suppression, but peak at 5 mM
Zoospore motility	*Py. aphanidermatum*	Mg^{2+} & K^+	Suppression
Zoospore motility	*Py. aphanidermatum*	Ca^{2+}	Suppression, but peak at 5 mM
Zoospore motility	*Ph. parasitica*	Ca^{2+}	Suppression
Zoospore motility	*Ph. parasitica*	K^+	Possible suppression
Subsequent cyst germination	*Py. aphanidermatum*	Ca^{2+}	Complex
Subsequent cyst germination	*Py. aphanidermatum*	Mg^{2+}	No effect
Subsequent cyst germination	*Py. aphanidermatum*	K^+	Peak at 10 mM
Subsequent cyst germination	*Ph. parasitica*	Ca^{2+}	Peak at 5 mM
Subsequent cyst germination	*Ph. parasitica*	K^+	Suppression
Germination of vortex encysted zoospores	*Py. aphanidermatum*	Ca^{2+}	Complex
Germination of vortex encysted zoospores	*Py. aphanidermatum*	Mg^{2+} & K^+	Enhance
Germination of vortex encysted zoospores	*Ph. parasitica*	Ca^{2+}	Enhance
Germination of vortex encysted zoospores	*Ph. parasitica*	K^+	No effect

3.10. The effect of zoospore density on zoospore motility.

To establish the effect of the density of zoospores of *Py. aphanidermatum* on the proportion of zoospores that remain motile, a population of zoospores was first diluted with various quantities of SDW. Prior to incubation, an aliquot of zoospores from each dilution was taken and the zoospore density enumerated by heamocytometer counts. After 2h incubation the proportion of motile zoospores was determined (as in Section 2.3.6). Eight dilutions were used and for each dilution two Petri dishes were incubated. The lowest density of zoospores was 66 zoospores.μl^{-1} of which 9.8% were motile after the incubation period (Figure 3.73). The highest population density was 685 zoospores.μl^{-1} of which 19.3% were motile at the termination of the experiment. There was a very significant positive correlation (y = 10.89 + 0.015x, F = 0.0043, R = 0.64, n = 18) between zoospore density and the proportion of zoospores motile after the incubation period.

Figure 3.73. The effect of zoospore density on zoospore motility*.



* Means ± s.e.m. for 2 replicates per sub-population, based on three fields of view per replicate.

3.11. Modification of the potassium to calcium ratio.

Cooper (1979) suggested that the ideal concentrations of principal ions in a NFT system are 200 ppm (14.3 mM) for nitrogen, 300 ppm (7.7 mM) for potassium and 170 ppm (4.3 mM) for calcium. Therefore, the suggested millimolar potassium to calcium ratio is 7.7:4.3. The cumulative effect of this potassium to calcium ratio (control) on the biology of zoosporic fungi was determined and compared with a modified (test) ratio of 13.3:1.5. The method was as in Section 3.8. The nitrate concentration was 16.3 mM in both the control and test treatments. The cumulative effect of the test solution on the biology of *Ph. parasitica* was not significantly (t-tests, $P \geq 0.21$) different from the control for most aspects of zoospore biology (Table 3.11). However, the test treatment (high potassium to calcium ratio) caused a very highly significant ($P = 9.00 \times 10^{-5}$) suppression of zoospore motility compared to the control. In the control 59% of spores were motile after incubation, whereas only 5% were motile in the test treatment.

The same protocol was employed, but using *Py. aphanidermatum*. The experiment was repeated twice and analysed by two-way ANOVA with the experiment being the second factor (Table 3.12). However, for the second experiment cyst germination was not assessed and consequently the data from experiment 1 was analysed by a t-test. In all assessments no significant ($P \geq 0.234$) interaction between the experiments was observed, although significant differences between the experiments were found for the assessment of zoospore motility ($P_{experiment} = 9.11 \times 10^{-7}$) and subsequent cyst germination ($P_{experiment} = 8.79 \times 10^{-6}$).

The test treatment (high potassium to calcium ratio) significantly ($P = 0.037$) suppressed zoospore release in comparison to the control (low potassium to calcium ratio). In experiment 1 the test suppressed zoospore release to 51% of the control and in experiment 2 this value was 71%. Also in both experiments there was some reduction by the test treatment of zoospore motility and enhancement of subsequent cyst germination, although the effects were not quite significant ($P = 0.0589$ and $P = 0.0749$, respectively).

Table 3.11. The effect of modifying the potassium to calcium ratio on the biology of *Ph. parasitica*.

Aspect	Units	Control[†]	Test[††]	*P* (t-test)
Post-shock zoospore release[a]	Zoospores.μl^{-1}	5.1 ± 1.7	7.7 ± 2.2	3.87×10^{-1}
Pre-shock empty sporangia [b]	% (and Arcsine)	3.2 ± 1.9 (8.5 ± 3.5)	6.1 ± 2.5 (13.4 ± 3.0)	(3.27×10^{-1})
Post-shock empty sporangia [b]	% (and Arcsine)	14.8 ± 5.8 (19.6 ± 6.9)	11.9 ± 2.0 (20.0 ± 1.8)	(9.59×10^{-1})
Sporangial density [c]	Sporangia per video screen	12.2 ± 4.1	15.3 ± 1.2	4.93×10^{-1}
Zoospore motility [d]	% (and Arcsine)	58.4 ± 6.4 (50.0 ± 3.8)	4.9 ± 1.0 (12.6 ± 1.3)	(9.00×10^{-5})
Subsequent cyst germination [e]	% (and Arcsine)	23.3 ± 9.3 (25.1 ± 9.0)	5.1 ± 1.8 (11.4 ± 3.8)	(2.10×10^{-1})

[a] Means ± s.e.m. for 4 replicates, based on 2 haemocytometer counts per replicate.
[b] Means ± s.e.m. for 4 replicates, based on 3 fields of view per replicate.
[c] Means ± s.e.m. for 4 replicates, based on 6 fields of view per replicate. Three fields of view observed prior to cold shock and three fields of view observed post cold shock.
[d] Means ± s.e.m. for 4 replicates, based on 3 fields of view per replicate.

[†] 7.7 mM K^+, 4.3 mM Ca^{2+}, 16.3 mM NO_3^-
[††] 13.3 mM K^+, 1.5 mM Ca^{2+}, 16.3 mM NO_3^-

Table 3.12. The effects of modifying the potassium to calcium ratio on the biology of *Py. aphanidermatum*.

	Zoospore release [a]	Cyst germination [b]	Motile zoospores [c]	Subsequent cyst germination [c]
Units	Zoospores.μl^{-1}	% (and Arcsine)	% (and Arcsine)	% (and Arcsine)
Control[†] - Experiment 1	148.6 ± 31.6	57.7 ± 8.8 (49.7 ± 5.3)	73.3 ± 5.5 (59.2 ± 3.6)	13.7 ± 3.8 (20.8 ± 3.7)
Control[†] - Experiment 2	193.1 ± 34.3	n.d.	29 ± 5 (32.6 ± 3.8)	46.2 ±5.8 (52%)
Test[††] - Experiment 1	76.4 ± 24.8	70.5 5 ± 2.3 (7.2 ± 1.5)	68.8 ± 3.5 (56.2 ± 2.2)	16.7 ± 3.6 (23.7 ± 2.9)
Test[††] - Experiment 2	137.5 ± 15.8	n.d.	14 ± 4 (21.8 ± 3.5)	59.5 ± 3.6 (74%)
P (Treatment)	3.73×10^{-2}	(2.19×10^{-1})	(5.89×10^{-2})	(7.49×10^{-2})
P (Experiment)	8.34×10^{-2}	(NA)	(9.11×10^{-7})	(8.79×10^{-6})
P (Interaction)	7.87×10^{-1}	(NA)	(2.67×10^{-1})	(2.34×10^{-1})

[a] Means ± s.e.m. for 4 replicates, based on 2 haemocytometer counts per replicate.
[b-] Means ± s.e.m. for 4 replicates, based on 100 counts per replicate.
[c] Means ± s.e.m. for 4 replicates, based on 3 fields of view per replicate.

[†] 7.7 mM K^+, 4.3 mM Ca^{2+}, 16.3 mM NO_3^-
[††] 13.3 mM K^+, 1.5 mM Ca^{2+}, 16.3 mM NO_3^-

3.12. Comparison of the effects of the anions: Cl^- v NO_3^-.

A noticeable trend observed in the results presented in this chapter was that generally, when calcium was added as a nitrate the effect on the biology of zoosporic fungi was greater than when calcium is added as a chloride. For example, calcium suppressed the fraction of zoospores motile at 2h, but the result was more noticeable with $Ca(NO_3)_2$ than for $CaCl_2$ (Figures 3.32 and 3.33). Calcium also increased the fraction of vortex-encysted zoospores that germinated, but the result for $Ca(NO_3)_2$ was greater than for $CaCl_2$ (Figures 3.48 and 3.49). To confirm this trend several experiments were performed that directly compared the effects of $Ca(NO_3)_2$ and $CaCl_2$. The net charge on each species of anion (Cl^- and NO_3^-) is the same so straightforward comparisons were made.

The effect on motility was assessed as in Section 2.3.6. The proportion of zoospores motile in 10 mM $CaCl_2$ was 10.8%, and, of the encysted zoospores, 7.8% had germinated (Table 3.13.). The corresponding values in $Ca(NO_3)_2$ were not significantly different from those with $CaCl_2$ (t-tests, $P \geq 0.156$).

Table 3.13. Direct comparison between the effect of Cl^- and NO_3^- on suppression of zoospore motility and subsequent spontaneous cyst germination* for spores of *Pythium aphanidermatum* in the presence of 10 mM Ca^{2+}.

Treatment	Motile zoospores	Germinated cysts
	% (and Arcsine)	% (and Arcsine)
20 mM Cl^-	10.8 ± 1.1 (19.1 ± 1.0)	7.8 ± 1.0 % (16.1 ± 1.1)
20 mM NO_3^-	8.6 ± 1.4 (16.9 ± 1.4)	10.3 ± 1.2 (18.6 ± 1.1)
P (t-test)	(2.25×10^{-1})	(1.56×10^{-1})

* Means ± s.e.m. for 5 replicates, assessed after 2h, based on scoring 3 fields of view per replicate.

The effect on the germination of vortex-encysted zoospores was assessed as in Section 2.3.7. There was a very significant (t-test, $P = 1.03 \times 10^{-3}$) difference between the proportion of vortex-encysted zoospores that germinated in $Ca(NO_3)_2$ and $CaCl_2$ (Table 3.14). Likewise, there was a very significant (t-test, $P = 5.74 \times 10^{-3}$) difference between the level of

germination in $Mg(NO_3)_2$ and $MgCl_2$. In both cases germination was higher when the nitrate salt was used.

Table 3.14. Comparison of the effects of Cl^- and NO_3^- salts of Ca^{2+} and Mg^{2+*} (30 mM) on germination cysts of *Pythium aphanidermatum*.

	30 mM Ca^{2+}	15 mM Mg^{2+}
Units	% (and Arcsine)	% (and Arcsine)
Cl^-	25.1 ± 2.1 (30.0 ± 1.4)	33.7 ± 2.2 (35.5 ± 1.3)
NO_3^-	41.8 ± 2.6 (40.2 ± 1.5)	47.2 ± 2.4 (43.4 ± 1.4)
P (t-test)	(1.04×10^{-3})	(5.74×10^{-3})
n	5	4

* Means ± s.e.m. for n replicates, assessed after 2h, based on scoring at least 100 cysts per replicate. T-tests performed on arcsine transformed data.

It has already been observed that the presence of 5 mM Ca^{2+} during sporangiogenesis increased sporangial discharge and subsequent zoospore release, whereas 40 mM Ca^{2+} suppressed sporangial discharge and zoospore release compared to the calcium-free control. The effect of 5 mM and 40 mM Ca^{2+} with Cl^- and NO_3^- as the counter-ion was tested as in Section 2.3.4. There were no significant differences between 5 mM $CaCl_2$ and 5 mM $Ca(NO_3)_2$ on sporangiogenesis and subsequent zoospore release from *Ph. parasitica* (Table 3.15), although pre-cold shock zoospore release was almost significantly ($P = 0.09$) greater when calcium was added as a nitrate. However, 40 mM $Ca(NO_3)_2$ did significantly ($P = 0.048$) reduce post-shock sporangial discharge when compared to the 40 mM $CaCl_2$ treatment. All other differences were not significant ($P \geq 0.15$).

Table 3.16 shows a summary of the direct comparisons between the effect of Cl^- and NO_3^- on aspects of *Phytophthora parasitica* and *Pythium aphanidermatum* biology. Also shown are predications based on previous observations reported in this chapter. The arithmetic differences in the direct comparisons, although mostly not significant, were as predicted. The only comparison that was not as predicted concerned post-shock sporangial discharge in 5 mM Ca^{2+}. It was predicted that higher discharge would be seen in the $Ca(NO_3)_2$ treatment than for the $CaCl_2$ treatment, but the direct comparison showed otherwise.

Table 3.15. Direct comparison between the effect of Cl^- and NO_3^- on aspects of *Phytophthora parasitica* sporangiogensis[1] and subsequent zoospore release in the presence of 5 mM and 40 mM Ca^{2+} salts.

	$[Ca^{2+}]$	Pre-shock zoospore release [a]	Post-shock zoospore release [a]	Pre-shock sporangial discharge [b].	Post-shock sporangial discharge [b]	Sporangial density [c]
Units	mM	Zoospores.μl^{-1}	Zoospores.μl^{-1}	% (and Arcsine)	% (and Arcsine)	Sporangia per video screen
10 mM Cl^-	5	68.1 ± 10.0	55.7 ± 5.8	17.8 ± 1.9 (24.9 ± 1.4)	19.2 ± 3.3 (25.7 ± 2.3)	7.4 ± 0.7
10 mM NO_3^-	5	91.5 ± 6.8	59.1 ± 3.1	20.9 ± 4.2 (26.8 ± 3.0)	17.8 ± 3.4 (24.8 ± 2.5)	7.5 ± 0.8
P (t-test)		9.0×10^{-2}	6.2×10^{-1}	(5.7×10^{-1})	(7.5×10^{-1})	9.0×10^{-1}
80 mM Cl^-	40	6.5 ± 1.0	10.9 ± 1.2	5.5 ± 1.2 (13.2 ± 1.5)	6.3 ± 1.4 (14.2 ± 1.6)	8.7 ± 0.7
80 mM NO_3^-	40	4.6 ± 0.7	10.2 ± 1.4	2.2 ± 0.4 (8.3 ± 0.9)	2.8 ± 0.8 (9.3 ± 1.4)	7.7 ± 0.9
P (t-test)		1.5×10^{-1}	7.2×10^{-1}	(2.2×10^{-2})	(4.8×10^{-2})	3.9×10^{-1}

[a] Means ± s.e.m. for 5 replicates, based on 2 haemocytometer counts per replicate.
[b] Means ± s.e.m. for 5 replicates, based on 3 fields of view per replicate.
[c] Means ± s.e.m. for 5 replicates, based on 6 fields of view per replicate. Three fields of view observed prior to cold shock and three fields of view observed post cold shock.

Table 3.16. Summary of direct comparisons between the effect of Cl^- and NO_3^- on aspects of *Phytophthora parasitica* and *Pythium aphanidermatum* zoosporic infection sequences in various concentrations of Ca^{2+} and Mg^{2+} salts.

Aspect	Fungi	[Cation]	Predicted Result	Outcome	Significant difference
Zoospore motility	*Py. aphanidermatum*	10 mM Ca^{2+}	$Cl^- > NO_3^-$	As expected	
Subsequent cyst germination	*Py. aphanidermatum*	10 mM Ca^{2+}	$NO_3^- > Cl^-$	As expected	
Cyst germination (vortex encysted)	*Py. aphanidermatum*	30 mM Ca^{2+}	$NO_3^- > Cl^-$	As expected	✓
Cyst germination (vortex encysted)	*Py. aphanidermatum*	15 mM Mg^{2+}	$NO_3^- > Cl^-$	As expected	✓
Sporangiogenesis - Pre-shock zoospore release	*Ph. parasitica*	5 mM Ca^{2+}	$NO_3^- > Cl^-$	As expected	
Sporangiogenesis - Post-shock zoospore release	*Ph. parasitica*	5 mM Ca^{2+}	$NO_3^- > Cl^-$	As expected	
Sporangiogenesis - Pre-shock discharged sporangia	*Ph. parasitica*	5 mM Ca^{2+}	$NO_3^- > Cl^-$	As expected	
Sporangiogenesis - Post-shock discharged sporangia	*Ph. parasitica*	5 mM Ca^{2+}	$NO_3^- > Cl^-$	Not as expected	
Sporangiogenesis - Sporangial density	*Ph. parasitica*	5 mM Ca^{2+}	$NO_3^- = Cl^-$	$P = 0.90$	n.a.
Sporangiogenesis - Pre-shock zoospore release	*Ph. parasitica*	40 mM Ca^{2+}	$Cl^- > NO_3^-$	As expected	
Sporangiogenesis - Post-shock zoospore release	*Ph. parasitica*	40 mM Ca^{2+}	$Cl^- > NO_3^-$	As expected	
Sporangiogenesis - Pre-shock discharged sporangia	*Ph. parasitica*	40 mM Ca^{2+}	$Cl^- > NO_3^-$	As expected	✓
Sporangiogenesis - Post-shock discharged sporangia	*Ph. parasitica*	40 mM Ca^{2+}	$Cl^- > NO_3^-$	As expected	✓
Sporangiogenesis - Sporangial density	*Ph. parasitica*	40 mM Ca^{2+}	$Cl^- = NO_3^-$	$P = 0.39$	n.a.

n.a. not applicable.

✓ when $P \leq 0.05$.

3.13. Discussion

The modification of the cationic concentrations in irrigation solutions is an elegant method for disease control, especially when the effect on the crop is negligible. This method of disease control is economically attractive, straightforward in terms of legislation and implementation, and highly compatible with present biological control of insects in British horticultural glasshouse irrigation systems. Nutrient solution manipulation does not target a single site, but is broad acting and consequently has long term potential.

The results presented in this chapter show that suppression of mycelial growth of *Py. aphanidermatum* is not solely mediated by increased osmotic potential, nor is growth retarded by nitrate toxicity. This was illustrated by supplements up to 80 mM KNO_3 failing to suppress mycelial growth of both fungi. This result supports other findings (for example, Money & Harold, 1993) that mycelial growth in Oomycetes is not solely driven by turgor pressure. In contrast, 80 mM $Ca(NO_3)_2$ or $Mg(NO_3)_2$ markedly suppressed growth of *Py. aphanidermatum*, but not *Ph. parasitica*. Moreover, there is evidence in the results to suggest that $Ca(NO_3)_2$ enhances mycelial growth of *Ph. parasitica*. In an infection, mycelial growth that is *in planta* would be buffered from cationic supplements by the host tissue.

Halsall and Forester (1977) found maximal sporangial numbers of *Ph. cinnamomi* in solutions with sub-millimolar concentrations of Ca^{2+}, Mg^{2+} and K^{+}. The various supplements presented in this Chapter had no noticeable effect on sporangial density of *Ph. parasitica*, nor any effect on sporangiogenesis of *Py. aphanidermatum* when measured indirectly by subsequent zoospore release. Leaching of salts from the fungal mats could raise the concentration of cations in the bathing medium of the SDW controls to a level where maximum sporangiogenesis occurs, with additional supra-optimal supplements having negligible effect on sporangial density. The exception being that MSS with a calcium concentration of 5 mM increased subsequent zoospore release from *Py. aphanidermatum* four-fold, compared to the Ca^{2+}-free MSS (Table 3.3). Similarly, MSS supplemented with 5 mM Ca^{2+} increased zoospore release from sporangia of *Ph. parasitica*. This increased level of zoospore release was also reflected in the proportion of sporangia that discharged their contents. Therefore, it appears that circa. 5 mM Ca^{2+} is the optimum concentration for sporangiogenesis of both species. The average concentration of calcium in the glasshouses of Stockbridge House was approximately 8 mM (Appendices 8 to 13). However, during the first few weeks of the season calcium levels were near 5 mM.

Von Broembsen and Deacon (1997) found that increasing concentrations of calcium (up to 50 mM) visually increased sporangial cleavage of *Ph. parasitica*, but suppressed zoospore release. The observations made in this chapter are that concentrations up to 40 mM Ca^{2+} do not affect sporangial density, but do generally suppress sporangial discharge. Therefore, although increasing concentrations of calcium do not affect sporangia formation, increasing concentrations of calcium do facilitate the cleavage of cytoplasm into zoospores, but inhibit the ability of sporangia to subsequently liberate zoospores.

In contrast to sporangiogenesis, the motile zoospore is the target of many potential approaches to disease control in glasshouse irrigation systems (for example, Deacon & Mitchell, 1985; Ghaouth *et al.*, 1994; Stanghellini *et al.*, 1996a & 1996b). Suppression of sporangiogenesis and the mechanisms of zoospore release are also potential targets for disease control.

Hill *et al.* (1998) found that 5 mM $CaCl_2$ suppressed both the direct and indirect (zoospore liberation) germination of sporangia of *Ph. infestans.* Results presented in this chapter show this to also be true for *Ph. parasitica* and *Py. aphanidermatum.* All salts tested did suppress zoospore release (indirect germination) in a concentration dependent manner. Whether this is due to increased electrical conductivity or osmotic potential cannot be answered here. Grayson (1999) found that 10 mM $CaCl_2$ completely suppressed zoospore release of two strains (81/1/1 and EUPi1) of *Ph. infestans*, but found that this concentration of calcium did not affect the direct germination of sporangia. In contrast, complete suppression of zoospore release from pre-formed sporangia of *Ph. parasitica* was not achieved with the highest concentration of calcium tested (40 mM). If the linear relationship between calcium concentration and zoospore release were assumed then approximately 80 mM Ca^{2+} would be required to completely suppress zoospore release from pre-formed sporangia.

The suppression by cationic supplements of zoospore release from *Py. aphanidermatum* is greater than for *Ph. parasitica.* This may be due to the differing physiology of zoospore release. Zoospores are released from sporangia of *Phytophthora* by the dissolution of the papillar plug (Gisi, 1983), whereas zoospore release from sporangia of *Pythium* first requires the formation of a membrane-bound vesicle that contains undifferentiated cytoplasm. After the cytoplasm has differentiated into zoospores their release can proceed. Therefore, cationic suppression of zoospore release has potentially more points to target in zoospore release

from *Pythium* than from *Phytophthora*. The ability of 10 mM calcium to completely suppress sporangial discharge by *Ph. infestans* as reported by Grayson (1999) could either be an indication of the sensitivity of this species to calcium or be a reflection on the different methodologies employed to assess sporangial discharge.

Cations have been implicated in the regulation of termination of zoospore motility in the water mould *Achlya heterosexualis* (Thomas & Butler, 1989). For zoospores of *Ph. cinnamomi* Bryt *et al.* (1982) also found that cations curtailed zoospore motility. In general, they found that the sensitivity of zoospores to the cationic species was related to the charge density of the cation. Of the anions tested by Bryt *et al.* (1982) only F^- and CH_3COO^- inhibited zoospore motility, whereas nitrate, chloride and sulphate - the major three anions found in irrigation systems - were found not to affect zoospore motility. The results of this chapter found that for all three cations the degree of suppression of motility was directly related to salt concentration. *Py. aphanidermatum* was slightly more sensitive to cation supplements than *Ph. parasitica*. This is similar to findings for *Ph. cinnamomi* (Bryt *et al.*, 1982) and *Ph. palmivora* (Bimpong and Clerk, 1970). Soll and Sonneborn (1972) concluded that ionic effects on the monoflagellate zoospores of *Blastocladiella emersonii* were not due to osmotic shock, as a sugar had no effect in the test system. Bimpong and Clerk (1970) also found that of the three sugars tested none noticeably suppressed motility of zoospores of *Ph. palmivora*. However, one of the nitrogen sources tested, L-glutamine, did suppress zoospore motility.

Both monovalent and divalent cations act as counterions to the net negative charge caused by the presence of acidic phospholipids in the plasmamembrane (Gennis, 1989). Increased concentrations of cations reduce the transmembrane potential of the zoospore and this has the potential to induce zoospore encystment. Monovalent potassium was more effective than divalent calcium or magnesium at suppressing motility of zoospores of both species when the concentration of ionic products is taken into account. This was clearly demonstrated when the potassium to calcium ratio was modified (and the anion concentration stayed constant). Altering the potassium to calcium ratio from 7.7:4.3 to 13.3:1.5 halved the proportion of zoospores of *Py. aphanidermatum* that remained motile in both experiments (Table 3.12). The effect in the corresponding experiment with *Ph. parasitica* was that the proportion of zoospores remaining motile dropped from 59% to 5%. This large increase in

suppression of zoospore motility was achieved by only slightly increasing the sum of ionic conductivities from 224 $m^2.nS.mol^{-1}$ to 232 $m^2.nS.mol^{-1}$†. Therefore, there is potential to suppress zoospore motility in irrigation systems without increasing electrical conductivity by manipulating the potassium to calcium ratio, and this modified ratio is only marginally (2%) more expensive then the control ratio (based on prices from Anon, 2000a). The effect of modifying the potassium to calcium ratio without altering the electrical conductivity of the solution on the biology of *Py. aphandidermatum* and *Ph. parasitica* demonstrates that the observed effect is not due to altering the ionic strength of the bathing solution. Bouchibi *et al.* (1990) also found that variable Ca^{2+} concentration in a constant ionic concentration (varied Na^+ to Ca^{2+} ratio) was negatively correlated with zoospore motility and cyst germination, whereas zoospore lysis and encystment were positively correlated with calcium concentration.

The level of subsequent germination of cysts after spontaneous zoospore encystment was dependent on the concentration of cations in the media. Increasing concentrations of calcium caused increased levels of germination of cysts of both species. Calcium is known to have a central role in cyst germination (Donaldson & Deacon, 1992). Many *in vitro* studies have shown Ca^{2+} to trigger germination of *Phytophthora* (for example, Bryt *et al.*, 1982; Grant *et al.*, 1986; Deacon & Saxena, 1998). The observation that increasing concentrations of Mg^{2+} also increase subsequent cyst germination of *Py. aphanidermatum* could be attributed to the cation acting as a surrogate for calcium (Donaldson & Deacon, 1992). The observation of a peak in subsequent cyst germination of *Py. aphanidermatum* between 5 and 10 mM KNO_3 might be discarded as erroneous if the same phenomenon had not been observed in a later experiment (see Figures 3.38 and compare with Figure 3.65).

Germination of vortex-encysted zoospores in the absence of any salt was lower for *Ph. parasitica* than for *Py. aphanidermatum*. This may be due to the higher densities of *Py. aphanidermatum* cysts elevating the exogenous salt concentrations through leaching, and consequently raising the control level of germination. Potassium had a large influence on the germination of cysts of *Ph. parasitica*. In SDW the germination level was 3.7% and in 5 mM KNO_3 the level was increased to 30.5%, although higher concentrations of K^+ did not significantly improve this, suggesting that a low concentration of K^+ acts as a germination

---

† The molar (equivalent) conductivity λ (10^{-4} $m^2.S.mol^{-1}$) at infinite dilution at 25 °C for K^+ is 73.48, ½Ca^{2+} is 59.47 and 71.42 for NO_3^-.

trigger. However, potassium had no noticeable effect on cyst germination of *Py. aphanidermatum* which is consistent with the previous findings by Donaldson (1992) who worked with the same isolate (CBS 634.70). Magnesium, like potassium, appears to trigger the germination of *Ph. parasitica* at a low concentration (less than 5 mM). This contrasts with other work where magnesium was found not to promote the germination of spores of *Ph. palmivora* (Bryt *et al.*, 1982).

From a theoretical standpoint, based on *in vitro* results described in this chapter, the compound effect of potassium is superior to that of calcium. The maximum supplement that would be commercially acceptable would be approximately 10 mM. However, this concentration could be further increased to the detriment of another cation. The compound effect of 10 mM K^+ was to reduce zoospore release of *Py. aphanidermatum* to 23% of the control and suppress zoospore motility to 7% of the control, the equivalent values for calcium being 58% and 42%, respectively. The ionic conductivity of KNO_3 is 55% of that of $Ca(NO_3)_2$. Therefore, a commercial irrigation system could tolerate a higher KNO_3 supplement mole-for-mole than $Ca(NO_3)_2$.

A constant supplement of 5 mM $Ca(NO_3)_2$ clearly promotes zoospore release from *Py. aphanidermatum* and *Ph. parasitica*. Whether this phenomenon is observed in commercial irrigation systems is yet to be established. The presence of calcium in irrigation solutions is justified on two fronts. First, it is essential for healthy plant growth, and second, it facilitates the addition of nitrogen (as nitrate) to the system. Nitrogen can be added in other forms, although ammonium can increase pH to a phytotoxic level. Potassium or magnesium nitrates are possible substitutes for calcium nitrate. Calcium is required by vegetable crops as a component of cell walls and for meristematic activity (Fordham & Biggs, 1985). Calcium deficiency in tomatoes is typically shown as blossom-end rot. Blossom-end rot can also be induced by high concentrations of magnesium or potassium, because these elements compete with calcium (Adams & Ho, 1993). In addition to these factors, excess potassium can also cause a reduction in magnesium and calcium uptake (Fordham & Biggs, 1985). The recommendation of ideal concentrations of the principal cations in irrigation solutions is beyond the scope of this thesis. However, from a mycological perspective the elevation of potassium levels to the detriment of calcium concentration theoretically would suppress inoculum density and zoospore motility. Bres and Weston (1992) have already found that elevating K^+ levels from 3.8 mM to 5.8 mM in a NFT system had no effect on lettuce

(*Lactuca sativa* L.) yield or tipburn incidence. Tipburn in lettuce is the equivalent of blossom-end rot in tomatoes.

The correlation between zoospore release numbers and the proportion of zoospores remaining motile in the experiment on cumulative effects of Ca^{2+} (Section 3.8.1) can only be partially explained by the observation in another experiment (Section 3.10) that there is positive correlation between zoospore density and the proportion of zoospores remaining motile. This correlation is consistent with the observation of Ho and Hickman (1967) that dilution of zoospores induces zoospore encystment. The relationship between zoospore density and the proportion of zoospores remaining motile is not strong enough to explain the phenomenon observed in the cumulative effect assay. The usual effect of calcium on zoospore motility is that increased cation concentration is associated with suppressed motility (for example, Figure 3.33). The most likely explanation is that these zoospores are conditioned to high calcium concentrations by virtue of the sporangia and mycelia being previously exposed to elevated calcium concentrations. Based on this explanation, these zoospores would be expected to have modified plasma membranes. That is, the environment of zoosporangiogenesis may affect the behaviour of the zoospores that are subsequently liberated.

Bryt *et al.* (1982) observed that the germination of *Ph. cinnamomi* was higher in $Ca(NO_3)_2$ than in $CaCl_2$. Inspection of the data presented by von Broembsen and Deacon (1997) shows that $CaCl_2$ suppressed sporangial discharge of *Ph. parasitica*, but the effect was more noticeable when calcium nitrate was added. Similarly, they found that calcium suppressed diplanetism of *Ph. parasitica* and increased the percentage germination of cysts. The effect was more pronounced with calcium nitrate than with calcium chloride. This trend is confirmed by the results of this chapter. The observation of this phenomenon can now be extended to the magnesium cation - germination of cysts of *Py. aphanidermatum* was 33.7% in 15 mM $MgCl_2$, but germination (47.2%) was very significantly higher in $Mg(NO_3)_2$ (Table 3.14). These observations cannot be simply explained by the relative ionic conductivities of these two anions: λ for Cl^- is 763 $m^2.mS.mol^{-1}$ and 714 $m^2.mS.mol^{-1}$ for NO_3^-.

The ATP-dependent acidification of the interior of vesicles of barley (*Hordeum vulgare* L.) roots is strongly dependent on the presence of chloride and nitrate (Yamashita *et al.*, 1996). However, the same research group later found that vesicles were more permeable to nitrate

than chloride (Yamashita *et al.*, 1997). The increased permeability of nitrate was associated with greater activity of H^+-ATPase and H^+-transport. If the same is true in zoosporic fungi then anion-induced disruption of the transmembrane potential could account for the differing effects of chloride and nitrate, although in other species the differences in the mechanism of uptake of chloride and nitrate cannot be resolved. The permeability of rat liver lysosomes to inorganic anions does not seem to be mediated by any known anion transporter (Klemm *et al.*, 1998). The ability of anions to permeate the membrane was attributed to their position in the lyotropic series, but chloride is slightly higher than nitrate in the lyotropic series. The lamprey (*Lampetra fluviatilis*) erythrocyte membrane is suggested to have an electroneutral furosemide-sensitive anion-exchange pathway that accepts both chloride and nitrate (Bogdanova *et al.*, 1998). Therefore, there appears to be little consistency in the interaction with anions and biological membranes.

The work presented in this chapter confirms and extends the findings of von Broembsen and Deacon (1997). That is, calcium at a supplement of 20 mM affects several stages of the infection sequence and the cumulative effect has the potential to reduce the inoculum for primary infection and the secondary spread of infection. The results in this Chapter have also demonstrated the magnitude of the speculated cumulative effect. *Py. aphanidermatum* was more sensitive to cation supplements *in vitro* than *Ph. parasitica,* which has already been shown to be controlled effectively by calcium supplements in small-scale glasshouse trials (von Broembsen and Deacon, 1997), although the level of supplement found to be effective by von Broembsen and Deacon (1997) could be detrimental to water up-take in large horticultural crops (tomatoes and cucumbers). However, no effects were detected in the small vinca plants of their trial. Ideally the modification of the nutrient solution for disease control purposes should not increase the electrical conductivity of a nutrient solution.

Cooper (1979) recommends that the electrical conductivity of irrigation solutions should be above 2.0 $m\Omega^{-1}$, but less than 3.0 $m\Omega^{-1}$. The electrical conductivity of the nutrient solutions at Stockbridge House ranged from 2.1 to 4.3 $m\Omega^{-1}$, with the mean being 3.16 $\pm$ 0.05 $m\Omega^{-1}$ (Appendices 8 to 13). Concentrations of the principal cations (Ca^{2+}, Mg^{2+} and K^+) and nitrogen were also greater than recommended by Cooper (1979). The average concentrations of these ions were 8.2 mM for Ca^{2+}, 9.6 mM for K^+, 5.2 mM for Mg^{2+} and 22.4 mM for nitrogen. Virtually all nitrogen was present in the nitrate form, with only 26 μM of nitrogen present as ammonium. Ultimately, the upper limit of cation supplements for the control of pathogenic Oomycetes will depend on agronomic factors. The results of this chapter suggest

that the greater the supplement the greater the disease suppression. Therefore, there will be trade-off between disease control and plant stress through increased salt concentrations.

Calcium is essential for normal zoospore behaviour (Halsall and Forester, 1977; Gubler *et al.*, 1989; Donaldson and Deacon, 1992; Deacon and Donaldson, 1993; Donaldson and Deacon, 1993a; von Broembsen and Deacon, 1996; Warburton and Deacon, 1998) and supra-optimal levels profoundly interfere with zoospore biology. However, similar supplements of either potassium or magnesium in most cases also have a noticeable effect on the infection sequence. Magnesium has been reported to surrogate calcium in several physiological processes in *Pythium* (Donaldson & Deacon, 1992). In this study Mg^{2+} was generally less effective than Ca^{2+}, although $Mg(NO_3)_2$ was the only salt tested that significantly suppressed biomass gain of *Ph. parasitica*. Magnesium, unlike calcium, did not elevate sporangial discharge at concentrations that are commercially used. The effectiveness of K^+ in suppressing the infection sequence prompted the manipulation of the potassium to calcium ratio in favour of potassium, but without increasing the overall nitrate level. This manipulation suppressed zoospore release from *Py. aphanidermatum* and suppressed zoospore motility in both species, thus demonstrating that simple manipulation of nutrient balance has potential as a disease control method. Coincidentally, certain complete fertilisers designed for growing of tomatoes or cucumbers in NFT based systems by amateurs lack calcium (Anon, 2000b).

Generally *Py. aphanidermatum* was more susceptible to cationic manipulation than *Ph. parasitica*. A strong contrast was seen between the effect of salt supplements on suppression of biomass gain in *Py. aphanidermatum* and the relative insensitivity of *Ph. parasitica*. The compound effect of 5 mM KNO_3 clearly suppressed the theoretical infection potential of *Py. aphanidermatum*, but the effect on the biology of *Ph. parasitica* was only marginal. In laboratory conditions *Ph. parasitica* grows slower and liberates fewer zoospores than *Py. aphanidermatum*. Most species of *Phytophthora*, including *Ph. parasitica*, are moderately resistant to hymexazol (Tay *et al.*, 1983), whereas all species of *Pythium* are sensitive to hymexazol (Masago *et al.*, 1977). These differences in biology may be indicative of life strategy.

Chapter 4.

The effects of recirculating horticultural irrigation solutions on the biology of zoosporic fungi.

4.1. Introduction.

Presently, an estimated 90 to 95% of British tomatoes and 80 to 85% of cucumbers are grown in glasshouse irrigation systems; together the area covers approximately 700 ha. All these irrigation installations presently operate 'run-to-waste' systems: that is, nutrient solution is applied to the crop and all the excess solution is discarded. Crops are irrigated to excess (25-35%) to counter both potential variation in the irrigation supply and the individual plant's demand for nutrients (McPherson *et al.*, 1995). The discarded solution contains a range of chemicals including nitrates, phosphates and potentially pesticides. Not only does this practice have environmental implications, but also it represents a waste of resources. The practice of run-to-waste is employed because of the relative ease of management and to minimise the danger of recirculating pathogen propagules.

Conventional wisdom suggests that the recirculation of irrigation solution containing disease propagules will increase disease pressure and result in increased disease incidence. This would consequently reduce the quantity and quality of the yield. However, it has been found that, contrary to expectation, the recirculation of contaminated irrigation solution does not necessarily increase the incidence of disease (McPherson *et al.*, 1995). That is, a closed, recirculating irrigation system can enable natural disease suppression to occur. This phenomenon is poorly understood, and perhaps unpredictable, so there is unwillingness by commercial operators to adopt recirculation technology.

Horticulture Research International (HRI) at Stockbridge House (North Yorkshire) has attempted to investigate the suppressive potential of irrigation solutions from closed (recirculating) irrigation systems against the tomato root pathogen *Ph. cryptogea*. The objectives of their work included the following (McPherson, 1998):

- To determine whether a suppressive potential develops in the irrigation solution in the absence of the pathogen.
- To determine how quickly a suppressive solution develops in 'closed' systems.
- To determine if the suppressive potential is enhanced by the application of specific treatments e.g. slow sand filtration.
- To determine if any applied treatments, e.g. pasteurisation, are detrimental to the suppressive potential.

- To investigate whether a suppressive solution, generated against *Ph. cryptogea*, is effective against other root pathogens.
- To determine the maximum dilution factor for continued efficacy of the suppressive solution.
- To determine whether irrigation solution from commercial 'closed' systems is equally suppressive.
- To determine the persistence of the suppressive potential of used solution.

A collaboration with Stockbridge House was agreed whereby irrigation samples were taken regularly from the trial and submitted to me for further analysis. The analyses in Edinburgh were intended to identify where the agents of suppression acted in the infection sequence of the zoospore-producing fungi (*Py. aphanidermatum* and *Ph. parasitica*).

4.2. The experimental protocol at Stockbridge House.

4.2.1. An overview.

In a large glasshouse (1000 m^2) all treatments were present in a semi-replicated format (see later). The treatments were:

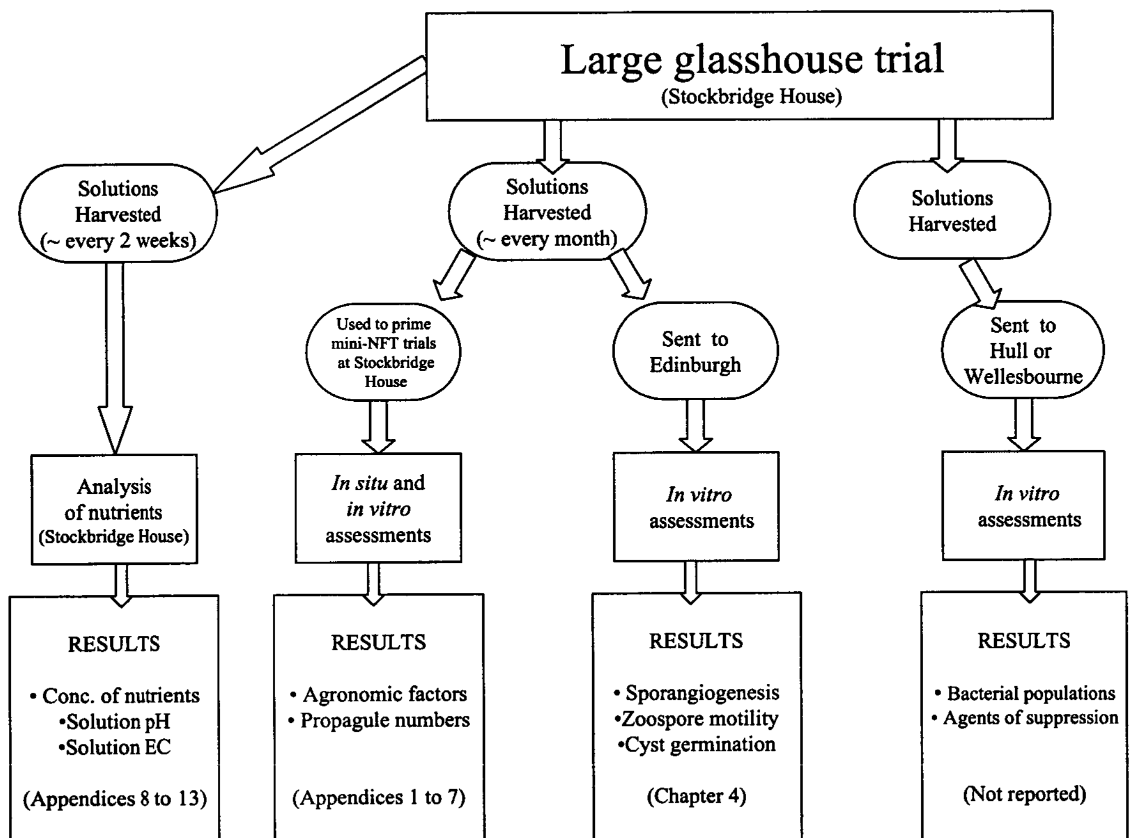
1. Uninoculated 'open' rockwool system.
2. Uninoculated 'closed' rockwool system.
3. Inoculated 'open' rockwool system.
4. Inoculated 'closed' rockwool system.
5. Inoculated 'closed' rockwool system, solution pasteurised.
6. Inoculated 'closed' rockwool system, solution slow-sand filtered.

All inoculated treatments had *Ph. cryptogea* artificially introduced once the plants were established (see later). Two disinfection treatments were employed in this trial. Pasteurisation represented an active and physical form of disinfection, whereas slow-sand filtration was passive and biological. Rockwool is an inert solid rooting medium that is commonly used in commercial operations.

Once the plants were established and the relevant treatments inoculated with the pathogen the irrigation solution was 'harvested' from each treatment at regular intervals and used to 'prime' a series of mini-NFT (nutrient film technique) systems. Small samples of the

'harvested' solution were sent to Edinburgh, to test their effects on different stages of the zoosporic infection sequence. The mini-NFT systems were designed to determine the suppressive potential of each solution taken from the treatments in the main glasshouse. That is, the systems of the large glasshouse principally generated suppressive solutions for assessment in the mini-NFT trials, and at Edinburgh. At Stockbridge House the assessments were primarily agronomic, whereas the results presented in this Chapter focus on the effects of irrigation solutions on the zoosporic fungi *Ph. parasitica* and *Py. aphanidermatum*. In addition, F. Lewis and J. Friend (University of Hull), and J. Taylor (HRI, Wellesbourne) sought to partially characterise the bacterial populations present in the treatments and tentatively identify the mechanism of suppression. In Figure 4.1, a flow diagram represents schematically how the work at Edinburgh, Hull and HRI are related.

Figure 4.1. Schematic representation of the relationship between the work performed at various locations.



I was not involved with the experimental design, trial implementation and results gathering at Stockbridge House. However, I solely performed all the analyses at Edinburgh along with the interpretation of the results derived from both Edinburgh and Stockbridge House that are presented in this thesis.

4.2.2. The experimental set up of the large glasshouse.

Tomato (cultivar Ferrari) seed was sown into small rockwool blocks during mid April of 1997. These blocks were transferred to the large glasshouse in mid May. Three plants were planted out into each rockwool trough (Figure 4.2). Each treatment was present as four randomly allocated double rows of plants. Each double row contained 20 rockwool troughs, with each plant being individually drip irrigated and each slab draining into the channel between the double row (Figures 4.3, 4.4 and 4.5).

Figure 4.2. The arrangement of rockwool blocks on rockwool slabs.

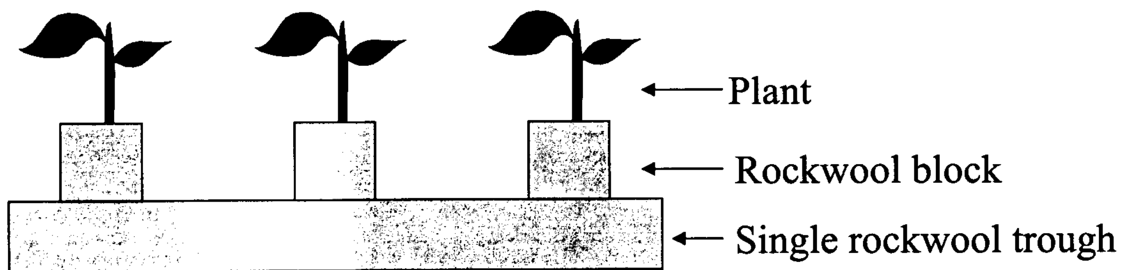


Figure 4.3. The arrangement of rockwool slabs in a double row.

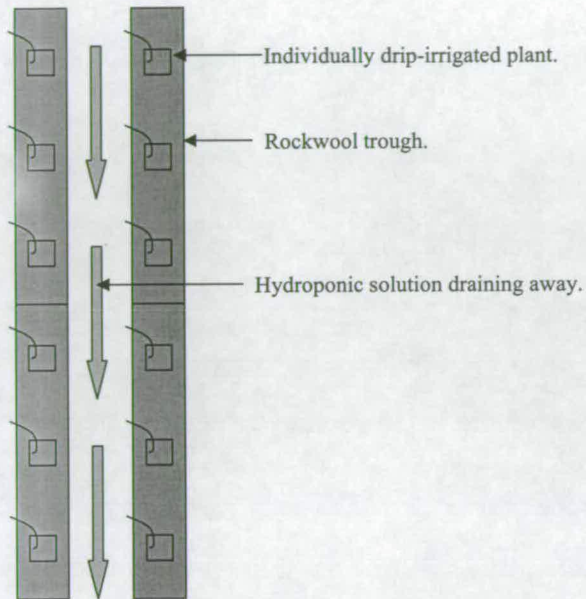
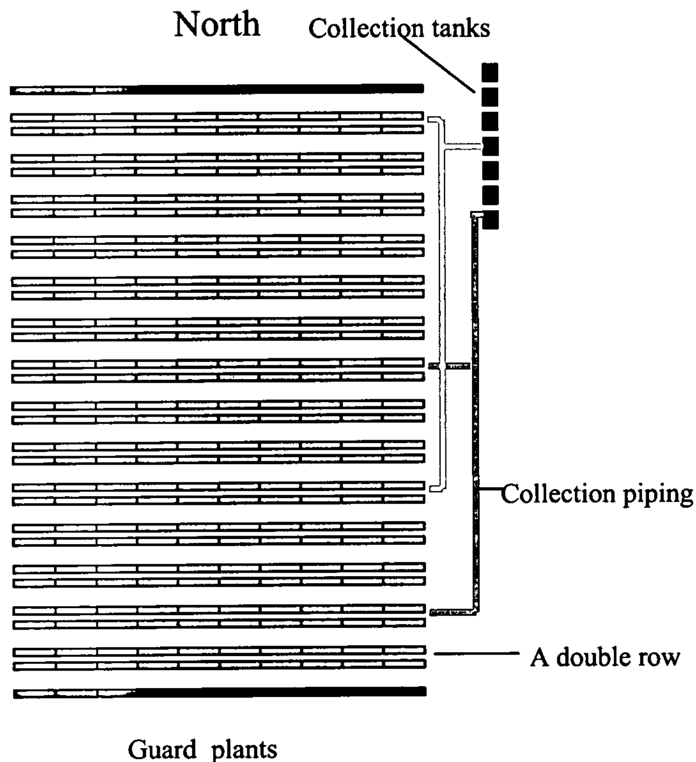


Figure 4.4. Photograph of young tomato plants in the double row arrangement.

The glasshouse contained a total of 28 double rows with a north-south pathway dividing the double rows into two sides of 14 rows. The single rows at the north and south ends of the glasshouse were not included in the trial, but acted as guard rows. Plants grown in these outer rows perform better than the other rows because of increased exposure to solar radiation. Each treatment was represented by two double rows in each side of the glasshouse; row allocation was fully randomised. The run-off from each treatment was captured and pooled in a collection tank according to treatment (Figure 4.5). Therefore, the trial was not fully replicated - this was due to financial constraints. A couple of weeks after planting (at the end of May) the appropriate plants were inoculated with a virulent isolate of *Ph. cryptogea* by placing a 2 × 1 cm agar block containing the fungus beneath the propagation block of the central plant in each slab.

Figure 4.5. Diagram of the layout of the double rows (east half of the glasshouse only) and the collection of irrigation solution.



From the collection tanks the irrigation solution was pumped to, and stored in, 1000 l covered containers. Once a container was full the contents were then pumped to a second 1000 l covered container. However, for the slow sand filtered and pasteurised treatments the pumping from the first to the second container was via the relevant apparatus (that is, either a sand filter or a pasteuriser). Irrigation solution in the second container was then discarded for the 'open' run-to-waste treatments or recirculated for the 'closed' treatments. All recirculated irrigation solutions before being re-irrigated passed through a Vocom unit which adjusted the pH of irrigation solution by adding nitric acid, and the electrical conductivity of the solution was adjusted by either diluting with fresh water or adding concentrated nutrient solution.

At fortnightly intervals irrigation solution from each treatment was sampled and analysed for nutrient content, electrical conductivity and pH (Appendices 8 to 13). From these analyses any nutrient deficiencies could be rectified. As the tomato plants grew they were trained onto supports according to standard commercial practice.

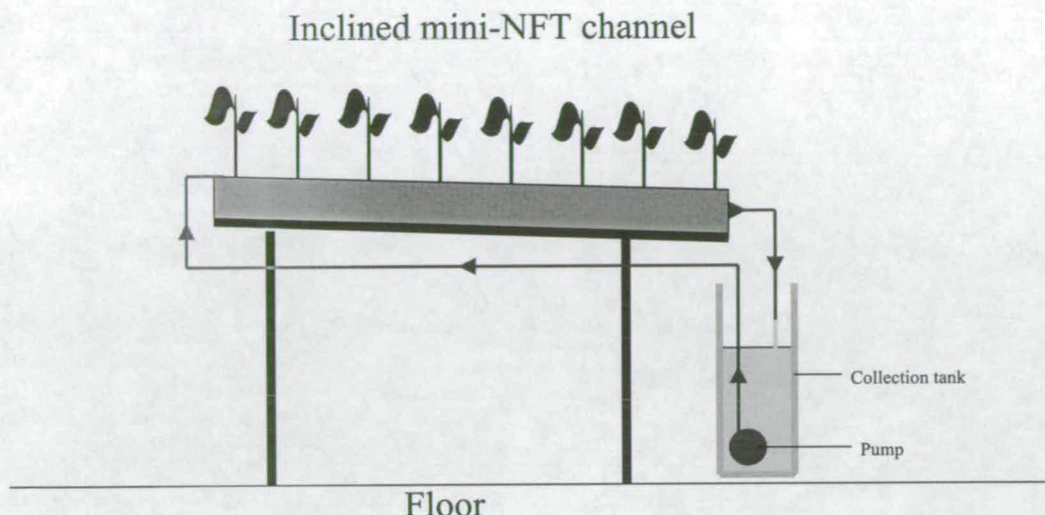
Irrigation solutions were harvested from each treatment at five time points: early July (1st), mid July (15th), late July (30th), early September (9th) and late October (24th). The irrigation solution was harvested from the second container - that is, prior to adjustment for pH and electrical conductivity - but after slow sand filtration and pasteurisation in the treatments where these processes occurred. The volume of solution harvested from each treatment was approximately 250 l, of which 250 ml to 500 ml was sent to Edinburgh.

4.2.3. The set up of the mini-NFT systems.

In the large glasshouse the tomato plants were grown in rockwool slabs for the duration of the trial. Roots growing in this solid substrate cannot be assessed for development or indications of disease. Similarly, the height of the plants would be difficult to obtain in the large glasshouse because of the spiral training of the plants. However, in mini-NFT systems, which lack solid substrate, it is easy to make observations of root development and disease incidence.

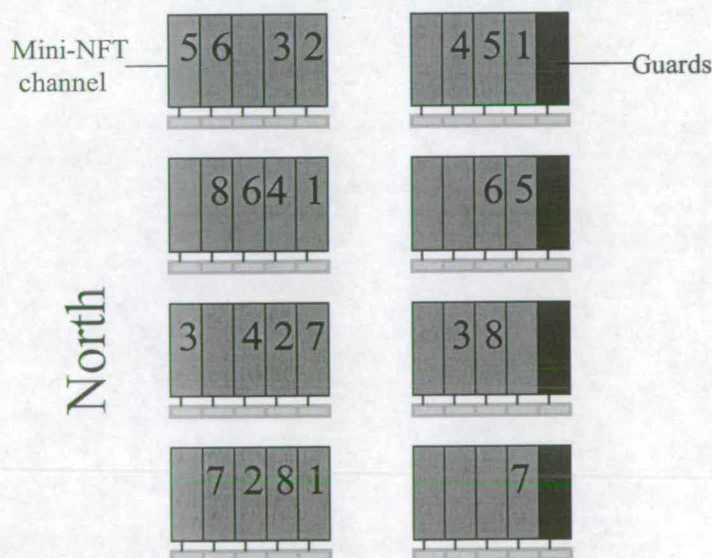
The harvested irrigation solution from each treatment was used to prime three fully replicated mini-NFT systems. Each mini-NFT system was a single channel and contained six to eight plants (Figure 4.6). The irrigation solution was recirculated for the duration of the experiment, which was typically about a month.

Figure 4.6. The set up of a single mini-NFT system.



The positioning of the mini-NFT systems was completely randomised within a small glasshouse that contained eight tables. Each table supported five mini-NFT systems (Figure 4.7). Guard rows were employed at the south end of the four south tables. In these short-term experiments using mini-NFT systems it was occasionally necessary to top-up the solution reservoirs with fresh solution. This mirrors what would be done in a commercial NFT system.

Figure 4.7. Diagram of the allocation of the mini-NFT trials*.



*Trials that are unnumbered or numbered 7 and 8 are not relevant to this thesis.

4.2.4. Assessment of plants grown in the mini-NFT systems and number of *Phytophthora* propagules recirculating.

At the initiation of each mini-NFT trial the number of *Ph. cryptogea* propagules per litre of irrigation solutions was determined by two different methods performed by staff at HRI. The first method used a serological probe (dipstick) and the second, more reliable but more time-consuming method, used a dilution plate method. In this second method a solution sample was passed through a membrane filter, then the filter was resuspended in a small quantity of water before diluting and plating out on selective media. However, the results from both techniques reported low propagule numbers - for example, the membrane method failed to find a zoospore in any of the 9th September samples. Consequently all treatments (except the fresh nutrient solution control) were inoculated with *Ph. cryptogea* at the initiation of the NFT trial and propagule numbers reassessed at the termination of the mini-trial. Propagule numbers determined by the probe method are in Appendix 1 and the propagule numbers as determined by the membrane method are in Appendix 2.

At the termination of the mini-NFT trials an index of root development (0 to 100) was determined by randomly taking four roots per plant from each channel (replicate) and measuring the length of the roots (Appendix 3). Similarly, for these randomly selected roots the length of discolouration was measured to allow the determination of the percentage root area with *Ph. cryptogea* infection (Appendix 4.). Also from these roots the number of visible lesions per unit area of root was determined (Appendix 5). Five 1 cm root pieces per plant were also taken and plated onto a *Phytophthora* selective medium and the number of infection sites of *Phytophthora* determined by the number of plated root pieces from which the fungus grew (Appendix 6).

In the final mini-NFT trial that started on the 24th October additional agronomic data were obtained (Appendix 7). The dry weight of the roots was determined. A single leaf per plant from a standard position on each plant was selected in all NFT channels (replicates) and then leaf length was measured *in situ* from the stem to the tip of the leaf. The leaf was then excised and the leaf area was determined using a Planimeter. Plant height and stem diameter were also determined.

4.2.5. Summary of results from Stockbridge House.

Root infection by *Ph. cryptogea* in the main glasshouse was extensive, but disease symptoms did not become visibly apparent in the aerial portion of the crop until September, although the high temperatures and high incidence of solar radiation in August subjected the crops to stressful conditions. The aerial symptoms were limited to wilting of the tomato plant 'heads' during bright sunny days with recovery being made at night. With the advance of the disease, increased incidence of wilting was observed, but only a few plant losses were reported by the termination of the experiment. The most severe wilt was reported in the two inoculated plots (treatments 3 and 4) with the higher incidence of wilting occurring in the closed system (treatment 4). During the whole growing season low incidence of wilting was reported when the irrigation solution passed through the slow sand filter (treatment 6), whereas noticeably more wilting was reported in the plots where the solution was pasteurised prior to recirculation (treatment 5).

A reason cited by Stockbridge House for the lack of build up of natural suppression in the closed system is that the removal of large quantities of potentially suppressive irrigation solution for the mini-NFT trials caused a dilution of the potentially suppressive solution by the introduction of fresh non-suppressive nutrient solution. Earlier studies had observed that when a system is switched from 'closed' to 'open' and refreshed with new nutrient solution a peak in zoospore release is observed. In an auxiliary treatment during the trial, evidence was found to suggest that the removal of irrigation solution may have compromised the build up of natural suppression.

The number of *Ph. cryptogea* propagules enumerated at the termination of the mini-NFT trials was consistently higher when determined by the membrane method. Generally, lower propagule numbers were recovered from the 'closed' solutions than from the 'open' solutions. Towards the end of the season there were ten times as many zoospores recorded in the uninoculated open mini-NFT trial than in the uninoculated closed trial (treatments 1 and 2). A similar effect was not observed in the corresponding solutions from the inoculated treatments (treatments 3 and 4). Higher levels of zoospores were observed in the solutions from the pasteurised treatment than the slow-sand filtered treatment (treatments 5 and 6). Low levels of *Ph. cryptogea* were recorded in the uninoculated fresh irrigation solution control.

In general, the infections of *Phytophthora* spp. per 1 cm of root followed a similar trend to propagule numbers in the solution. There was lower infection from 'closed' solutions than 'open' solutions. However, in solutions from the inoculated closed (treatment 4) treatment there was higher infection than in the corresponding open treatment (3). There was little difference in root infection between the two disinfection treatments.

Overall, root development was highest in the fresh irrigation solution. Greater root development was seen in the uninoculated closed solutions than in the open solutions, with the trend, again, reversing for the corresponding inoculated solutions. Like root infection incidence, there was little difference in root development between the two disinfection treatments. With the exception of the fresh irrigation solution control, root development was lowest in all treatments during the final mini-NFT trial.

No root discolouration was observed on any roots from the fresh irrigation solution control. Greater discolouration was observed generally in the uninoculated open solutions than the closed solutions, with the overall trend being reversed for the corresponding inoculated solutions. Root discolouration for all treatments was highest during the final mini-NFT trial.

In the fresh irrigation solutions no lesions per unit area of root were observed. The greatest number of lesions for all other treatments was found in the final mini-NFT trial. Again, generally a higher incidence of lesions was observed in the uninoculated open solutions than the closed solutions, and in the corresponding inoculated solutions the trend was reversed. The numbers of lesions observed in the two disinfection treatments were similar.

There was high correlation between the five agronomic factors assessed in the final mini-NFT trial. Overall, the healthiest plants were in the control trial with the plants being the tallest, having the largest leaves and the heaviest roots. The least healthy plants were in the trial with uninoculated open solution, with the plants in the uninoculated closed solution being slightly healthier. There were only marginal differences between the indices of health for the plants grown in the two corresponding inoculated solutions. The plants grown in the solution from the slow sand filtered treatment were noticeably healthier than plants grown in the pasteurised solution.

4.3. The work performed at Edinburgh.

When irrigation samples from Stockbridge House were received they were stored at 4 °C in the dark until required. When solutions were required the amount needed was filter sterilised through a 0.22 µm millipore membrane immediately before the experiment. The effects of the irrigation solutions on the various aspects of the infection sequences of *Py. aphanidermatum* and *Ph. parasitica* were determined using the standard protocols (see Chapter 2). The assays were:

1. The proportion of zoospores of *Ph. parasitica* that remain motile in irrigation solutions during 1h incubation.
2. The proportion of cysts (from 1) that spontaneously germinated during the 1h incubation.
3. The proportion of zoospores of *Py. aphanidermatum* that remain motile in irrigation solutions during 2h incubation.
4. The proportion of cysts (from 3) that spontaneously germinated during the 2h incubation.
5. The number of zoospores of *Ph. parasitica* released into irrigation solutions from pre-formed sporangia.
6. The proportion of vortex-encysted zoospores of *Py. aphanidermatum* that had germinated after 2h in irrigation solutions.
7. The subsequent release of zoospores into SDW from sporangia of *Ph. parasitica* that had formed in irrigation solutions.
8. The density of sporangia of *Ph. parasitica* after incubation in irrigation solutions.
9. The proportion of sporangia of *Ph. parasitica* that had discharged their contents after incubation in irrigation solutions

4.3.1. Summary of 'Edinburgh' data.

In Section 4.4 details of the various analyses are reported. However, neither temporal nor other trends were clearly identified in the data series. Therefore, the results are summarised here.

To summarise the overall suppressive effect of each treatment on the biology of *Py. aphanidermatum* and *Ph. parasitica* the means of each treatment were ranked between 1 and

8 (Table 4.1). The highest rank (1) was attributed to the solution that was theoretically the least suppressive for the particular aspect of zoospore biology over the sampling period. A theoretically suppressive solution is a solution that suppresses zoospore motility, sporangia formation, sporangial discharge and the number of zoospores liberated. Promotion in cyst germination levels was also categorised as an indication of a suppressive solution. This is based on the assumption that all cysts are viable and that a germinated cyst only has the potential to infect locally, whereas a non-germinated cyst has the potential to release a secondary zoospore and infect a distant host plant.

Table 4.1. Ranking of irrigation solutions based on theoretical suppressiveness.

	Treatment							
Aspect of zoospore biology	SDW	Fresh irrigation solution	Uninoculated Open	Uninoculated Closed	Inoculated Open	Inoculated Closed	Inoculated closed (pasteurised)	Inoculated closed (filtered)
Zoospore motility (*Ph. parasitica*) - Table 4.4.	1	3	6	6	5	2	8	3
Subsequent cyst germination (*Ph. parasitica*) - Table 4.5.	1	3	2	6	3	7	8	5
Zoospore motility (*Py. aphanidermatum*) - Table 4.6.	4	1	6	7	2	5	8	3
Subsequent cyst germination (*Py. aphanidermatum*) - Table 4.7.	4	1	2	5	8	6	7	3
Zoospore release (*Ph. parasitica*) - Table 4.8.	2	1	3	5	4	7	8	6
Germination of encysted spores (*Py. aphanidermatum*) - Table 4.9.	2	7	5	6	1	4	8	3
Zoospore release (sporangiogenesis) (*Ph. parasitica*) - Table 4.10.	8	2	3	1	4	5	7	6
Sporangial density (*Ph. parasitica*) - Table 4.11.	1	4	5	3	7	2	6	8
Sporangial discharge (*Ph. parasitica*) - Table 4.12.	1	3	7	8	5	4	2	6
Mean rank	**3.2**	**2.7**	**4.2**	**4.8**	**4.3**	**4.7**	**6.9**	**4.9**

Within each treatment the range of theoretical suppressiveness was large. For example, the uninoculated closed treatment was the most suppressive of sporangial discharge and also the least suppressive of the numbers of zoospores released from pre-formed sporangia. However, overall the most suppressive treatment was the inoculated closed (pasteurised) treatment with a mean rank of 6.9, and the lowest mean rank of 2.7 was for the fresh irrigation solution control.

In Table 4.2 the mean rank is displayed along with the mean pH of the treatment (from Table 4.3). Overall the four most theoretically suppressive treatments were the four that were from

closed systems, and the least overall suppressive treatments were the two control treatments. The treatments from the two open systems both had the same level of overall theoretical suppressiveness.

Table 4.2. Overall theoretical suppressiveness in each treatment and associated mean solution pH.

Overall theoretical suppressiveness	Treatment	Mean rank	PH
Most	Pasteurised (Inoculated and closed)	6.9	4.8
↓	Filtered (Inoculated and closed)	4.9	6.4
	Uninoculated Closed	4.8	6.7
Moderate	Inoculated Closed	4.7	6.2
	Inoculated Open	4.3	6.7
↓	Uninoculated Open	4.2	7.0
Least	SDW	3.2	6.8
	Fresh Irrigation solution	2.7	7.0

4.4. Details of 'Edinburgh' results.

4.4.1. Determination of the pH of irrigation solutions.

The pH of irrigation solutions was determined at Edinburgh with an AGB-75 laboratory pH/mV/temperature meter (Table 4.3, page 136). The pH range of irrigation solutions was from 4.3 to 7.5. For each of the sampling times the inoculated closed (pasteurised) solution had the lowest pH with the mean pH for the four sampling times being 4.8. In the inoculated closed (pasteurised) solution there was a negative correlation (R = 93%) between pH and sampling date. In all other irrigation solutions no temporal trends were observed. Overall the solutions from closed systems were more acidic than solutions from the non-recirculated systems, that is, from open systems and the two controls (SDW and fresh irrigation solution).

4.4.2. The effects of irrigation solutions on the motility of zoospores of *Ph. parasitica* and subsequent cyst germination.

Two ml of zoospore suspension of *Ph. parasitica* (prepared as in Section 2.3.1.1) was diluted with 2 ml of irrigation solution and then incubated for 1h. After incubation the proportion of spores that were motile was determined (see Section 2.3.6) (Table 4.4, page 137), along with the proportion of cysts that had subsequently germinated (Table 4.5, page 138). The duration

of the incubation was only one hour because in a 2h incubation almost all zoospores encyst (results not shown).

The proportion of motile zoospores in the SDW control varied significantly ($P = 6.9 \times 10^{-3}$) between the dates when irrigation solutions were collected. Also, in the fresh irrigation solution control there was significant ($P = 4.8 \times 10^{-3}$) variation in the proportion of motile zoospores between the dates of solution harvesting. However, there was no significant ($P \geq 1.8 \times 10^{-1}$) difference in zoospore motility between the sampling times for the uninoculated open, uninoculated closed, inoculated open and inoculated closed (pasteurised) treatments. The other two treatments (inoculated closed (filtered) and inoculated closed) both showed significant ($P \leq 8.1 \times 10^{-5}$) differences in zoospore motility between the sampling times.

Although there were no significant temporal trends in any of the treatments, overall the lowest levels of zoospore motility were found in the inoculated closed (pasteurised) treatments, and the highest levels of zoospore motility were found in the SDW controls. The proportion of motile zoospores in inoculated closed (filtered) irrigation solutions collected on 1st July and 15th July was 5.4% and 15.0%, respectively, whereas in closed (filtered) irrigation solutions for the last three sampling times no motile zoospores were observed. In the inoculated closed irrigation solutions harvested on 30th July and 9th September the level of zoospore motility was less than 1%. However, in the sample harvested on 24th October the level of motility was 14.6%.

The proportion of cysts spontaneously germinating in the SDW control did not significantly differ ($P = 0.29$) between the harvesting dates. In the uninoculated open and inoculated open treatments there were also no significant differences ($P > 0.20$) in the proportion of cysts germinating between the dates of harvesting. In the other five treatments, including the fresh irrigation solution control, significant ($P \leq 0.015$) differences in cyst germination were observed between the dates of solution harvesting. The overall highest levels of cyst germination were observed in samples from the inoculated closed (pasteurised) system. The lowest overall levels of cyst germination were observed in the SDW control. Generally, higher germination levels were observed in solutions from closed systems.

4.4.3. The effects of irrigation solutions on the motility of *Py. aphanidermatum* and subsequent cyst germination.

One ml of zoospores suspension of *Py. aphanidermatum* (prepared as in Section 2.3.1.1) was diluted with 3 ml of irrigation solution and then incubated for 2h. After incubation the proportion of spores that were motile was determined (see Section 2.3.6) (Table 4.6, page 139), along with the proportion of cysts that had subsequently germinated (Table 4.7, page 140). Only one ml of zoospore suspension was required because *Py. aphanidermatum* produces more zoospores than *Ph. parasitica*.

The proportion of motile zoospores in the SDW control varied very highly significantly ($P = 5.6 \times 10^{-4}$) between the dates of irrigation solution harvesting. However, in the control consisting of fresh irrigation solution there was no significant ($P = 0.23$) variation in the proportion of motile zoospores between the dates of solution harvesting. Similarly, there was no significant ($P \geq 0.10$) variation in the proportion of zoospores motile between dates of solution harvesting for solutions from the uninoculated open, uninoculated closed, inoculated open and inoculated closed systems. In contrast, there were highly significant ($P \leq 1.2 \times 10^{-3}$) differences in the proportion of motile zoospores between sampling dates for solutions from the pasteurised and slow sand filtered inoculated closed systems. The mean proportion of zoospores motile over the growing season in each treatment was greater than 42%, except for the inoculated closed (pasteurised) treatment where the mean was 2%.

There were significant ($P \leq 0.028$) differences in the proportion of cysts germinating between sampling dates in SDW controls, fresh irrigation solution controls, inoculated open solutions, inoculated closed solutions and inoculated closed (pasteurised) solutions. In contrast, there were no significant ($P \geq 0.17$) differences between the proportion of cysts germinating in the other three treatments (uninoculated open, uninoculated closed and inoculated closed (filtered)).

4.4.4. The effects of irrigation solutions on the release of zoospores from pre-formed sporangia of *Ph. parasitica*.

After washing with aliquots of SDW, pre-chilled irrigation solutions were added to sporangia-bearing fungal mats of *Ph. parasitica*. Then after the cold shock and release period, an aliquot of zoospore suspension was taken, fixed and enumerated (as in Section 2.3.5 (Table 4.8, page 141).

In all but two treatments and in both the controls there were no significant ($P \geq 0.11$) differences in zoospore release between the dates of solution harvesting. There were significant ($P \leq 0.011$) differences in zoospore release between harvesting dates for the inoculated closed and inoculated closed (filtered) treatments. Although temporal trends were not observed there were very highly significant ($P = 3.4 \times 10^{-6}$) differences between the treatments. Overall the greatest zoospore release numbers were observed in the fresh irrigation solutions and the lowest values were observed in the inoculated closed (pasteurised) treatment. Generally lower numbers of zoospores were released into solutions from closed systems than from the corresponding open systems.

4.4.5. The effects of irrigation solutions on germination of vortex-encysted zoospores of *Py. aphanidermatum.*

To Eppendorf tubes, 200 µl of encysted zoospores of *Py. aphanidermatum* were added with 800 µl of irrigation solution. The tubes were inverted to mix the liquids and then incubated for 2h. After the incubation period the spores were fixed and later the proportions of cysts that had germinated were determined (see Section 2.3.7) (Table 4.9, page 142).

There were no significant ($P = 0.71$) differences in the proportion of spores germinating between the dates of harvesting for the SDW control. In the second control (fresh irrigation solutions) there were significant ($P = 8.7 \times 10^{-3}$) differences in the proportion of spores germinating between harvesting dates. In five out of the six other treatments there were significant ($P \leq 0.046$) differences in the proportion of spores germinating between harvesting dates. Out of the treatments only the inoculated closed treatment had no significant ($P = 0.078$) difference between harvesting dates. Overall the highest germination levels were seen in the inoculated closed (pasteurised) treatment with a mean germination level of 62%, whereas the overall lowest level of 27% was seen in the inoculated open treatment. Generally higher levels of germination were observed in solutions from closed systems than from the corresponding open systems.

4.4.6. The effects of irrigation solutions on sporangiogenesis of *Ph. parasitica*.

The standard protocol for inducing sporangiogenesis (Section 2.3.1.1) was followed, but the MSS was substituted with an irrigation solution. The sporangia were induced to liberate zoospores into SDW, and then an aliquot (500 μl) of zoospore suspension was removed and fixed. The zoospore concentration was enumerated later by haemocytometer counts (Table 4.10, page 143). Direct video recordings were made of the mycelial mats to determine sporangial density (Table 4.11, page 144) and the proportion of sporangia with discharged contents (as in Section 2.3.4) (Table 4.12, page 145).

There were no significant (P = 0.50) differences in the number of zoospores subsequently released between the harvesting dates for the SDW control. Similarly, in the uninoculated open, uninoculated closed, inoculated open, inoculated closed and inoculated closed (pasteurised) treatments there were no significant ($P \geq 0.11$) differences in the number of zoospores subsequently released between the harvesting dates. However, in the fresh irrigation solutions and inoculated closed (filtered) treatment there were significant ($P \leq$ 0.028) differences in the number of zoospores subsequently released between the harvesting dates. The lowest overall level of zoospore release was 18.6 zoospores.μl^{-1}, which was in the SDW control. Out of the seven irrigation solutions the lowest overall level of zoospore release was 36.4 zoospores.μl^{-1} which were released into the inoculated closed (pasteurised) treatment. The overall highest level of zoospore release was in the uninoculated closed treatment with a mean zoospore release level of 96.7 zoospores.μl^{-1}.

There were no significant ($P \geq 0.094$) differences in the sporangial density between the harvesting dates within any of the treatments. However, there was a very highly significant ($P = 4.8 \times 10^{-4}$) difference between the treatments. The highest overall sporangial density was in the SDW control with 48.6 sporangia per video monitor screen and the lowest overall sporangial density was in the inoculated closed (filtered) treatment with 20.1 sporangia per screen. Generally higher sporangial densities were observed when mats were bathed in solutions from closed systems than the corresponding open systems.

There were no significant (P = 0.35) differences in the proportion of sporangia that had discharged between the harvesting dates for the SDW control. The fresh irrigation solution, uninoculated open, inoculated closed and inoculated closed (pasteurised) treatments had

significant ($P \leq 0.046$) differences in the proportion of sporangia that had discharged between the harvesting dates. The uninoculated closed, inoculated open and inoculated closed (filtered) did not have significant ($P \geq 0.095$) differences in the proportion of sporangia that had discharged between the harvesting dates. Overall the highest level of sporangial discharge was in the SDW control with 23%, and the lowest overall level of sporangial discharge was in the uninoculated closed treatment with 8%.

Table 4.3. The pH of irrigation solutions from HRI.

Solution	Date of harvesting irrigation solution from large glasshouse.					
	1st July	15th July	30th July	9th September	24th October	Mean
Sterile distilled water	6.8	6.8	6.8	6.8	6.8	**6.8**
Fresh Irrigation Solution	7.2	7.5	6.7	7.5	5.9	**7.0**
Uninoculated Open	n.d.	n.d.	7.3	7.0	6.8	**7.0**
Uninoculated Closed	6.7	6.4	6.7	6.9	6.8	**6.7**
Inoculated Open	n.d.	6.9	6.7	6.8	6.4	**6.7**
Inoculated Closed	n.d.	n.d.	6.2	6.3	6.0	**6.2**
Inoculated Closed (Pasteurised)	n.d.	5.5	4.9	4.7	4.3	**4.8**
Inoculated Closed (Filtered)	n.d.	6.0	6.2	6.9	6.6	**6.4**
Mean	**6.9**	**6.5**	**6.4**	**6.6**	**6.2**	**6.4**

n.d. Not done.

Table 4.4. Percentage of zoospores of *Ph. parasitica* that were motile after 1 h incubation in various irrigation solutions*.

Solution	Date of harvesting irrigation solution from large glasshouse.					Mean	P (ANOVA)	σ_d
	1st July	15th July	30th July	9th September	24th October			
Sterile distilled water	12.6 ± 1.2 (20.7 ± 1.0)	3.5 ± 0.4 (10.7 ± 0.6)	5.4 ± 0.1 (13.4 ± 0.2)	4.8 ± 1.3 (12.3 ± 2.0)	3.6 ± 1.8 (10.3 ± 2.8)	**6.0 ± 1.0** **(13.5 ± 1.2)**	(6.9×10^{-3})	(2.3)
Fresh Irrigation Solution	0.9 ± 0.5 (4.5 ± 2.3)	11.9 ± 2.4 (20.0 ± 2.1)	5.8 ± 2.3 (13.0 ± 3.5)	0.5 ± 0.3 (3.1 ± 1.8)	1.6 ± 0.8 (6.0 ± 3.0)	**4.1 ± 1.3** **(9.3 ± 2.0)**	(4.8×10^{-3})	(3.7)
Uninoculated Open	0.7 ± 0.4 (3.8 ± 1.9)	0.6 ± 0.0 (4.5 ± 0.2)	0.7 ± 0.3 (4.5 ± 1.0)	0.5 ± 0.2 (4.2 ± 0.6)	0.3 ± 0.3 (1.9 ± 1.9)	**0.6 ± 0.1** **(3.8 ± 0.6)**	(6.1×10^{-1})	(1.8)
Uninoculated Closed	0.8 ± 0.0 (5.0 ± 0.0)	0.8 ± 0.4 (4.1± 2.1)	0.5 ± 0.4 (3.3 ± 1.8)	0.6 ± 0.4 (3.5 ± 1.9)	0.5 ± 0.3 (3.3 ± 1.7)	**0.6 ± 0.1** **(3.9 ± 0.7)**	(9.4×10^{-1})	(2.4)
Inoculated Open	n.d.	0.4 ± 0.2 (3.0 ± 1.5)	0.4 ± 0.2 (2.9 ± 1.4)	0.7 ± 0.4 (3.8 ± 2.0)	1.2 ± 0.4 (6.2 ± 1.0)	**0.7 ± 0.2** **(4.0 ± 0.8)**	(4.3×10^{-1})	(2.2)
Inoculated Closed	n.d.	n.d.	0.9 ± 0.2 (5.4 ± 0.5)	0.4 ± 0.4 (2.2 ± 2.2)	14.6 ± 0.6 (22.4 ± 0.5)	**5.2 ± 2.3** **(10.0 ± 3.2)**	(8.1×10^{-5})	(1.9)
Inoculated Closed (Pasteurised)	n.d.	0.9 ± 0.5 (4.5 ± 2.3)	0.3 ± 0.0 (3.4 ± 0.1)	0.3 ± 0.2 (2.7 ± 1.4)	0.0 ± 0.0 (0.0 ± 0.0)	**0.4 ± 0.1** **(2.6 ± 0.8)**	(1.8×10^{-1})	(1.9)
Inoculated Closed (Filtered)	5.4 ± 0.8 (13.3 ± 1.0)	15.0 ± 2.6 (22.7 ± 2.1)	0.0± 0.0 (0.0 ± 0.0)	0.0 ± 0.0 (0.0 ± 0.0)	0.0 ± 0.0 (0.0 ± 0.0)	**4.1 ± 1.6** **(7.2 ± 2.5)**	(4.8×10^{-8})	(1.5)
Mean (Mean of arcsine transformed data).	**4.1 ± 1.3** **(9.5 ± 1.9)**	**4.7 ± 1.3** **(9.9 ± 1.8)**	**1.8 ± 0.5** **(5.8 ± 1.1)**	**1.0 ± 0.3** **(4.0 ± 0.9)**	**2.7 ± 1.0** **(6.3 ± 1.5)**	**2.7 ± 0.4** **(6.8 ± 0.7)**	(1.7×10^{-2})	
P (ANOVA)	(3.5×10^{-5})	(1.8×10^{-6})	(7.2×10^{-4})	(4.2×10^{-3})	(1.9×10^{-6})	(9.3×10^{-6})		
σ_d	(2.1)	(2.4)	(2.3)	(2.3)	(2.5)			

* Means ± s.e.m. for three replicates, based on three random fields of view per replicate. Results in parentheses are arcsine transformed data.
n.d. Not done.

Table 4.5. Percentage of encysted zoospores of *Ph. parasitica* that had germinated after 1 h incubation in various irrigation solutions*.

Solution	1st July	15th July	30th July	9th September	24th October	Mean	P (ANOVA)	σ_d
	Date of harvesting irrigation solution from large glasshouse.							
Sterile distilled water	7.4 ± 2.5 (15.3 ± 2.8)	3.4 ± 2.5 (8.3 ± 4.8)	7.0 ± 0.8 (15.3 ± 0.8)	8.2 ± 1.1 (16.6 ± 1.2)	4.8 ± 1.7 (12.4 ± 2.2)	**6.2 ± 0.8 (13.6 ± 1.3)**	(2.9×10^{-1})	(3.9)
Fresh Irrigation Solution	2.6 ± 0.3 (9.3 ± 0.5)	5.4 ± 2.4 (12.9 ± 3.0)	5.1 ± 1.0 (13.0 ± 1.3)	3.0 ± 0.6 (9.9 ± 1.1)	19.1 ± 5.2 (25.5 ± 3.8)	**7.1 ± 1.9 (14.1 ± 1.8)**	(3.1×10^{-3})	(3.2)
Uninoculated Open	3.6 ± 1.8 (8.9 ± 4.5)	4.7 ± 1.2 (12.3 ± 1.7)	6.2 ± 1.3 (14.3 ± 1.6)	8.0 ± 2.6 (16.0 ± 2.7)	10.7 ± 2.5 (18.8 ± 2.5)	**6.6 ± 1.0 (14.1 ± 1.4)**	(2.0×10^{-1})	(3.9)
Uninoculated Closed	5.6 ± 3.1 (11.1 ± 5.7)	4.8 ± 1.5 (12.3 ± 1.9)	23.7 ± 5.7 (28.8 ± 3.9)	9.9 ± 1.6 (18.2 ± 1.6)	16.5 ± 0.9 (24.0 ± 0.7)	**12.1 ± 2.2 (18.9 ± 2.2)**	(1.5×10^{-2})	(4.7)
Inoculated Open	n.d.	5.9 ± 1.1 (13.9 ± 1.4)	6.3 ± 2.2 (14.1 ± 2.6)	10.0 ± 0.5 (18.4 ± 0.5)	6.2 ± 2.0 (14.1 ± 2.3)	**7.1 ± 0.9 (15.1 ± 1.0)**	(3.3×10^{-1})	(2.7)
Inoculated Closed	n.d.	n.d.	25.4 ± 5.2 (30.0 ± 3.6)	7.9 ± 0.5 (16.3 ± 0.5)	9.5 ± 2.2 (17.8 ± 2.0)	**14.3 ± 3.2 (21.4 ± 2.5)**	(1.3×10^{-2})	(3.4)
Inoculated Closed (Pasteurised)	n.d.	9.7 ± 1.5 (18.0 ± 1.5)	12.2 ± 1.9 (20.4 ± 1.6)	51.3 ± 5.6 (45.8 ± 3.2)	14.3 ± 2.0 (22.1 ± 1.6)	**21.9 ± 5.3 (26.6 ± 3.5)**	(4.7×10^{-5})	(3.0)
Inoculated Closed (Filtered)	5.4 ± 2.1 (12.8 ± 3.0)	7.4 ± 0.9 (15.7 ± 1.1)	10.7 ± 1.0 (19.1 ± 1.0)	13.0 ± 1.3 (21.1 ± 1.1)	13.5 ± 0.8 (21.5 ± 0.7)	**10.0 ± 1.0 (18.0 ± 1.1)**	(1.3×10^{-2})	(2.2)
Mean (Mean of arcsine transformed data).	**4.9 ± 0.9 (11.5 ± 1.5)**	**5.9 ± 0.7 (13.3 ± 1.0)**	**12.1 ± 1.8 (19.4 ± 1.5)**	**13.9 ± 3.1 (20.3 ±2.2)**	**11.8 ± 1.2 (19.5 ±1.1)**	**10.2 ± 0.9 (17.3 ± 0.8)**	(2.1×10^{-4})	
P (ANOVA)	(5.0×10^{-1})	(2.6×10^{-1})	(2.4×10^{-4})	(9.0×10^{-9})	(6.0×10^{-3})	(3.8×10^{-5})		
σ_d	(5.3)	(3.5)	(3.3)	(2.5)	(3.1)			

* Means ± s.e.m. for three replicates, based on three random fields of view per replicate. Results in parentheses are arcsine transformed data.
n.d. Not done.

Table 4.6. Percentage of zoospores of *Py. aphanidermatum* that were motile after 2 h incubation in various irrigation solutions*.

Solution	Date of harvesting irrigation solution from large glasshouse. 1st July	15th July	30th July	9th September	24th October	Mean	P (ANOVA)	σ_d
Sterile distilled water	23.9 ± 4.9 (29.1 ± 3.3)	67.6 ± 4.8 (55.4 ± 3.0)	35.6 ± 5.7 (36.5 ± 3.5)	49.4 ± 3.7 (44.7 ± 2.1)	58.0 ± 4.1 (49.6 ± 2.4)	**46.9 ± 4.5** **(43.1 ± 2.7)**	(5.6×10^{-4})	(4.1)
Fresh Irrigation Solution	65.3 ± 6.7 (54.0 ± 4.0)	52.6 ± 2.6 (46.5 ± 1.5)	59.3 ± 3.8 (50.4 ± 2.2)	49.3 ± 3.9 (44.6 ± 2.2)	49.0 ± 8.2 (44.4 ± 4.8)	**55.1 ± 2.7** **(48.0 ± 1.6)**	(2.3×10^{-1})	(4.5)
Uninoculated Open	n.d.	57.6 ± 1.9 (49.4 ± 1.1)	41.9 ± 5.1 (40.3 ± 2.9)	41.8 ± 4.1 (40.3 ± 2.9)	42.5 ± 6.0 (40.6 ± 3.5)	**42.5 ± 4.3** **(42.6 ± 1.6)**	(1.0×10^{-1})	(3.7)
Uninoculated Closed	47.6 ± 2.3 (43.6 ± 1.3)	54.4 ± 3.0 (47.5 ± 1.7)	43.1 ± 3.8 (41.0 ± 2.2)	26.2 ± 6.5 (30.4 ± 4.4)	39.8 ±11.6 (38.6 ± 7.2)	**42.2 ± 3.5** **(40.2 ± 2.2)**	(1.1×10^{-1})	(5.7)
Inoculated Open	n.d.	58.1 ± 4.7 (49.7 ± 2.7)	58.7 ± 6.6 (50.1 ± 3.9)	43.1 ± 3.9 (41.0 ± 2.3)	46.2 ± 3.8 (42.8 ± 2.2)	**51.5 ± 3.0** **(45.9 ± 1.7)**	(1.2×10^{-1})	(4.1)
Inoculated Closed	n.d.	n.d.	51.1 ± 2.0 (45.6 ± 1.1)	42.8 ± 5.6 (40.8 ± 3.2)	37.2 ± 9.8 (37.3 ± 5.8)	**43.7 ± 3.9** **(41.3 ± 2.3)**	(3.7×10^{-1})	(5.5)
Inoculated Closed (Pasteurised)	n.d.	5.2 ± 1.1 (13.0 ± 1.5)	2.5 ± 1.1 (8.4 ± 2.5)	0.1 ± 0.1 (1.2 ± 1.2)	0.4 ± 0.0 (3.8 ± 0.1)	**2.1 ± 0.7** **(6.6 ± 1.5)**	(3.5×10^{-5})	(2.2)
Inoculated Closed (Filtered)	53.6 ± 2.7 (47.1 ± 1.6)	68.3 ± 2.8 (55.8 ± 1.7)	50.0 ± 4.4 (45.0 ± 2.5)	35.7 ± 5.5 (36.6 ± 3.3)	46.5 ± 0.3 (43.0 ± 0.2)	**50.8 ± 3.1** **(45.5 ± 1.9)**	(1.2×10^{-3})	(3.0)
Mean (Mean of arcsine transformed data).	**47.6 ± 4.9** **(43.5 ±3.0)**	**52.0 ± 4.6** **(45.3 ± 3.1)**	**42.8 ± 3.8** **(39.7 ±2.8)**	**36.1 ± 3.5** **(34.9 ± 2.9)**	**40.0 ± 3.9** **(37.5 ± 3.0)**	**42.6 ± 1.9** **(39.7 ± 1.4)**	(1.1×10^{-1})	
P (ANOVA)	(1.4×10^{-3})	(7.0×10^{-9})	(2.2×10^{-7})	(1.2×10^{-7})	(2.4×10^{-5})	(2.6×10^{-26})		
σ_d	(3.9)	(2.8)	(3.9)	(4.0)	(5.7)			

* Means ± s.e.m. for three replicates, based on three random fields of view per replicate. Results in parentheses are arcsine transformed data.
n.d. Not done.

Table 4.7. Percentage of encysted zoospores of *Py. aphanidermatum* that had germinated after 2 h incubation in various irrigation solutions*.

Solution	Date of harvesting irrigation solution from large glasshouse. 1st July	15th July	30th July	9th September	24th October	Mean	*P* (ANOVA)	σ_d
Sterile distilled water	31.4 ± 2.6 (34.1 ± 1.6)	18.8 ± 2.8 (25.6 ± 2.0)	37.5 ± 6.9 (37.6 ± 4.1)	16.8 ± 4.1 (23.9 ± 3.2)	22.1 ± 2.4 (28.0 ± 1.7)	**25.3 ± 2.6** **(29.8 ± 1.7)**	(2.2×10^{-2})	(3.8)
Fresh Irrigation Solution	27.1 ± 4.2 (31.3 ± 2.7)	20.9 ± 6.7 (26.5 ± 5.0)	16.5 ± 3.5 (23.7 ± 2.8)	7.6 ± 2.4 (15.5 ± 2.9)	28.6 ± 3.1 (32.2 ± 2.0)	**20.1 ± 2.6** **(25.8 ± 2.0)**	(2.8×10^{-2})	(4.6)
Uninoculated Open	n.d.	17.4 ± 5.9 (23.9 ± 4.8)	31.8 ± 3.1 (34.3 ± 1.9)	21.0 ± 1.2 (27.2 ± 0.8)	18.2 ± 6.1 (24.7 ± 4.4)	**22.3 ± 2.4** **(27.5 ± 1.9)**	(2.1×10^{-1})	(4.8)
Uninoculated Closed	31.2 ± 5.1 (33.8 ± 3.3)	28.2 ± 2.7 (32.0 ± 1.7)	36.6 ± 2.7 (37.2 ± 1.6)	23.4 ± 1.1 (28.9 ± 0.8)	31.2 ± 4.0 (33.9 ± 2.4)	**30.1 ± 1.7** **(33.2 ± 1.1)**	(1.7×10^{-1})	(3.0)
Inoculated Open	n.d.	45.2 ± 2.4 (42.2 ± 1.4)	34.4 ± 0.6 (35.9 ± 0.3)	26.2 ± 3.8 (30.7 ± 2.6)	38.6 ± 2.7 (38.4 ± 1.6)	**36.1 ± 2.4** **(36.8 ± 1.5)**	(7.3×10^{-3})	(2.4)
Inoculated Closed	n.d.	n.d.	12.5 ± 0.4 (20.7 ± 0.3)	34.8 ± 3.8 (36.1 ± 2.3)	53.1 ± 6.8 (46.8 ± 3.9)	**33.4 ± 6.3** **(34.5 ± 4.0)**	(1.3×10^{-3})	(3.8)
Inoculated Closed (Pasteurised)	n.d.	62.6 ± 3.7 (52.4 ± 2.2)	33.4 ± 1.7 (35.3 ± 1.0)	20.7 ± 1.3 (27.0 ± 0.9)	19.5 ± 2.6 (26.1 ± 1.8)	**34.0 ± 5.3** **(35.2 ± 3.2)**	(8.7×10^{-6})	(2.2)
Inoculated Closed (Filtered)	27.4 ± 3.8 (31.5 ± 2.5)	27.4 ± 10.7 (30.9 ± 6.7)	28.6 ± 5.2 (32.1 ± 3.4)	21.8 ± 3.5 (27.7 ± 2.4)	19.4 ± 4.1 (25.8 ± 3.1)	**24.9 ± 2.5** **(29.6 ± 1.6**	(7.6×10^{-1})	(5.6)
Mean (Mean of arcsine transformed data).	**28.9 ± 1.7** **(32.6 ± 1.2)**	**31.5 ± 3.9** **(33.4 ± 2.5)**	**28.9 ± 2.1** **(32.1 ± 1.4)**	**21.5 ± 1.7** **(27.1 ± 1.3)**	**28.8 ± 2.7** **(32.0 ± 1.7)**	**27.7 ± 1.2** **(31.3 ± 0.8)**	(8.0×10^{-2})	
P (ANOVA)	(8.0×10^{-1})	(1.4×10^{-3})	(4.2×10^{-4})	(6.0×10^{-4})	(4.9×10^{-4})	(2.6×10^{-3})		
σ_d	(3.6)	(5.5)	(3.3)	(3.1)	(4.0)			

* Means ± s.e.m. for three replicates, based on three random fields of view per replicate. Results in parentheses are arcsine transformed data.
n.d. Not done.

Table 4.8. Number of zoospores (zoospores.μl^{-1}) of *Ph. parasitica* released into various irrigation solutions from pre-formed sporangia*.

Solution	Date of harvesting irrigation solution from large glasshouse.					Mean	*P* (ANOVA)	σ_d
	1st July	15th July	30th July	9th September	24th October			
Sterile distilled water	13.2 ± 1.8	13.2 ± 2.1	16.5 ± 2.9	14.0 ± 3.5	16.9 ± 2.1	**14.7 ± 1.1**	7.4×10^{-1}	3.6
Fresh Irrigation Solution	25.7 ± 9.0	10.3 ± 1.7	30.7 ± 10.4	19.3 ± 2.4	7.4 ± 2.2	**18.7 ± 3.4**	1.1×10^{-1}	9.0
Uninoculated Open	18.9 ± 3.9	16.7 ± 2.5	15.6 ± 2.9	8.8 ± 1.4	12.3 ± 0.6	**14.5 ± 1.3**	1.2×10^{-1}	3.6
Uninoculated Closed	11.9 ± 1.4	12.6 ± 2.6	13.0 ± 0.9	7.6 ± 0.8	7.4 ± 2.3	**10.5 ± 0.9**	1.2×10^{-1}	2.5
Inoculated Open	n.d.	15.2 ± 1.8	16.7 ± 4.9	15.6 ± 2.4	8.2 ± 1.8	**13.9 ± 1.6**	2.6×10^{-1}	4.3
Inoculated Closed	13.2 ± 1.8	n.d.	3.7 ± 0.7	7.8 ± 2.5	3.3 ± 1.3	**7.0 ± 1.4**	1.1×10^{-2}	2.4
Inoculated Closed (Pasteurised)	n.d.	5.3 ± 2.4	6.8 ± 1.2	6.2 ± 0.6	7.0 ± 0.5	**6.3 ± 0.6**	8.4×10^{-1}	2.0
Inoculated Closed (Filtered)	14.8 ± 0.4	10.5 ± 2.2	6.2 ± 0.7	9.1 ± 1.5	6.0 ± 0.2	**9.3 ± 1.0**	3.0×10^{-3}	1.8
Mean	**16.3 ± 1.8**	**12.0 ± 1.0**	**13.6 ± 2.1**	**11.1 ± 1.1**	**8.6 ± 0.9**	**12.1 ± 0.7**	8.3×10^{-3}	
P (ANOVA)	2.5×10^{-1}	4.9×10^{-2}	1.2×10^{-2}	3.9×10^{-3}	6.6×10^{-4}	3.4×10^{-6}		
σ_d	5.9	3.1	6.2	3.0	2.2			

*Means ± s.e.m. for three replicates, based on four haemocytometer counts per replicate.
n.d. Not done.

Table 4.9. Percentage of vortex-encysted zoospores of *Py. aphanidermatum* that had germinated after 2 h incubation in various irrigation solutions*.

Solution	Date of harvesting irrigation solution from large glasshouse. 1st July	15th July	30th July	9th September	24th October	Mean	P (ANOVA)	σ_d
Sterile distilled water	33.0 ± 5.7 (34.9 ± 3.5)	27.1 ± 5.7 (31.1 ± 3.7)	39.9 ± 3.5 (33.1 ± 2.2)	31.7 ± 2.0 (34.2 ±1.3)	35.2 ± 2.9 (36.4 ± 1.7)	**31.4 ± 1.8 (33.9 ± 1.1)**	(7.1×10^{-1})	(3.8)
Fresh Irrigation Solution	64.6 ± 2.3 (53.5 ± 1.4)	42.8 ± 4.0 (40.8 ± 2.3)	43.0 ± 6.0 (40.9 ± 3.5)	45.3 ± 1.8 (42.3 ± 1.0)	39.7 ± 4.4 (39.0 ± 2.6)	**47.1 ± 2.8 (43.3 ± 1.6)**	(8.7×10^{-3})	(3.3)
Uninoculated Open	31.9 ± 2.4 (34.4 ± 1.5)	33.3 ± 2.8 (35.2 ± 1.7)	38.5 ± 3.1 (38.3 ± 1.8)	21.5 ± 3.5 (27.5 ± 2.5)	47.6 ± 7.5 (43.6 ± 4.4)	**34.6 ± 2.8 (35.8 ± 1.7)**	(1.6×10^{-2})	(3.7)
Uninoculated Closed	53.4 ± 3.3 (46.9 ± 1.9)	40.8 ± 3.2 (39.7 ± 1.9)	31.6 ± 2.6 (34.2 ± 1.6)	43.5 ± 8.5 (41.2 ± 5.0)	34.0 ± 1.6 (35.7 ± 1.0)	**40.6 ± 2.7 (39.5 ± 1.6)**	(4.6×10^{-2})	(3.8)
Inoculated Open	n.d.	25.1 ± 4.7 (29.8 ± 3.2)	17.4 ± 0.8 (24.7 ± 0.6)	27.2 ± 2.8 (31.4 ± 1.8)	39.4 ± 0.5 (38.9 ± 0.3)	**27.3 ± 2.7 (31.2 ± 1.7)**	(4.8×10^{-3})	(2.7)
Inoculated Closed	36.4 ± 3.8 (37.0 ± 2.3)	n.d.	31.0 ± 7.5 (33.5 ± 4.7)	25.7 ± 0.9 (30.5 ± 0.6)	45.0 ± 2.8 (42.1 ± 1.6)	**34.5 ± 2.9 (35.8 ± 1.8)**	(7.8×10^{-2})	(3.9)
Inoculated Closed (Pasteurised)	49.2 ± 2.4 (44.5 ± 1.4)	52.9 ± 2.9 (46.7 ± 1.7)	72.4 ± 3.0 (58.3 ± 2.0)	74.3 ± 2.0 (59.6 ± 1.3)	60.2 ± 5.3 (51.0 ± 3.1)	**61.8 ± 3.0 (52.0 ± 1.8)**	(9.8×10^{-4})	(2.8)
Inoculated Closed (Filtered)	48.8 ± 1.8 (44.3 ± 1.0)	33.1 ± 1.3 (35.1 ± 0.8)	21.5 ± 1.1 (27.6 ± 0.8)	42.2 ± 3.6 (40.5 ± 2.1)	26.4 ± 1.4 (30.9 ± 0.9)	**34.4 ± 2.8 (35.7 ± 1.7)**	(1.5×10^{-5})	(1.8)
Mean (Mean of arcsine transformed data).	**45.3 ± 2.7 (42.2 ± 1.6)**	**36.4 ± 2.3 (36.9 ± 1.4)**	**35.7 ± 3.5 (36.3 ± 2.1)**	**38.9 ± 3.5 (38.4 ± 2.1)**	**40.9 ± 2.3 (39.7 ± 1.4)**	**39.4 ± 1.3 (38.7 ± 0.8)**	(1.6×10^{-1})	
P (ANOVA)	(6.5×10^{-5})	(2.6×10^{-3})	(2.5×10^{-6})	(1.2×10^{-6})	(7.8×10^{-4})	(1.8×10^{-14})		
σ_d	(2.8)	(3.4)	(3.5)	(3.3)	(3.3)			

* Means ± s.e.m. for three replicates, based on counts of 100 spores per replicate. Results in parentheses are arcsine transformed data.
n.d. Not done.

Table 4.10. Subsequent zoospore release (Zoospores.μl^{-1}) into SDW from sporangia of *Ph. parasitica* that had formed in various irrigation solutions*.

Solution	Date of harvesting irrigation solution from large glasshouse.					Mean	*P* (ANOVA)	σ_d
	1st July	15th July	30th July	9th September	24th October			
Sterile distilled water	14.5 ± 1.4	16.8 ± 4.2	21.4 ± 1.3	21.4 ± 4.5	18.7 ± 2.8	**18.6 ± 1.4**	5.0×10^{-1}	4.5
Fresh Irrigation Solution	139.6 ± 15.0	79.5 ± 9.6	137.9 ± 16.2	60.1 ± 10.5	33.7 ± 2.9	**90.2 ± 12.1**	2.4×10^{-4}	16.7
Uninoculated Open	88.2 ± 13.2	83.2 ± 8.2	58.2 ± 13.1	135.3 ± 37.4	64.0 ± 8.6	**85.8 ± 10.3**	1.1×10^{-1}	27.5
Uninoculated Closed	117.8 ± 23.8	131.6 ± 41.7	93.5 ± 14.2	71.4 ± 21.6	69.3 ± 8.3	**96.7 ± 11.4**	3.5×10^{-1}	34.9
Inoculated Open	n.d.	81.8 ± 3.1	93.7 ± 22.5	90.2 ± 5.9	88.0 ± 21.4	**88.4 ± 6.9**	9.6×10^{-1}	22.4
Inoculated Closed	n.d.	n.d.	88.7 ± 8.6	75.6 ± 16.0	58.2 ± 8.6	**74.2 ± 7.3**	2.5×10^{-1}	16.4
Inoculated Closed (Pasteurised)	n.d.	57.1 ± 20.2	27.3 ± 1.2	25.7 ± 0.9	35.7 ± 17.7	**36.4 ± 6.9**	3.9×10^{-1}	19.1
Inoculated Closed (Filtered)	85.3 ± 13.6	83.2 ± 26.8	38.6 ± 11.8	82.6 ± 4.6	20.3 ± 4.3	**62.0 ± 9.2**	2.8×10^{-2}	20.8
Mean	**89.1 ± 12.7**	**76.2 ± 9.7**	**69.9 ± 8.8**	**70.3 ± 8.7**	**48.5 ± 5.9**	**69.1 ± 4.1**	4.6×10^{-2}	
P (ANOVA)	1.8×10^{-3}	5.8×10^{-2}	1.1×10^{-4}	5.5×10^{-3}	4.0×10^{-3}	3.1×10^{-9}		
σ_d	21.5	29.6	18.4	24.0	16.0			

* Means ± s.e.m. for three replicates, based on four haemocytometer counts per replicate.
n.d. Not done.

Table 4.11. The density of sporangia (sporangia per video monitor screen) of *Ph. parasitica* after incubation in various irrigation solutions*.

Solution	Date of harvesting irrigation solution from large glasshouse.					Mean	*P* (ANOVA)	σ_d
	1st July	15th July	30th July	9th September	24th October			
Sterile distilled water	53.7 ± 30.7	44.0 ± 6.4	60.0 ± 4.4	43.9 ± 8.6	41.2 ± 3.6	**48.6 ± 5.9**	8.8×10^{-1}	20.9
Fresh Irrigation Solution	45.5 ± 14.4	31.0 ± 5.3	49.5 ± 7.8	32.7 ± 6.7	16.4 ± 6.2	**35.0 ± 4.5**	1.3×10^{-1}	12.3
Uninoculated Open	31.4 ± 12.3	32.4 ± 1.4	5.8 ± 2.6	47.4 ± 15.0	24.5 ± 4.4	**28.3 ± 5.0**	9.4×10^{-2}	12.9
Uninoculated Closed	44.8 ± 6.8	53.6 ± 5.9	37.5 ± 10.0	27.7 ± 8.4	32.4 ± 11.1	**39.2 ± 4.1**	3.0×10^{-1}	12.3
Inoculated Open	n.d.	28.0 ± 11.4	25.5 ± 5.0	22.3 ± 6.9	35.2 ± 4.4	**27.8 ± 3.5**	6.7×10^{-1}	10.6
Inoculated Closed	n.d.	n.d.	41.5 ± 3.8	50.4 ± 15.9	28.0 ± 2.9	**40.0 ± 5.8**	3.2×10^{-1}	13.6
Inoculated Closed (Pasteurised)	n.d.	31.6 ± 12.1	27.7 ± 5.0	21.2 ± 7.7	31.7 ± 11.7	**28.1 ± 4.3**	8.5×10^{-1}	13.6
Inoculated Closed (Filtered)	20.3 ± 4.4	16.8 ± 2.6	29.0 ± 8.8	24.1 ± 8.1	10.2 ± 1.0	**20.1 ± 2.8**	2.7×10^{-1}	8.2
Mean	**39.1 ± 7.0**	**33.9 ± 3.4**	**34.6 ± 3.7**	**33.7 ± 3.8**	**27.4 ± 2.8**	**33.3 ± 1.8**	4.1×10^{-1}	
P (ANOVA)	6.5×10^{-1}	7.9×10^{-2}	8.3×10^{-4}	2.9×10^{-1}	8.1×10^{-2}	4.8×10^{-4}		
σ_d	23.3	10.8	9.1	14.5	9.5			

* Means ± s.e.m. for three replicates, based on three fields of view per replicate.
n.d. Not done.

Table 4.12. Percentage of sporangia of *Ph. parasitica* that had discharged contents after incubation in various irrigation solutions*.

Solution	Date of harvesting irrigation solution from large glasshouse. 1st July	15th July	30th July	9th September	24th October	Mean	*P* (ANOVA)	σ_d
Sterile distilled water	33.2 ± 12.2 (34.7 ± 7.3)	14.9 ± 3.3 (22.4 ± 2.8)	23.4 ± 4.5 (28.7 ± 3.1)	20.9 ± 3.0 (27.1 ± 2.1)	21.0 ± 1.8 (27.2 ± 1.3)	**22.7 ± 2.8** **(28.0 ± 1.8)**	(3.5×10^{-1})	(5.6)
Fresh Irrigation Solution	6.8 ± 1.8 (14.9 ± 2.0)	10.6 ± 0.6 (19.0 ± 0.6)	7.8 ± 2.0 (15.9 ± 2.3)	22.4 ± 5.8 (27.9 ± 3.9)	21.4 ± 4.6 (27.3 ± 3.2)	**13.8 ± 2.2** **(21.0 ± 1.8)**	(1.3×10^{-2})	(3.8)
Uninoculated Open	6.1 ± 1.6 (14.0 ± 1.8)	19.2 ± 8.5 (27.5 ± 8.7)	1.0 ± 1.0 (3.3 ± 3.3)	4.1 ± 1.2 (11.4 ± 1.9)	13.9 ± 1.2 (21.8 ± 1.0)	**8.9 ± 2.3** **(15.6 ± 2.8)**	(2.3×10^{-2})	(6.2)
Uninoculated Closed	7.7 ± 1.8 (15.9 ± 1.9)	6.1 ± 2.2 (13.8 ± 2.6)	10.4 ± 2.9 (18.5 ± 2.9)	4.5 ± 1.8 (11.7 ± 2.5)	12.3 ± 3.5 (20.1 ± 3.2)	**8.2 ± 1.2** **(16.0 ± 1.3)**	(2.4×10^{-1})	(3.7)
Inoculated Open	n.d.	5.5 ± 0.3 (13.5 ± 0.4)	19.6 ± 10.7 (24.8 ± 7.6)	16.5 ± 3.0 (23.8 ± 2.2)	3.9 ± 1.0 (11.2 ± 1.4)	**11.4 ± 3.1** **(18.3 ± 2.5)**	(9.5×10^{-2})	(5.7)
Inoculated Closed	n.d.	n.d.	5.8 ± 0.5 (13.9 ± 0.6)	8.3 ± 3.2 (16.2 ± 3.2)	25.9 ± 5.5 (30.4 ± 3.6)	**13.3 ± 3.7** **(20.2 ± 2.9)**	(1.2×10^{-2})	(4.0)
Inoculated Closed (Pasteurised)	n.d.	20.3 ± 1.9 (26.7 ± 1.3)	16.4 ± 0.7 (23.9 0.5)	26.6 ± 4.0 (30.9 ± 2.6)	15.3 ± 2.4 (22.9 ± 1.9)	**19.6 ± 1.7** **(26.1 ± 1.2)**	(4.6×10^{-2})	(2.5)
Inoculated Closed (Filtered)	12.5 ± 5.3 (19.8 ± 4.6)	12.4 ± 5.0 (19.8 ± 4.4)	8.1 ± 4.4 (13.5 ± 6.9)	8.6 ± 2.9 (16.6 ± 2.9)	8.6 ± 2.0 (16.8 ± 2.2)	**10.0 ± 1.6** **(17.3 ± 1.8)**	(8.4×10^{-1})	(6.4)
Mean (Mean of arcsine transformed data).	**13.3 ± 3.6** **(19.9 ± 2.6)**	**12.7 ± 1.8** **(20.4 ± 1.7)**	**11.6 ± 2.0** **(17.8 ± 2.0)**	**14.0 ± 2.0** **(20.7 ± 1.7)**	**15.3 ± 1.7** **(22.2 ± 1.4)**	**13.4 ± 0.9** **(20.2 ± 0.8)**	(5.1×10^{-1})	
P (ANOVA)	(2.9×10^{-2})	(1.4×10^{-1})	(1.5×10^{-2})	(3.6×10^{-4})	(7.9×10^{-4})	(3.9×10^{-5})		
σ_d	(5.9)	(5.7)	(5.9)	(3.9)	(3.4)			

* Means ± s.e.m. for three replicates, based on three random fields of view per replicate. Results in parentheses are arcsine transformed data.
n.d. Not done.

4.5. The effect of pH on zoospore motility and subsequent cyst germination.

4.5.1. Modification of irrigation solution pH.

In order to test the effect of irrigation solution pH on zoospore motility the October sample of the inoculated closed (pasteurised) irrigation solution with a pH of 4.3 (the lowest of all samples in the trial) was increased to pH 7.0 with NaOH. An equivalent amount of NaCl was added to a second portion of the irrigation solution to act as a control. Zoospores of *Py. aphanidermatum* were introduced to both the test and control solutions with the aim of later determining the proportion of zoospores motile after the incubation period. However, a white precipitate immediately formed in the control upon the addition of NaCl, and the precipitate rapidly immobilised the motile zoospores. It was hoped that the effect of neutralising the irrigation solution and comparing the level of zoospore motility with the control would have been ascertained by this experiment. A modified protocol lacking the NaCl supplement could not be performed because of the lack of suitable irrigation solution.

4.5.2. Zoospore motility and subsequent cyst germination in solutions of various pH.

In three experiments (summarised in Table 4.13) the effect of HNO_3 and $Ca(NO_3)_2$ concentration on the motility and subsequent germination of encysted zoospores of *Py. aphanidermatum* was determined. The data for the three experiments were pooled and trend lines were fitted to the data (Table 4.14). The expected pH of the nitric acid treatment was based on pH $\equiv - \log_{10}[H_3O^+]$. Both the observed pH of $Ca(NO_3)_2$ and the proportion of cysts germinating in nitric acid were unaffected by the concentration of $Ca(NO_3)_2$ and acid, respectively. Therefore, the fitted lines for these sets of data were the arithmetic means of the data set. A suitable line of best fit was not found for the observed pH in the nitric acid and consequently the mean pH at each concentration of nitric acid was used. For the other three lines (zoospore motility with nitric acid, zoospore motility with $Ca(NO_3)_2$ and cyst germination with $Ca(NO_3)_2$) the lines of best fit were all very highly significant ($F < 7.47 \times 10^{-4}$). The line of best fit for the proportion of zoospores motile in nitric acid treatment was one of exponential decay, whereas there was a sigmoid relationship between $Ca(NO_3)_2$ concentration and proportion of zoospores motile. The proportion of cysts germinating in

$Ca(NO_3)_2$ increased with $Ca(NO_3)_2$ concentration up to 16 mM, but this was followed by a very highly significant (P = 1.1 × 10^{-4}) decrease in germination with 32 mM $Ca(NO_3)_2$. Therefore, a quadratic function ($y = a + bx + cx^2$) was used to describe the line of best fit.

Table 4.13. Concentrations of HNO_3 and $Ca(NO_3)_2$ for the experiments on the effects of HNO_3 and $Ca(NO_3)_2$ concentration on zoospore motility and subsequent cyst germination.

Experiment	HNO_3 concentrations	$Ca(NO_3)_2$ concentrations	n
I	0, 4, 8,16 and 32 mM	0, 4, 8,16 and 32 mM	3
II	0, 0.05, 0.1, 0.25, 0.5, 1, 2 and 5 mM	0, 100, 250, 1000 and 5000 μM	2
III	0, 1, 2, 5, 10, 25, 50, 100 and 250 μM	n.d.	2

n.d. Not done.

Although the effects of HNO_3 and $Ca(NO_3)_2$ on zoospore motility and cyst germination were tested simultaneously the results are displayed separately (Figures 4.8 and 4.9). The proportion of motile zoospores and the proportion of cyst germination was constant in $Ca(NO_3)_2$ up to ~100 μM. From ~100 μM to 16 mM the proportion of zoospores motile declined while the proportion of cysts germinating increased. In nitric acid the proportion of cysts germinating was consistently low (1.5%) in all concentrations of acid up to 32 mM. The proportion of zoospores motile in 0 mM nitric acid was 20%, but the value decayed exponentially to ~0% in 250 μM nitric acid. An observed pH of ~7 was recorded in all treatments of nitric acid up to a 50 μM, then from 50 μM to 500 μM the observed pH tended towards the expected theoretical pH. Between 500 μM and 32 mM nitric acid the observed and expected pH values were similar.

Figures 4.8 and 4.9. The effect of increasing $Ca(NO_3)_2$ and nitric acid concentration on zoospore motility and subsequent zoospore release*.

Figure 4.8. Nitric acid

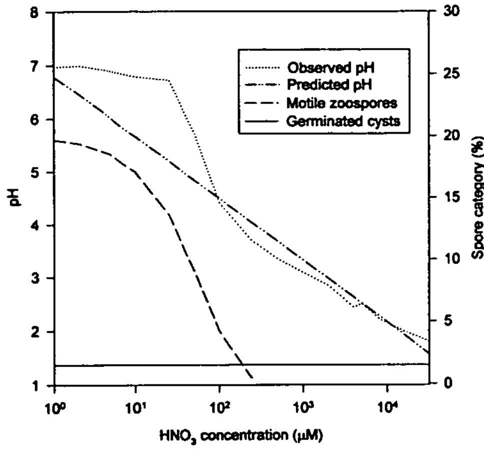
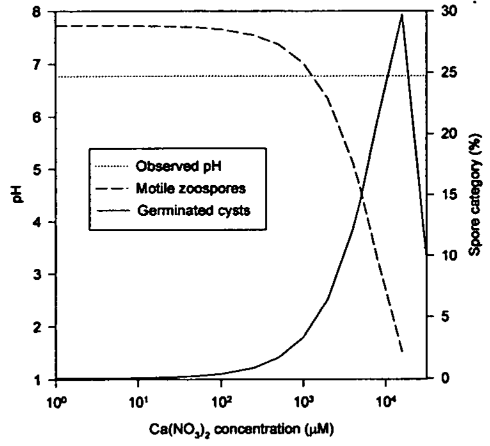


Figure 4.9. $Ca(NO_3)_2$



* All lines derived from relationships described in Table 4.14.

Table 4.14. The data transformation method, formula of relationship and correlation statistics for lines of best fit between either $Ca(NO_3)_2$ or HNO_3 concentration and zoospore motility, cyst germination, expected pH and observed pH*.

Line	Line type	Transformation method	Formula of fitted line $x=[H^+ \text{ or } Ca^{2+}]$ μM	Correlation between transformed line and concentration	Correlation between observed and fitted	n
Expected pH (HNO_3)	Inverse log	NA	$= -\log_{10}[10^{-6}x+10^{-7}]^{\dagger}$	n.a.	100.0% $F = 10^{-\infty}$	49
Observed pH (HNO_3)	Linear	None	n.a.	n.a.	99.4% $F = 9.61 \times 10^{-21}$	22
Observed pH ($Ca(NO_3)_2$)	Linear	Mean of data	$= 6.77 \pm 0.04$	n.a.	n.a.	10
Motile (HNO_3)	Exponential decay	$\log_{10}(x+0.01)$ [a]	$= 0.200 * 10^{-0.00675x}$	79.0% $F = 9.63 \times 10^{-5}$	85.9% $F = 5.08 \times 10^{-6}$	18
Motile ($Ca(NO_3)_2$)	Sigmoid	Probit[b]	n.a.	86.2% $F = 2.51 \times 10^{-7}$	66.4% $F = 7.47 \times 10^{-4}$	22
Germ (HNO_3)	Linear	Mean of data	$= 0.015 \pm 0.003$	n.a.	n.a.	49
Germ ($Ca(NO_3)_2$)	Quadratic	Excel Solver function	$= 3.32 \times 10^{-5} + 3.41 \times 10^{-5}x - 9.70 \times 10^{-10}x^2$	n.a.	89.7% $F = 1.33 \times 10^{-9}$	25

* Data for line fitting are from experiments I, II and III (Table 4.13).

† The addition of 10^{-7} is required for when x = 0 because there is no $\log_{10}$ for zero.

[a] Only data from experiment III was used because no motile zoospores were observed in the majority of treatments in experiments I and II.

[b] All data except when values are zero (that is, 32 mM $Ca(NO_3)_2$) were used. Zeros can not be probit transformed. The excel worksheet function NORMINV (x, μ = 5, σ = 1) was used to convert percentages into probits

[c] Excel Regression analysis tool used to predict probit values, these were then back transformed into percentages to give the fitted line. The conversion of probits into percentages was done by the Excel worksheet function NORMDIST (x, μ = 5, σ = 1, cumulative = TRUE) × 100.

n.a. - Not appropriate.

4.6. Correlations between characteristics of irrigation samples.

By combining results from Edinburgh and Stockbridge House up to 16 characteristics can be attributed to each irrigation sample (Table 4.15). These variables include the pH of the sample, the effects on sporangiogenesis, and the discolouration of roots of the plants grown in the irrigation samples. In total there were 35 irrigation samples (seven samples per month for five months).

Table 4.15. Summary of characteristics associated with each irrigation sample.

	Characteristics associated with irrigation samples	Location of characteristic determination.
	Physical chemistry of solution	
1.	Solution pH	Edinburgh
	Effects on zoospore motility and subsequent cyst germination.	
2.	Motility of zoospores of *Ph. parasitica.*	Edinburgh
3.	Subsequent germination of cysts of *Ph. parasitica.*	Edinburgh
4.	Motility of zoospores of *Py. aphanidermatum.*	Edinburgh
5.	Subsequent germination of cysts of *Py. aphanidermatum.*	Edinburgh
	Effect on zoospore release from pre-formed sporangia.	
6.	Release of zoospores from pre-formed sporangia of *Ph. parasitica.*	Edinburgh
	Effect on germination of vortex encysted zoospores.	
7.	Germination of vortex encysted spores of *Py. aphanidermatum.*	Edinburgh
	Effects on zoosporangiogenesis of *Ph. parasitica.*	
8.	Zoospore release from sporangia.	Edinburgh
9.	Sporangial density.	Edinburgh
10.	Fraction of sporangia that had discharged.	Edinburgh
	Number of *Ph. cryptogea* propagules in mini-NFT system.	
11.	Determined by membrane method.	Stockbridge House
12.	Determined by dip-stick method.	Stockbridge House
	Indicators of infection in mini-NFT systems.	
13.	Incidence of infection per 1cm of root.	Stockbridge House
14.	Index of root development.	Stockbridge House
15.	Index of root decolouration.	Stockbridge House
16.	Lesions per unit area of root.	Stockbridge House

The sixteen characteristics were all correlated against each other for all combinations (Table 4.16). For all correlation values greater than 50% the significance of regression value was also determined (Table 4.18). A square-root transformation was used on *Ph. cryptogea* (membrane method) propagule number because of the high variance within the data (Appendix 1). None of the other data sets was transformed.

Additional agronomic data were available from Stockbridge House for the October irrigation samples. These additional characteristics were plant height, stem diameter, leaf area, leaf length and root weight (Appendix 7). This increased the maximum number of characteristics per irrigation sample to 21. A separate analysis of the October solutions was carried out (Table 4.17). Significance of correlation was only determined for values of R greater than 75% for correlations between a characteristic determined in Edinburgh and one of the additional agronomic characteristics (Table 4.19).

Table 4.16. Correlation values for correlations between characteristics for all irrigation samples.

	Maximum observations	Irrigation solution pH	*Ph. para.* %motile	*Ph. para* %germ (motility exp.)	*Py. aph* %motile	*Py. aph* %germ (motility exp.)	*Ph.para.* zoospore release	*Py. aph* vortex-encysted %germ.	*Ph. para* % sporangial discharge	*Ph. para* sporangial density	*Ph. para* subsequent zoospore release	*Ph.crypt* propagules (membrane)*	*Ph.crypt* propagules (dip-stick)	Infection per 1cm root	Root development index	Root discoloration index	Lesion no/unit area
Maximum observations		28	31	31	31	31	31	31	31	31	31	27	29	29	29	29	29
Irrigation solution pH	28	100%															
Ph. parasitica. %motile	31	3%	100%														
Ph. parasitica %germinated (motility exp.)	31	**-53%**	-17%	100%													
Py. aphanidermatum %motile	31	**71%**	27%	**-52%**	100%												
Py. aphanidermatum %germ. (motility exp.)	31	-20%	16%	-11%	-20%	100%											
Ph. parasitica zoospore release	31	49%	-3%	-46%	**52%**	-27%	100%										
Py. aphanidermatum vortex-encysted %germ.	31	-49%	2%	30%	**-58%**	-1%	-1%	100%									
Ph. parasitica % sporangial discharge	31	-47%	20%	36%	-36%	12%	-18%	34%	100%								
Ph. parasitica sporangial density	31	10%	-12%	-20%	15%	-3%	23%	0%	-27%	100%							
Ph. parasitica subsequent zoospore release	31	**52%**	7%	-45%	57%	-7%	**59%**	-21%	-47%	**68%**	100%						
Ph.cryptogea propagules (membrane)*	27	-10%	1%	10%	-39%	8%	-39%	-7%	9%	-28%	-27%	100%					
Ph.cryptogea propagules (dip-stick)	29	10%	-5%	-2%	1%	30%	-13%	-31%	15%	-18%	-5%	18%	100%				
Infection per 1cm root	29	-12%	-9%	12%	-29%	8%	**-50%**	-31%	-7%	-16%	-22%	**51%**	28%	100%			
Root development index	29	17%	0%	-11%	28%	-13%	32%	5%	-11%	13%	26%	**-62%**	-41%	**-61%**	100%		
Root discoloration index	29	-19%	11%	13%	-24%	30%	**-50%**	-13%	6%	-25%	-32%	**59%**	44%	**68%**	**-88%**	100%	
Lesion no/unit area	29	-18%	32%	1%	-20%	32%	-37%	7%	23%	-20%	-29%	**60%**	34%	**51%**	**-82%**	**90%**	100%

Highlighted values are $|x| \geq 50\%$. ($P \leq 0.01$).

* Square root transformed.

Table 4.17. Correlation values for correlations between characteristics for October irrigation samples only.

	Maximum observations	Irrigation solution pH	*Ph. para.* %motile	*Ph. para* %germ (motility exp.)	*Py. aph* %motile	*Py. aph* %germ (motility exp.)	*Ph. para.* zoospore release	*Py. aph* vortex-encysted %germ.	*Ph. para* % sporangial discharge	*Ph. para* sporangial density	*Ph. para* subsequent zoospore release	*Ph.crypt* propagules (membrane)*	*Ph.crypt* propagules (dip-stick)	Infection per 1cm root	Root development index	Root discoloration index	Lesion no/unit area	Root dry weight	Plant height	Stem diameter	Leaf area	Leaf length
Maximum observations		7	7	7	7	7	7	7	7	7	7	6	7	7	7	7	7	7	7	7	7	7
Irrigation solution pH	7	100%																				
Ph. parasitica. %motile	7	-4%	100%																			
Ph. parasitica %germinated (motility exp.)	7	-18%	-32%	100%																		
Py. aphanidermatum %motile	7	**87%**	5%	-8%	100%																	
Py. aphanidermatum %germ. (motility exp.)	7	10%	**85%**	-41%	23%	100%																
Ph. parasitica zoospore release	7	29%	-66%	-5%	13%	-62%	100%															
Py. aphanidermatum vortex-encysted %germ.	7	**-76%**	11%	-11%	**-79%**	-6%	16%	100%														
Ph. parasitica % sporangial discharge	7	-31%	69%	34%	-14%	39%	-42%	35%	100%													
Ph. parasitica sporangial density	7	-21%	13%	-43%	-41%	39%	8%	50%	-14%	100%												
Ph. parasitica subsequent zoospore release	7	38%	14%	-61%	22%	48%	30%	6%	-29%	**78%**	100%											
Ph.cryptogea propagules (membrane)*	6	49%	28%	**-86%**	28%	-5%	34%	1%	-4%	6%	38%	100%										
Ph.cryptogea propagules (dip-stick)	7	46%	-1%	9%	15%	25%	-7%	-38%	-20%	43%	49%	-11%	100%									
Infection per 1cm root	7	2%	10%	-13%	-41%	-23%	20%	34%	13%	17%	2%	67%	22%	100%								
Root development index	7	-30%	-16%	69%	9%	-20%	-35%	-25%	24%	-67%	**-76%**	-69%	-41%	-63%	100%							
Root discoloration index	7	38%	35%	**-76%**	2%	29%	11%	6%	-18%	43%	59%	**81%**	34%	66%	**-92%**	100%						
Lesion no/unit area	7	29%	52%	-71%	3%	38%	15%	26%	14%	39%	56%	**86%**	12%	63%	**-86%**	**91%**	100%					
Root dry weight	7	-23%	-15%	67%	18%	-15%	-31%	-29%	23%	-65%	-69%	-68%	-38%	-70%	**99%**	**-93%**	**-86%**	100%				
Plant height	7	-25%	-8%	62%	13%	-18%	-40%	-29%	27%	**-76%**	**-81%**	-57%	-47%	-57%	**98%**	**-84%**	**-78%**	**97%**	100%			
Stem diameter	7	16%	-42%	73%	43%	-43%	6%	-52%	3%	**-80%**	-63%	-44%	-22%	-51%	**84%**	**-80%**	**-77%**	**86%**	**83%**	100%		
Leaf area	7	-6%	-15%	61%	30%	-20%	-33%	-47%	14%	**-80%**	-75%	-52%	-36%	-62%	**96%**	**-82%**	**-80%**	**96%**	**98%**	**90%**	100%	
Leaf length	7	-13%	-21%	49%	24%	-23%	-32%	-43%	2%	**-77%**	-74%	-50%	-45%	-66%	**95%**	**-80%**	**-81%**	**94%**	**96%**	**84%**	**98%**	100%

Values high-lighted are significant correlations ($P < 0.05$).

* Square root transformed.

Table 4.18. Correlation values (*R*) and corresponding values of significance (*F*) for the major correlations between characteristics of irrigation samples based on data from all months.

Pair of characteristics	*R*	*F*	n
Pp %germ (motility. exp.) & Irrigation solution pH	-53%	3.4×10^{-3}	28
Pa %motile & Irrigation solution pH	71%	2.0×10^{-5}	28
Pa %motile & *Pp* %germ (motility exp.)	-52%	2.8×10^{-3}	31
Pa zoospore release & *Pa* %motile	52%	2.4×10^{-3}	31
Pa vortex-encysted %germ. & *Pa* %motile	-58%	5.5×10^{-4}	31
Pp subsequent zoospore release & Irrigation solution pH	52%	4.7×10^{-3}	28
Pp subsequent zoospore release & *Pa* %motile	57%	7.7×10^{-4}	31
Pp subsequent zoospore release & *Pa* zoospore release	59%	4.4×10^{-4}	31
Pp subsequent zoospore release & *Pp* sporangial density	68%	2.4×10^{-5}	31
Infection per 1cm root & *Pa* zoospore release	-50%	1.0×10^{-2}	26
Infection per 1cm root & *Pc* propagules (membrane)	51%	6.0×10^{-3}	27
Root development index & *Pc* propagules (membrane)	-62%	6.1×10^{-4}	27
Root development index & Infection per 1cm root	-61%	4.0×10^{-4}	29
Root discolouration index & *Pa* zoospore release	-50%	9.0×10^{-3}	26
Root discolouration index & *Pc* propagules (membrane)	59%	1.1×10^{-3}	27
Root discolouration index & Infection per 1cm root	68%	5.8×10^{-5}	29
Root discolouration index & Root development index	-88%	4.0×10^{-10}	29
Lesion no/unit area & *Pc* propagules (membrane)	60%	9.1×10^{-4}	27
Lesion no/unit area & Infection per 1cm root	51%	4.8×10^{-3}	29
Lesion no/unit area & Root development index	-88%	4.0×10^{-10}	29
Lesion no/unit area & Root discolouration index	90%	1.8×10^{-11}	29

Pa *Py. aphanidermatum.*

Pp *Ph. parasitica.*

Pc *Ph. cryptogea.*

There was significant positive correlation (R = 0.71, F = 2.0 × 10^{-5}) between the irrigation solution pH and the proportion of zoospores of *Py. aphanidermatum* that remained motile after the 2h incubation. However, there was negligible (R = 0.03) correlation between irrigation solution pH and the comparable experiment using zoospores of *Ph. parasitica.* In all three experiments that measured the proportion of cysts germinating there was negative correlation with irrigation solution pH. However, only the germination of spontaneously encysted zoospores in the motility experiment using zoospores of *Ph. parasitica* had an R value greater than 50%. The only other characteristic for which the pH of irrigation solution had a significant correlation (R = 0.52, F = 4.7 × 10^{-3}) was the level of subsequent zoospore release in the zoosporangiogenesis experiment.

Generally, an irrigation solution that suppressed zoospore motility increased the level of cyst germination. There was a very highly significant correlation (R = 0.59, F = 4.7 × 10^{-4}) between zoospore release from pre-formed sporangia of *Ph. parasitica* and zoospore release from sporangia of *Ph. parasitica* that had formed in the irrigation solution. Similarly there was a very highly significant correlation (R = 0.68, F = 2.4 × 10^{-5}) between sporangial density and subsequent zoospore release from those sporangia. However, there was negative correlation (R = -0.47) between the proportion of sporangia liberating their contents and the number of zoospores liberated from those sporangia. That is, overall, from mycelial mats with dense sporangial populations the number of zoospores released per sporangium was less than from mats with a low density of sporangia.

There was a consistency in the correlation analysis of the Stockbridge House data. Very significant positive correlations ($R \geq 0.51$, $F \leq 6.0 \times 10^{-3}$) were found between the following characteristics: infections per unit length of root; lesions per unit area of root; root discoloration and the number of *Ph. cryptogea* propagules (membrane method). These four characteristics were all very significantly negatively correlated ($R \leq -0.61$, $F \leq 6.1 \times 10^{-4}$) with the root development index.

There were only two very significant correlations between the characteristics determined in Edinburgh and those determined at Stockbridge House. Both correlations (R = -0.50, $F \leq 0.01$) were negative and concerned zoospore release from pre-formed sporangia of *Ph. parasitica.*

These data correlated with the Stockbridge House determined characteristics of 'infections per 1 cm root' and 'root discolouration index'.

Table 4.19. Correlation values (*R*) and corresponding significance (*F*) for the major correlations between characteristics determined in Edinburgh and the additional agronomic factors for the October irrigation samples.

Pair of characteristics	*R*	*F*	n
Plant height & Sporangial density	-76%	4.7×10^{-2}	7
Plant height & Zoospore release (sporangiogenesis)	-81%	2.6×10^{-2}	7
Stem diameter & Sporangial density	-80%	3.0×10^{-2}	7
Leaf area & Sporangial density	-80%	2.9×10^{-2}	7
Leaf length & Sporangial density	-77%	4.3×10^{-2}	7

In the correlation analysis incorporating the additional agronomic data for October, five significant correlations were observed between these agronomic characteristics and Edinburgh determined characteristics. Sporangial density was negatively correlated ($R \leq -0.76$, $F \leq 0.047$) with plant height, stem diameter, leaf area and leaf length. The fifth correlation ($R = -0.81$, $F = 0.026$) was between plant height and zoospore release from sporangia of *Ph. parasitica* that had formed in the irrigation samples.

4.7. Discussion.

When the sampling of irrigation solutions from the Stockbridge House experiments was begun in 1997, for analysis of the effects of these solutions on zoospore producing fungi, it was expected that a build-up of disease, and of disease suppression, would occur in the glasshouse during the growing season. Then, in the experiments carried out at Edinburgh, it was hoped to determine which aspects of fungal biology were affected by the factors in irrigation solutions associated with disease conduciveness and suppressiveness. Unfortunately, there was very little disease development in the glasshouse trial at Stockbridge House in 1997, unlike 1996 when clear trends of disease progression and major differences between treatments (open versus closed) were observed. Even though the tomato plants in the treatments were artificially inoculated with a proven pathogenic strain of *Ph. cryptogea*, symptoms of the disease did not become apparent in the aerial portion of the crop until September, although the crop was subjected to stressful conditions (high temperatures and high incidence of solar radiation) during August.

Therefore, the principal objectives of the experimental work at Edinburgh could not be achieved. Nevertheless, the experiments presented in this chapter did reveal a number of differences in the effects of the irrigation solutions on different aspects of infection-related behaviour, such as zoospore production, zoospore motility and cyst germinability.

In three data sets (Tables 4.4, 4.6 and 4.7) there were significant differences within the five SDW controls. These significant variations within SDW controls complicate interpretation of these data sets. The finite quantity of irrigation solution supplied for analysis unfortunately did not permit the repetition of any experiments.

In six of the data sets there were also significant differences within the fresh irrigation solution controls. The fresh irrigation solutions harvested during early July would be approximately five months older than those harvested at the end of October. These solutions when shipped were effectively sterile. However, with the passing of time, microbial populations and their associated metabolites would presumably change, even though the samples were incubated at a low temperature. These differing incubation times coupled with variable initial micro-organisms populations might account for the different characteristics associated with the five fresh

irrigation samples. Similarly, the other treatments would be incubated for differing durations depending on date of harvesting. However, the microbial populations in these samples would be established by the date of harvesting, and therefore less prone to alter during storage.

The option of analysing each batch of samples (same harvesting date) at a fixed duration after the harvesting date would impose uniform incubation duration on all samples. However, this would be logistically hard and impose an impossibly rigid timetable. In addition, there would be difficulty in comparing the results between the different harvesting dates as zoospore density and consequently zoospore behaviour is highly variable between zoospore populations.

The observation that the disease did not become visibly apparent in the aerial portion of the crop until September is borne out in the results from the mini-NFT trials. The solutions harvested at the end of October generally contained the highest number of *Ph. cryptogea* propagules. The plants grown in these solutions also generally had the most infections per 1 cm of root tissue, the lowest level of root development, the greatest level of root discolouration and the greatest number of lesions per unit area of root. However, none of the Edinburgh determined characteristics consistently showed solutions harvested on the 24th October to be significantly different from the solutions harvested on the other four dates. Significant negative correlations between sporangial density and four of the agronomic characteristics (plant height, stem diameter, leaf area and leaf length) initially suggest that sporangiogenesis may be a target for the agent(s) of natural suppression. However, no significant differences in sporangial density were observed between solutions harvested at the end of October and solutions harvested during the preceding months.

The highly significant negative correlations between zoospore release and two measurements of infection: - infections per 1 cm of root and root discolouration - imply that irrigation solutions that support healthy roots also promote zoospore release from pre-formed sporangia.

In the calculation of theoretical suppressiveness of irrigation solutions two major assumptions were made. These were that each month's data had the same weighting and each characteristic also had equal weighting. However, some characteristics were similar and could consequently over represent that aspect of the infection sequence, for example; cyst germination was assessed

in three different experiments. Similarly, the characteristic that may represent the 'weak link' in the infection sequence could be under represented in the calculation. The assumption that a non-germinated cyst had the potential to undergo diplanetism was challenged by McPherson (1998) who speculates that cysts in suppressive solutions were less capable of regermination (germination or diplanetism) than cysts found in non-suppressive solutions. It was also suggested by McPherson (1998) that zoospore motility was maintained in suppressive irrigation solutions. Therefore, negative correlation between zoospore motility and cyst germination would have to be assumed. In all correlations between proportion of zoospores maintaining motility and the proportion of cysts that had germinated negative correlations were found. The exception was the positive correlation between motility of *Ph. parasitica* zoospores and germination of vortex-encysted zoospores of *Py. aphanidermatum*. In this exception the positive correlation was far from significant ($R = 0.02$, $F = 0.90$). Therefore, my assumption that a theoretically suppressive solution would increase direct germination of cysts is challenged, and McPherson's hypothesis is supported. Therefore, further experiments on the effect of suppressive irrigation solutions on cyst viability and diplanetism are required.

The observation that the two least theoretically suppressive treatments were the two controls, that the four most theoretically suppressive treatments were from closed systems, and that the two open treatments were intermediate in theoretical suppressiveness was consistent with expectation. That is, in closed systems suppressive compounds can accumulate, whereas in open systems accumulation was not possible, but trace levels of suppressive compounds could be present. In either SDW or fresh nutrient solution an absence of suppressive compounds would be expected.

The relationship between the mean rank of theoretical suppressiveness and mean solution pH suggests that reduced pH was a mechanism of suppression in the irrigation system. However, no significant correlations exist between solution pH and any of the characteristics determined at Stockbridge House. Additionally, pH is continuously monitored and adjusted in the irrigation system and therefore could not function in disease suppression. The optimal pH of the irrigation solution is between pH 6.0 and 6.5 (Cooper, 1979). The pH range of solutions excluding those that had passed through the pasteuriser is from pH 5.9 to 7.5. Both these extreme pHs were recorded in samples of fresh irrigation solutions. All the low pHs (5.5 and below) were recorded

in irrigation samples that had passed through the pasteuriser. Therefore, this active process of pasteurisation could modify the solution and render it more acidic. It is possible that acidity accumulation occurred during cold storage at Edinburgh, but this would not account for the acidity in the pasteurised samples to decrease with the age of the sample - the converse would be expected. The overall trend in five out of the seven treatments (SDW control excluded) is for pH to increase (tend to neutrality) with ageing of stored solutions.

Zoospore motility was completely curtailed when the observed pH in the experiment was reduced to pH 4 by the addition of nitric acid. Low pH has been reported to curtail zoospore motility in *Ph. palmivora* (Bimpong & Clerk, 1970). The established relationship between bathing medium pH and zoospore motility explains firstly the very highly significant positive correlation between zoospore motility (of *Py. aphanidermatum*) and irrigation solution pH, and secondly the very low motility levels of zoospores of *Py. aphanidermatum* in the irrigation solutions that had been pasteurised. The effect of irrigation solution pH on motility of zoospores of *Ph. parasitica* was less because the ratio of zoospore suspension to irrigation solution was one to one, whereas in the corresponding experiment that used *Py. aphanidermatum* the ratio was one to three. The high levels of germination of vortex-encysted spores were probably not the result of low pH. The proportion of cysts found germinating in various concentrations of nitric acid was constant and therefore independent of pH over the range tested (up to 32 mM nitric acid ≈ pH 1.5). Elevated germination levels in the pasteurised irrigation solutions was probably due to liberation of germination-promoters by the process of pasteurisation. During pasteurisation cell contents will be liberated and might contain the particular L-amino acids and sugars that are known to promote germination in *Py. aphanidermatum* (Jones *et al.*, 1991; Donaldson & Deacon, 1993b).

In commercial glasshouses unusual pH drift is generally attributed to a change in nutrient up-take by the plants, and this can be an indication of the presence of disease. However, the lack of correlation between solution pH and any plant health indicators suggests that irrigation solution pH is not an indicator of disease.

Holderness and Pegg (1986) observed greater density of sporangia of *Ph. nicotianae* var. *parasitica* (= *Ph. parasitica*) in irrigation solutions than in distilled water. They also observed

that sterilisation of the irrigation solution reduced the sporangial density, but not to the level found in distilled water. The irrigation samples received from Stockbridge House failed to increase sporangial density compared to the SDW control. However, it was observed that from these mats with less sporangial density significantly more zoospores were released. The irrigation solutions would contain cations (principally Ca^{2+}, Mg^{2+}, K^{+} and Fe^{3+}) that are known to increase sporangial density in *Phytophthora* (Halsall, 1977; Halsall & Forester, 1977). The presence of microorganisms and their metabolites also been found to increase sporangial numbers (Marx & Haasis, 1965; Marx & Bryson, 1969; Ayers, 1971; Ayres & Zentmyer, 1971; Ribeiro, 1983). The mechanism of increased sporangia numbers in the presence of micro-organisms is two fold. First, microbial metabolites promote sporangiogenesis - for example, of several nitrogen sources tested L-α-alanine was the greatest promoter of sporangiogenesis in *Ph. palmivora* (Singh, 1973). Second, micro-organisms can remove substances that are inhibitory to sporangiogenesis - for example, glucose (Ribeiro, 1983).

However, sugars also function as chemoattractants for zoospores (Donaldson & Deacon, 1993b). The metabolism of sugars by pseudomonads in the rhizoplane of target plants has a reportedly detrimental effect on zoospore chemotaxis (Zhou & Paulitz, 1993). In irrigation systems used for the production of tomatoes the principal heterotrophic bacterial genus present is *Pseudomonas* with *Ps. facilis* being the most abundant species (Berkelmann & Wohanka, 1993). F. Lewis (McPherson, 1998) found that bacterial populations in the Stockbridge House irrigation solutions were greater for closed systems than for open, and that pasteurisation markedly reduced population numbers. *In vivo* it was found by F. Lewis (McPherson, 1998) that fluorescent yellow pseudomonads displayed the strongest antagonism, and siderophores were implicated in the antagonism. If siderophores are an agent of antagonism then iron limitation is implicated. There is growing evidence that indigenous microflora suppress *Py. aphanidermatum* in cucumbers grown on Rockwool (Postma *et al.*, 2000). Also there is growing acceptance by commercial (Dutch) growers of the merits of recirculating irrigation solutions in the production of tomatoes and cucumbers (van Os, 1999).

Chapter 5.

The effects of ethanol on *Py. aphanidermatum* and *Ph. parasitica*.

5.1. Introduction.

The presence of ethanol in a horticultural irrigation system based on the nutrient film technique (NFT) will be as a by-product of fermentation. Ethanol released by plants can act as an indicator to Oomycete phytopathogens that plants are stressed and therefore susceptible to attack (Allen & Newhook, 1973; Cahill & Hardham, 1994). The zoosporic fungi (particularly *Phytophthora* and *Pythium* spp.) are suitably adapted to infecting plants in systems based on NFT. The effect of ethanol on these fungi is generally unknown. Positive chemotaxis to ethanol is established in several species of *Phytophthora* (Halsall, 1976). The induction of cyst germination in *Ph. cinnamomi* by ethanol also occurs, albeit at a higher concentration than for the threshold for chemoattraction (Bryt *et al.*, 1982).

Although zoospores are believed to be essential for successful host infection their existence is dependent on the production of sporangia, differentiation of sporangial cytoplasm into zoospores and the successful liberation of zoospores from the sporangia. Zoosporangia in turn require the mycelial network for nutrition. Consequently these pre-zoosporic stages of the infection sequence are also potential targets of any control approach.

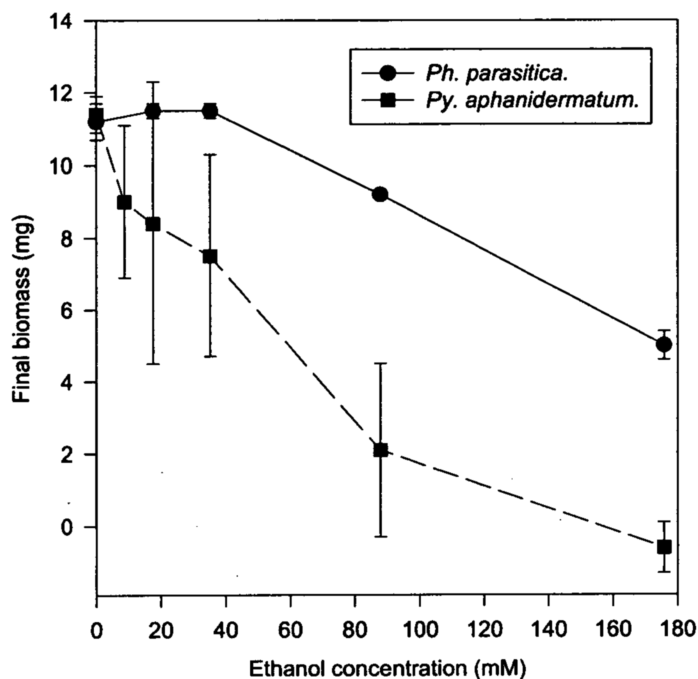
The results presented in this chapter summarise the effects of ethanol on several aspects of the infection sequence of two zoosporic fungi. Ethanol supplements to a closed irrigation solution would be a radical proposition. However, ethanol is a renewable, biodegradable and inexpensive chemical.

5.2. Effect on mycelial growth.

Mycelial fragments of *Py. aphanidermatum* when grown in CV8B in the absence of ethanol for 48h (as in Section 2.3.3) form a complete mat that covers the base of the Petri dish. Increasing the concentrations of ethanol supplemented to CV8B reduced the extent of the mycelial mat and the density of the mycelia. This was reflected in the biomass produced over the 48h incubation period (Figure 5.1). There was very highly significant negative correlation ($y = 9.83 - 0.065x$, $F = 3.73 \times 10^{-4}$, $R = -0.75$, $n = 18$) between ethanol concentration and final biomass. In a parallel experiment with *Ph. parasitica* many small dense colonies were observed in the ethanol-free control. Each colony was presumably derived from a small mycelial fragment. Again, increasing concentrations of ethanol suppressed biomass gain. Significant (ANOVA $P < 0.05$, $\sigma_d = 0.4$ mg) suppression of

biomass production was achieved with the 87.9 and 176 mM ethanol treatments compared to the ethanol-free control. In the 176 mM treatment the mean biomass production was 5.0 mg; this was less than half of the mean biomass recorded in the ethanol-free control, although this contrasts strongly with *Py. aphanidermatum* which did not grow in 176 mM ethanol.

Figure 5.1. The effect of supplementing CV8B with ethanol on final biomass* of *Py. aphanidermatum* and *Ph. parasitica*.



* Means ± s.e.m. for 3 replicates, assessed by arithmetic dry mass gain of pre-weighed Petri-dishes.

5.3. Effect of supplementing MSS with ethanol at different times during sporangiogenesis of *Py. aphanidermatum*.

Five different treatments were used in this experiment. The first treatment employed the standard protocol for producing sporangia-bearing mats of *Py. aphanidermatum* (Section 2.3.1.2), although an additional wash with MSS was included on the day between the second MSS wash and the induction of zoospore release. For the second treatment the MSS for all three washes was supplemented with 35.2 mM ethanol. In treatment three the first wash was with MSS supplemented with 35.2 mM ethanol, whereas the other two washes used the ethanol-free MSS. In the fourth treatment the first two washes were with MSS supplemented

with ethanol and the third wash was with ethanol-free MSS. In the fifth treatment the first two washes with ethanol-free MSS and the third wash was with MSS supplemented with 35.2 mM ethanol. The induction of zoospore release in all treatments employed the standard protocol. That is, the release solution was SDW. Table 5.1 shows the results of this experiment.

In the first treatment (all washes were with ethanol-free MSS) a mean zoospore density of 241 zoospores.μl^{-1} was found, whereas in treatment two (all washes with ethanol-supplemented MSS) no zoospores were observed. The addition of ethanol to the MSS wash of day 1 only (treatment 3) reduced the number of zoospores to 59% of that in the ethanol-free control (treatment 1), but this reduction was not significant ($P > 0.05$). When ethanol was added to MSS on day 1 and day 2 (treatment 4) or only on day 3 (treatment 5) then significant suppression was observed (t-tests, $P = 0.032$ and $P = 0.024$, respectively). The addition of ethanol to the MSS of day three reduced the number of zoospores released to 5% of the ethanol-free control (treatment 1).

Table 5.1. The effect of ethanol added to wash solutions at different times on sporangiogenesis of *Py. aphanidermatum* when assessed by subsequent zoospore release into SDW.*

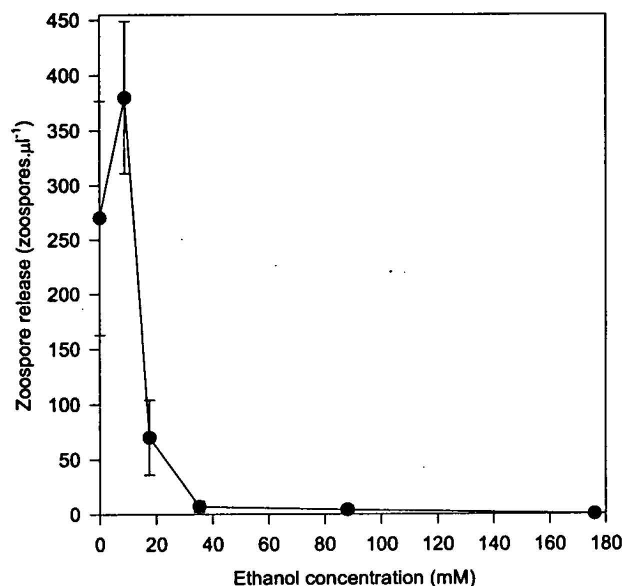
		MSS treatment		
Treatment	Day 1	Day 2	Day 3	Zoospores.μl^{-1}
1	-	-	-	241 ± 65
2	+	+	+	0 ± 0
3	+	-	-	143 ± 69
4	+	+	-	26 ± 18
5	-	-	+	13 ± 4
			P (ANOVA)	0.011
			σ_d	61

* Means ± s.e.m. for 3 replicates, determined by two haemocytometer counts per replicate.
- Ethanol free MSS
+ MSS supplemented with 35.2 mM ethanol.

5.4. Effect of various ethanol concentrations in MSS on zoospore release from *Py. aphanidermatum*.

The standard protocol for production of zoospores of *Py. aphanidermatum* (Section 2.3.1.2) was employed, but the MSS was supplemented with various concentrations of ethanol. After sporangiogenesis had occurred the ethanol supplemented MSS was removed by washes of SDW and the sporangia were induced to release zoospores into SDW. Increasing concentrations of ethanol in MSS to 35.2 mM or above significantly suppressed the numbers of zoospores released into SDW (Figure 5.2) with no zoospores being released when MSS was supplemented with 176 mM ethanol.

Figure 5.2. The effect of supplementing MSS with various concentrations of ethanol on subsequent zoospore release* from *Py. aphanidermatum* into SDW.



* Means ± s.e.m. for 3 replicates, determined by two haemacytometer counts per replicate.

5.5. Effect of various ethanol concentrations in MSS on zoospore release from *Ph. parasitica*.

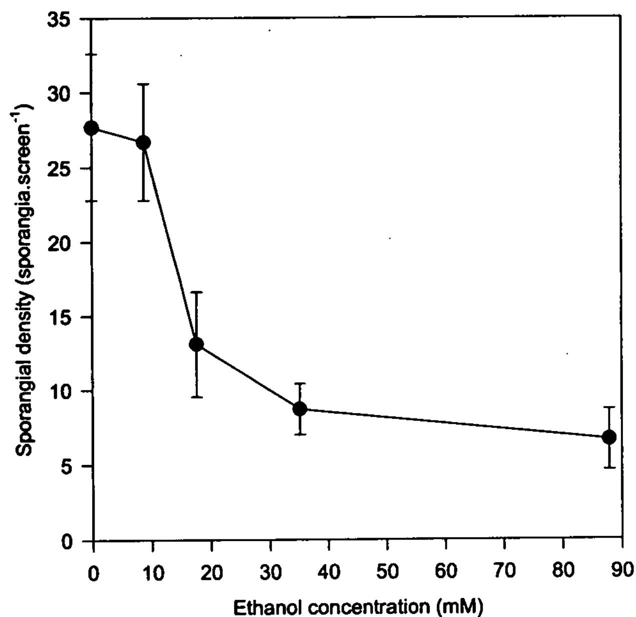
A parallel experiment was performed with *Ph. parasitica*. That is, the standard protocol for production of zoospores of *Ph. parasitica* (Section 2.3.1.1) was used, but the MSS was supplemented with various concentrations of ethanol. Because the sporangial morphology of *Ph. parasitica* is distinct and zoospores are released pre-shock into MSS then additional characteristics of sporangiogenesis were recorded. These additional characteristics were

sporangial density, pre-shock zoospore release (into ethanol supplemented MSS), and percentage pre- and post-shock sporangial discharge.

MSS supplemented with various concentrations of ethanol affected sporangial density, sporangial morphology, proportion of sporangia discharging their contents, number of zoospores released and specific zoospore release (that is, zoospores released per sporangium) in *Ph. parasitica* (Figures 5.3 to 5.8). Concentrations of 17.6 mM ethanol and above significantly ($P < 0.032$ determined by t-tests) reduced sporangial density compared to the ethanol-free control (Figure 5.3). In MSS supplemented with 87.9 mM ethanol the sporangial density was 27% of that of the ethanol-free control.

Sporangia that form in ethanol-free MSS were distinct, terminal, ovoid structures (Figure 5.4). This is typical morphology for this species (Waterhouse, 1956). However, sporangia that had formed in MSS supplemented with 176 mM ethanol were visibly deformed and frequently the anterior of the sporangium was elongated and curved (Figure 5.5).

Figure 5.3. The effect of supplementing MSS with various concentrations of ethanol on sporangial density* of *Ph. parasitica*.



* Means ± s.e.m. for 3 replicates, based on 6 random fields of view (3 fields prior to cold-shock and 3 fields after zoospore release) per replicate.

Figure 5.4. The morphology of sporangia of *Ph. parasitica* that formed in ethanol-free MSS (100× total magnification).

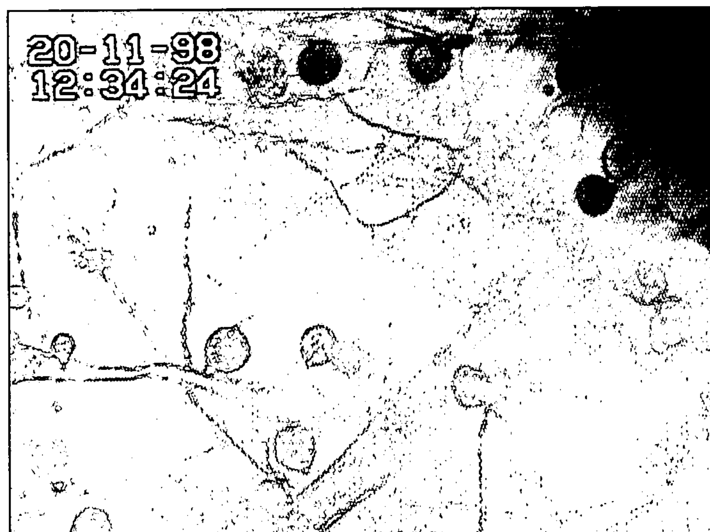
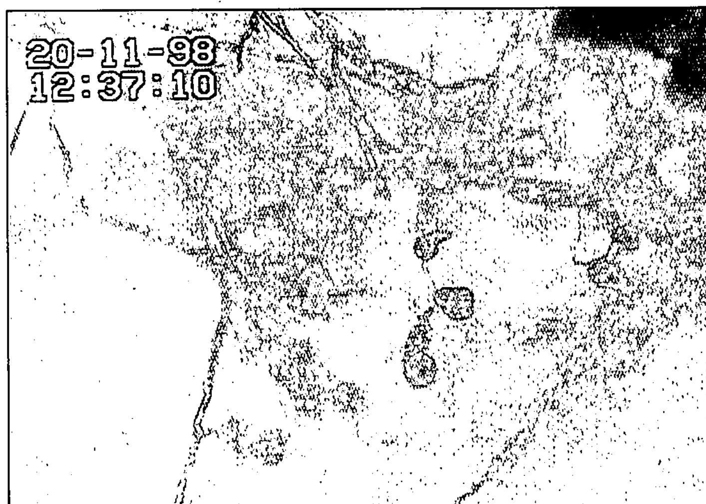


Figure 5.5. The morphology of sporangia of *Ph. parasitica* that formed in MSS supplemented with 176 mM ethanol (100× total magnification).

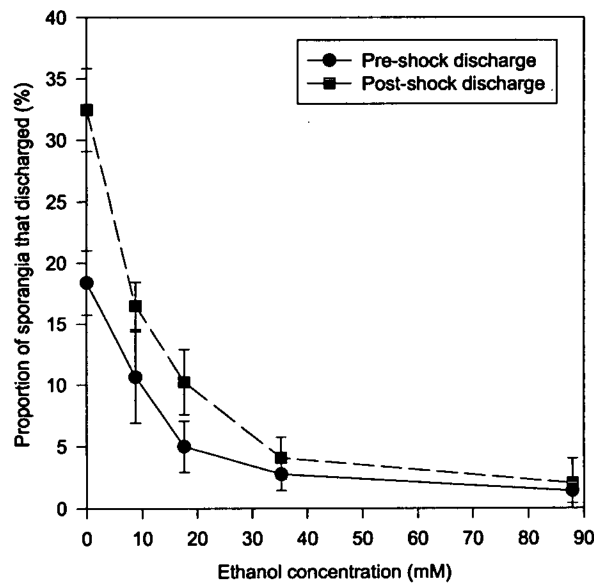


Increasing ethanol concentrations suppressed the proportion of sporangia that discharged zoospores both before and after the cold shock (Figure 5.6). In the control, before the cold shock, 18.4% of sporangia had discharged, but only 1.4% of sporangia had discharged their contents in the 87.9 mM ethanol treatment. A similar pattern occurred for post-cold shock

sporangial discharge. In the ethanol free-control 32.5% of sporangia were observed to have discharged their contents, whereas in the 87.9 mM treatment only 2.0% had discharged.

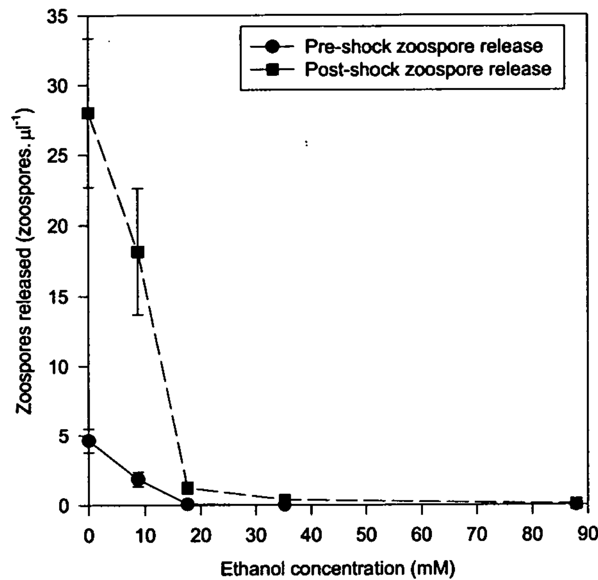
Zoospore release into the pre-shock ethanol-free control was significantly greater than into MSS supplemented with either 8.8 mM or 17.6 mM ethanol (t-tests, P = 0.026 and P = 0.006, respectively) (Figure 5.7). When MSS was supplemented with either 35.2 or 87.9 mM ethanol no zoospores were observed to have been released. The post-shock ethanol-free control had a significantly ($P < 0.05$) greater concentration of zoospores than in all the treatments with ethanol supplements greater than 8.8 mM. In the treatment with an ethanol supplement of 87.9 mM the number of zoospores released was less than 0.5% of the ethanol-free control.

Figure 5.6. Effect of ethanol concentration in MSS on the proportion of sporangia of *Phytophthora parasitica* that discharge their contents* before and after the induction of zoospore release.



* Means ± s.e.m. for 3 replicates, assessed before the cold shock and after zoospore release, based on scoring 3 fields of view per replicate.

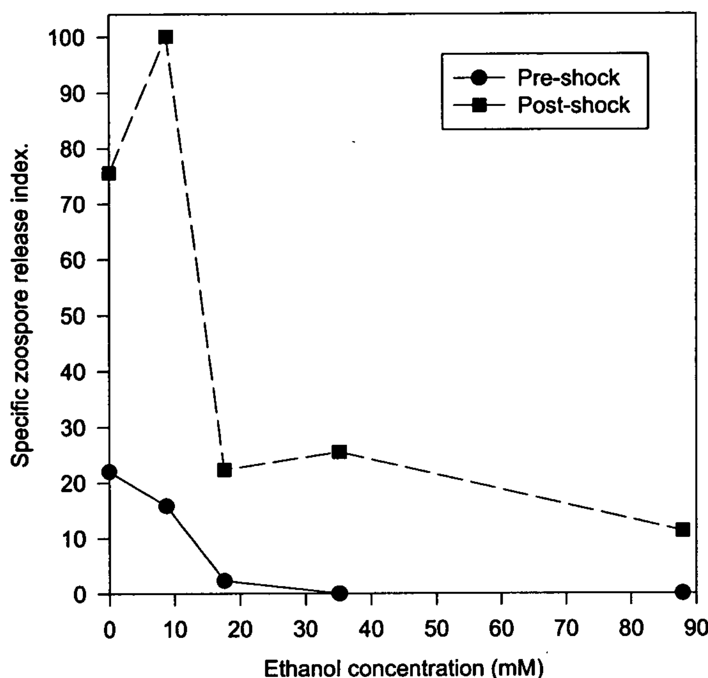
Figure 5.7. Effect of ethanol concentration in MSS on the zoospore release from sporangia of *Phytophthora parasitica* * before and after the induction of zoospore release.



* Means ± s.e.m. for 3 replicates, assessed before the cold shock and after zoospore release, based on 2 heamocytometer counts per replicate.

An index of specific zoospore release (an indication of the number of zoospores released per sporangium) was calculated from the above results (sporangial density, sporangial discharge and zoospore release). The calculation was the mean zoospore release divided by the product of sporangial density and percentage of sporangia that had discharged their contents. The results were then scaled so that the maximum value was 100. The overall trend was that increasing ethanol concentration suppressed the number of zoospores released per sporangium irrespective of whether it was pre- or post-shock (Figure 5.8). Although the trends are similar for pre- and post-shock the magnitude of the effect on specific zoospore release is greater for post-shock than for pre-shock.

Figure 5.8. Effect of ethanol concentration in MSS on the specific zoospore release of zoospores from sporangia of *Phytophthora parasitica*[*] before and after the induction of zoospore release.



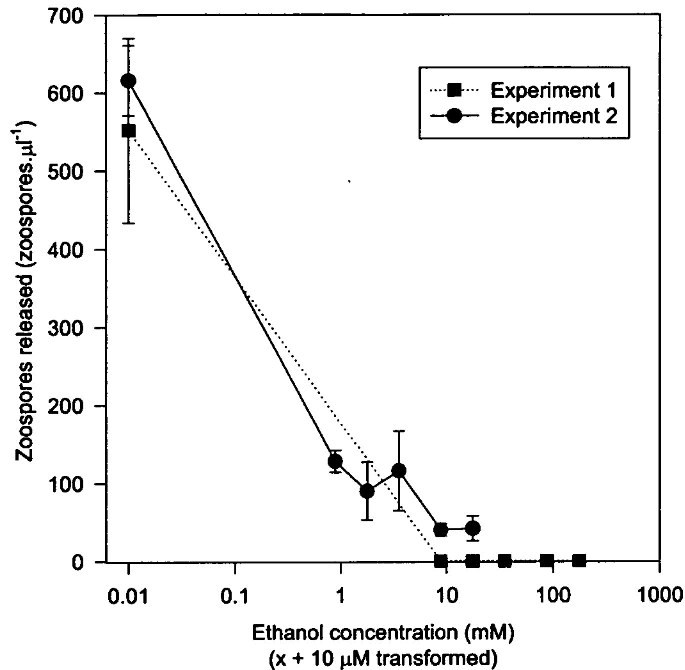
[*] Specific zoospore release at each supplement of ethanol was calculated by dividing mean zoospore release by the product of mean sporangial density and mean proportion of sporangia that had discharged their contents. The values were then linearly scaled so that the highest value of specific zoospore release was 100.

5.6. Effect of various ethanol concentrations in the release solution on zoospore release from pre-formed sporangia of *Py. aphanidermatum* and *Ph. parasitica.*

Pre-formed sporangia of *Py. aphanidermatum* were produced (Section 2.3.1.2) and then the release solution (SDW) was supplemented with various concentrations of ethanol. After the standard release period the number of zoospores released into the ethanol-supplemented SDW were enumerated by haemocytometer counts. This experiment was repeated twice.

The results of the second of two experiments showed that 880 μM ethanol significantly ($P < 0.05$) suppressed zoospore release from pre-formed sporangia to 21% of the control (Figure 5.9) Concentrations of ethanol of 8.8 mM and above in the first experiment completely suppressed zoospore release; that is, no zoospores were detected.

Figure 5.9. Effect of ethanol concentration in the release solution on zoospore release from pre-formed sporangia of *Py. aphanidermatum* *.



* Means ± s.e.m. for 3 replicates, based on 2 heamocytometer counts per replicate.

The same experimental design was employed, but with pre-formed sporangia of *Ph. parasitica* being exposed to various ethanol concentrations. A solution of up to 1.4 M ethanol did not significantly ($P > 0.05$) suppress percentage sporangial discharge or the number of zoospores released when compared to the ethanol-free control (Figure 5.10 and 5.11). In both experiments sporangial discharge and zoospore release peaked in the 87.9 mM ethanol solution. The only peak that was significantly (t-test, $P = 0.0027$) different from the ethanol-free control was the number of zoospores released in experiment 1.

Figures 5.10 and 5.11. The effect of ethanol concentration in the release solution on zoospore release from pre-formed sporangia of *Ph. parasitica* *.

Figure 5.10 Experiment 1.

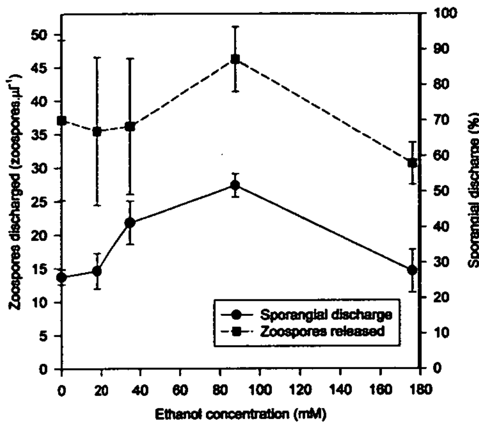
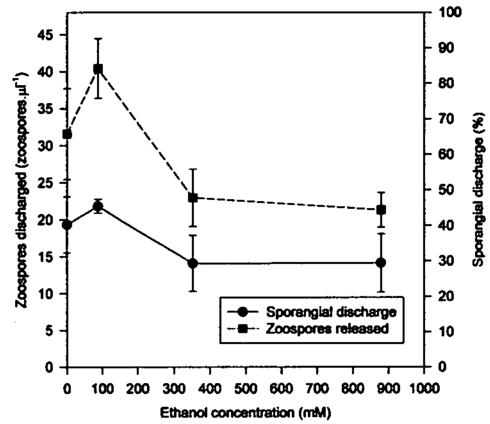


Figure 5.11 Experiment 2.



* Means ± s.e.m. for 3 replicates, based on 2 heamocytometer counts per replicate for zoospore release numbers or three fields of view for fraction of sporangia that had discharged their contents.

5.7. Effects of various ethanol concentrations on zoospore motility and cyst germination of *Py. aphanidermatum*.

Zoospores of *Py. aphanidermatum* were introduced into SDW or SDW supplemented with 176 mM ethanol. After 2h the proportion of zoospores motile and the proportion of cysts that had germinated was determined (as in Section 2.3.6). The proportion of zoospores that remained motile in 176 mM ethanol during the 2h incubation period was not significantly (P = 0.48) different from the ethanol-free control (Table 5.2). Similarly, the proportion of cysts that subsequently germinated in the 176 mM ethanol was not significantly (P = 0.29) different from the ethanol-free control. In a separate experiment it was found that the proportion of vortex-encysted zoospores that germinated in the 176 mM ethanol treatment did not significantly (P = 0.46) differ from the SDW control (method as in Section 2.3.7).

Table 5.2. The effect of 176 mM ethanol on the proportion of zoospores of *Py. aphanidermatum* that remain motile, that subsequently germinate and the proportion of vortex-encysted zoospores that germinate.

	Motile zoospores*	Subsequent cyst germination*	Vortex encysted zoospore germination**
Units	% (and Arcsine)	% (and Arcsine)	% (and Arcsine)
Ethanol free	35.6 ± 6.5 (36.5 ± 3.9)	2.7 ± 0.7 (9.3 ± 1.2)	44.2 ± 2.7 (41.6 ± 2.7)
176 mM Ethanol	41.0 ± 3.3 (39.8 ± 1.9)	4.2 ± 1.0 (11.6 ± 1.4)	47.0 ± 2.1 (43.2 ± 2.1)
P (t-test)	(0.48)	(0.29)	(0.46)

* Means ± s.e.m. for 3 replicates, based on 3 fields of view per replicate.
** Means ± s.e.m. for 3 replicates, assessed after 2h, based on score of at least 100 spores per replicate.

5.8. The effect of ethanol on zoospore and cyst nutrient induced germination of *Py. aphanidermatum*.

Naked, motile zoospores or encysted zoospores of *Py. aphanidermatum* were introduced into various concentrations of ethanol in SDW. Then after 20 minutes concentrated CV8B was added. The addition of the broth caused all motile zoospores to lose motility and encyst. The high concentration of nutrients caused most cysts to germinate (fuller details of this method are described in Section 6.3). The proportion of cysts that germinated was then scored.

The mean nutrient supplemented germination of zoospores and mature cysts (vortex-encysted) of *Py. aphanidermatum* in the ethanol-free control was 84% for zoospores and 79% for mature cysts (Figure 5.2). In 2.25 M ethanol a few spores were recorded to be viable however, these spores were likely to have spontaneously encysted and germinated during the release period and prior to the addition of ethanol. The relationship between spore germination and ethanol concentration was sigmoid in both cases. The regression of the probit transformed lines was very highly significant in both cases ($F \leq 3.67 \times 10^{-8}$) (Table 5.3 and Figure 5.13). The LD_{50} was calculated to be 633 mM ethanol for zoospores and 862 mM for mature cysts. Before fitting of the lines all data were linearly scaled so that germination in the ethanol-free controls was 100%.

Figures 5.12. and 5.13. The effect of ethanol concentration on zoospore and cyst viability of *Py. aphanidermatum.**

Figure 5.12 Non-transformed data.

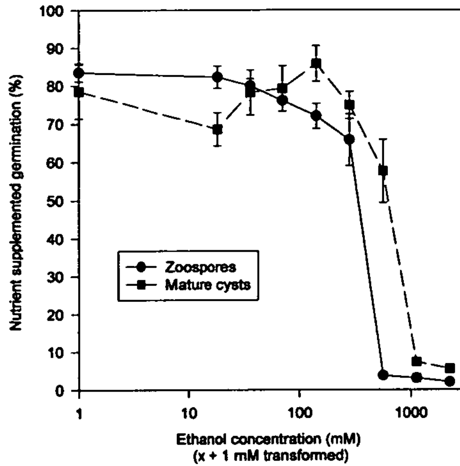
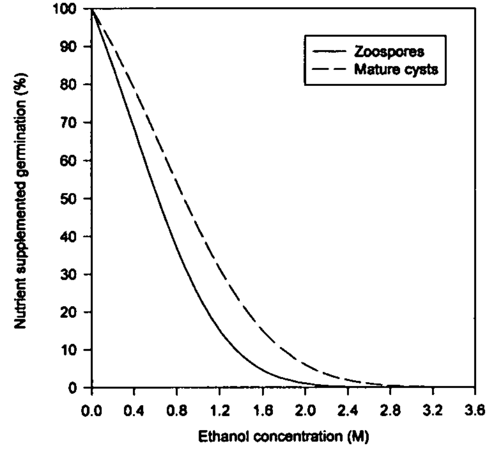


Figure 5.13 Fitted lines.



* Means ± s.e.m. for 3 replicates, assessed after 2h, based on score of at least 100 spores per replicate.

Table 5.3. Correlation statistics for lines of best fit for the effect of ethanol concentration on zoospore and cyst nutrient induced germination of *Py. aphanidermatum*.

	Zoospores	Cysts
F	3.67×10^{-8}	6.67×10^{-10}
R (transformed and concentration)	0.80	0.90
R (Fitted and observed)	0.93	0.93
df	26	26

5.9. Summary of the effects of ethanol on the biology of *Py. aphanidermatum* and *Ph. parasitica*.

In Table 5.4 the effects of ethanol on the biology of the two fungi is summarised. The most sensitive stages to ethanol were sporangiogenesis of *Ph. parasitica* and the release of zoospores from pre-formed sporangia of *Py. aphanidermatum*. High concentrations of ethanol did not affect zoospore release from pre-formed sporangia of *Ph. parasitica*. Mycelial growth of *Ph. parasitica* was less affected by ethanol than *Py. aphanidermatum*. Motile zoospores and cysts were not affected by concentrations of ethanol used in the assay; this is reflected in the relatively high LD_{50}.

Table 5.4. Summary of the effects of ethanol on the biology of *Py. aphanidermatum* and *Ph. parasitica*.

Aspect	*Py. aphanidermatum*	*Ph. parasitica*
Mycelial growth	176 mM completely reduced growth	176 mM reduced to 50% of control*
Sporangiogenesis		
Sporangial density	n.d.	35 mM reduced to 30% of control*
Proportion of sporangia discharging.	n.d.	35 mM reduced to 20% of control*
Zoospores subsequently released	35 mM reduced to 1% of control*	35 mM reduced to 10% of control*
Specific zoospore release	n.d.	18 mM reduced to 30% of control*
Zoospore release	35 mM completely suppresses release	880 mM had no affect.
Proportion of zoospores remaining motile	176 mM had no effect	n.d.
Cyst germination	176 mM had no effect	n.d.
Zoospore viability	LD_{50} = 633 mM	LD_{50} = 862 mM

* Control refers to the ethanol-free solution.
n.d. Not done.

5.10. Discussion.

These experiments show that ethanol is generally toxic to both *Py. aphanidermatum* and *Ph. parasitica*. However, the degree of toxicity is dependent on species and stage of the infection sequence.

Wall-less zoospores, believed to be the stage of the infection sequence most susceptible to disruption, have been targeted by several novel approaches, for example, by non-ionic surfactants (Stanghellini *et al.*, 1996a and 1996b). However, zoospores are not as sensitive to ethanol as are the pre-zoospore stages. Also, zoospore motility was not significantly suppressed by 176 mM ethanol. Likewise encysted zoospores were relatively insensitive to ethanol. However, the LD_{50} for zoospores was slightly lower than for cysts, which suggests that zoospores are slightly more sensitive to ethanol than cysts.

Overall, the most susceptible stage of the infection sequence to ethanol was zoosporangiogenesis. Although the release of zoospores from pre-formed sporangia of *Ph. parasitica* was insensitive to ethanol, release from *Py. aphanidermatum* was highly sensitive, with a sub-millimolar concentration of ethanol noticeably suppressing zoospore release. This difference between genera can possibly be attributed to differing physiology of zoospore release. *Pythium* produces a vesicle prior to zoospore release. Undifferentiated cytoplasm enters this vesicle where it differentiates into zoospores. In *Phytophthora* the vesicular stage is absent and cytoplasmic differentiation occurs within the sporangia. Therefore, the dissolution of the papillar plug might be assumed to be relatively insensitive to ethanol.

The sporangia of *Py. aphanidermatum* were able to recover from ethanol toxicity, although for almost full recovery a period of at least 48 h was needed in an ethanol free media. From a commercial perspective the longevity of ethanol toxicity on the zoosporic fungi will need to be ascertained. A factor yet to be determined is the effect of nutrient solution supplemented with ethanol on the crop plants, although ethanol vapour is known to affect the ripening of tomatoes (Beaulieu and Saltveit, 1997).

When tomatoes (*Lycopersicon esculentum* M.) were hypoxically induced with an external sucrose source, ethanol accumulation in the roots reached 30 $nmol.mg^{-1}$ of fresh tissue (Germain *et al.*, 1997). This equates to an approximately 50 mM concentration. The results

of this chapter suggest that the cumulative effect of 50 mM ethanol *in vitro* on the infection sequence of both fungi would be to completely inhibit zoospore release.

The tolerance of both *Ph. parasitica* and *Py. aphanidermatum* to ethanol is low compared to other fungi, particularly isolates of *Saccharomyces cerevisiae* which can grow in ethanol solutions up to 3 M for short durations (Chi *et al.*, 1999). Oral isolates of *Candida albicans* can tolerate an ethanol concentration of 475 mM, with growth completely suppressed by circa. 1.3 M (Botha *et al.*, 1997). Isolates of *Clostridium thermocellum* can tolerant up to ~850 mM (6% v/v) ethanol (Rani and Seenayya, 1999).

In general terms the degree of membrane fluidity confers ethanol tolerance. In *S. cerevisae* the sterols and not the phospholipids specifically confer ethanol tolerance (Agudo, 1992). Sterols, especially ergosterol, promote ethanol tolerance by increasing the barrier effect of the plasma membrane to entry of ethanol into the cell (Thomas *et al.*, 1978). However, work by Novotny *et al.* (1992) failed to find positive correlation between ethanol tolerance in a strain of *S. cerevisae* and δ–5,7–sterol content. However, it has been known for a long time that ethanol tolerance is highly variable within each genus of yeast and even between strains of a single species (Gray, 1941).

The pythiaceae require an exogenous supply of β-hydroxyl sterols for reproduction (including asexual) because of their inability to synthesise sterols *de novo* (Elliott, 1983). The sterols present in the membranes of the pythiaceae will consequently be ultimately of host (plant) origin. These sterols are used as precursors to satisfy various sterol synthesis pathways. The superior protectant properties to ethanol of ergosterol in *S. cerevisae* over cholesterol are attributed to the Δ^{22} unsaturated alkyl chain (Thomas *et al.*, 1978). The sterols prevalent in the Oomycetes are cholesterol which has a Δ^{22} saturated alkyl chain and fucosterol which is unsaturated, but at carbon-28 and not at carbon-22 as in ergosterol.

Phospholipids are the major components of all membranes and also confer tolerance to ethanol in yeast (Chi *et al.*, 1999). In yeast, unsaturated fatty acids are crucial for enhanced ethanol tolerance. In three species representative of the Pythiaceae the most abundant polar lipids were phosphatidylethanolamine and phosphatidylcholine (Moreau *et al.*, 1998b). Both these types of phospholipids are mono-unsaturated (Gennis, 1989). Of the major classes of inositol sphingophospholipids from *Ph. parasitica* all were mono-unsaturated (Bruneteau *et al.*, 1997). Any explanation of ethanol tolerance based on degree of sterol and phospholipid

saturation, and relative sterol abundance, fails to explain the tolerance of naked zoospores to ethanol, which is comparable to the ethanol tolerant-yeasts, and the high sensitivity of sporangiogenesis to ethanol.

It is generally agreed that the toxicity of ethanol involves the interaction of ethanol with membrane processes. One hypothesis is that ethanol acts by directly binding to membrane proteins (for example see, Covarrubias and Rubin, 1993). A second hypothesis suggests that the phospholipid membrane-water interface is a potential site for non-specific interactions with ethanol (for example see, Barry and Gawrisch, 1994). The work presented here clearly indicates that certain stages of the zoosporic infection sequence are highly susceptible to ethanol toxicity. The naked zoospore stage is relatively insensitive to ethanol, yet the plasma membrane is highly exposed, whereas the walled zoosporangiogenesis stage of *Ph. parasitica* is sensitive and the wall-less vesicle of *Py. aphanidermatum* is also highly sensitive. The deformation of *Phytophthora* sporangia in ethanol demonstrates that ethanol also interferes with the formation of sporangia. These findings consequently accord with the hypothesis of Covarrubias and Rubin (1993) that ethanol toxicity is mediated by direct interaction with particular membrane bound proteins.

It is to be noted that the trend identified in chapter 3 is also present in the results of this chapter. That is, *Py. aphanidermatum* is much more susceptible to ethanol supplements than *Ph. parasitica*.

The suggestion that recirculating irrigation systems could be supplemented with ethanol is novel, but much research is still required. Once an effective concentration has been determined, then the detriment, if any, to crop yield and quality of yield will also need to be determined. Whether the zoosporic fungi can adapt to high ethanol environments has yet to be considered.

The addition of ethanol to an irrigation system would undoubtedly affect the microfauna and microflora of the system, with the possible selection of organisms that can use ethanol as an energy or carbon source. The rates of ethanol degradation and evaporation from the system would require constant monitoring and appropriate supplements would have to be made if ethanol were ever to be used as a component of control of zoosporic fungi in NFT.

Chapter 6.

The effects of natural toxins on aspects of the infection sequences of *Py. aphanidermatum* and *Ph. parasitica*.

6.1. Introduction.

For commercial reasons fungicides are not registered for use in irrigation systems based on nutrient film technology. Therefore, there is potential for exploitation of natural compounds to control the most economically important diseases in these systems - the diseases caused by zoospore-producing Oomycetes. β-escin and gramicidin S are both naturally occurring toxins that have the potential to control zoospore-mediated infection in soil-less irrigation systems. Although these compounds cause cell death by membrane disruption, their origin, structure and mode of action are different. Zoospores of the major pathogens in these systems (*Pythium* spp. and *Phytophthora* spp.) are wall-less and potentially highly susceptible to disruption by gramicidin S and β-escin.

The target site of gramicidin S is the phospholipid component of the membrane (Katsu, *et al.*, 1988) whereas β-escin (triterpeneglycoside saponin from *Castanea sativa*) targets the membrane-bound sterols (Osbourn, 1996). So there is potential for synergism in using these two naturally occurring toxins in the control of zoosporic fungi in glasshouse irrigation systems. This chapter looks at the susceptibility of various stages of the zoosporic infection sequence to these two naturally occurring toxins and assesses the potential for their use in soil-less irrigation systems. Both chemicals were obtained from Sigma. Gramicidin S is a misnomer and really a tyrocidine (cyclic decapeptide). All other gramicidins are linear peptides.

The inhibition of unpurified solutions is also investigated. Saponins are present in oats and the inhibition of crude oat extracts is presented in this chapter, as is the inhibition of culture filtrates of *Brevibacillus brevis*[1], which is known to produce gramicidin S.

6.2. The effects of dimethy sulphoxide on aspects of biology of zoosporic fungi.

Neither β-escin nor gramicidin S is water soluble. In previous work with β-escin, ethanol was used as the solvent (Deacon & Mitchell, 1985). However, results presented in this thesis (Chapter 5) clearly demonstrate that ethanol affects sporangiogenesis. Consequently ethanol was not used as the solvent for either β-escin or gramicidin S in the experiments reported in

[1] *Brevibacillus brevis* until recently was know as *Bacillus brevis* (Shido *et al.*, 1996).

this chapter. The solvent chosen was dimethyl sulphoxide (DMSO). A DMSO concentration of 28 mM did not significantly affect the proportion of zoospores of *Py. aphanidermatum* remaining motile (P = 0.899), the proportion of cysts that subsequently germinated (P = 0.075), the density of sporangia of *Ph. parasitica* that formed in the presence of DMSO (P = 0.341), the proportion of sporangia that subsequently liberated their contents into SDW (P = 0.907) or the number of zoospores released pre- and post-shock ($P \geq 0.570$) (Table 6.1).

Table 6.1. The effects of 28 mM DMSO on zoospore motility and subsequent cyst germination of *Py. aphanidermatum* and on sporangiogenesis of *Ph. parasitica*.

	Units	0 mM DMSO	28 mM DMSO	*P* (t-test)
Zoospore motility [a]	Arcsine	34.5 ± 1.5	34.2 ± 1.5	0.899
Subsequent cyst germination [a]	Arcsine	8.0 ± 0.1	9.8 ± 0.8	0.075
Pre-shock zoospore release [b]	Zoospores.μl^{-1}	16.7 ± 2.0	15.9 ± 3.0	0.832
Post-shock zoospore release [b]	Zoospores.μl^{-1}	31.3 ± 4.9	27.4 ± 3.9	0.570
Post-shock empty sporangia [c]	Arcsine	22.2 ± 1.8	21.9 ± 1.6	0.907
Sporangial density [c]	Sporangia.$screen^{-1}$	93.5 ± 2.7	114.7 ± 19.4	0.341

[a] Means ± s.e.m. for 3 replicates, assessed after 2h, based on scoring 3 fields of view per replicate. (Data for *Py. aphanidermatum*; see 2.3.6 for methods).
[b] Means ± s.e.m. for 3 replicates, based on 2 haemocytometer counts per replicate. (Data for *Ph. parasitica*; see 2.3.4 for methods).
[c] Means ± s.e.m. for 3 replicates, assessed after 2h, based on scoring 3 fields of view per replicate. (Data for *Ph. parasitica*; see 2.3.4 for methods).

6.3. Effects of β-escin and gramicidin S on zoospore, immature cyst and mature cyst nutrient induced germintation.

To 1 ml of zoospores of *Py. aphanidermatum* or 2 ml of zoospores of *Ph. parasitica* various concentrations of β-escin and gramicidin S were added, so that the total volume was 4 ml. After a 20 min incubation in the dark at 23 °C, 444 μl of concentrated CV8B was added to each Petri dish. The addition of the broth to the Petri dishes immediately induced encystment of all motile zoospores. The proportion of encysted zoospores that subsequently germinated was assessed after a further 2h incubation by microscopically examining a random selection of spores (see section 2.3.7). The nutrient content of the broth consistently caused high germination levels in populations of spores in control (toxin-free) treatments.

In the β-escin-free control 70% of zoospores germinated (Figure 6.1). The addition of 32 μM β-escin reduced this value to 23%; 128 μM β-escin reduced nutrient induced germination to less than 2%. A concentration of 2.0 μM gramicidin S was required for complete suppression

of nutrient induced germination of zoospores of *Py. aphanidermatum* (Figure 6.2). Zoospores of *Ph. parasitica* were more tolerant to both β-escin and gramicidin S (Figures 6.3 and 6.4). Although 32 μM β-escin reduced zoospore nutrient induced germination to 10%, this still represented substantial relative germination because only 25% of zoospores were germinable in the β-escin-free control of the experiment. For complete suppression of nutrient induced germination of zoospores of *Ph. parasitica* a gramicidin S concentration of 4 μM was required

The nutrient induced germination of pre-encysted zoospores in various concentrations of β-escin and gramicidin S was also assessed. The same protocol as above was used, but immature cysts or mature cysts were substituted for zoospores. Immature cysts were classed as cysts that were immediately added to the experiment after vortexing. Cysts that were added 10 minutes post-vortexing were classed as mature. The primary difference between immature and mature cysts was that immature cysts had cell walls that were still forming, whereas mature cysts were presumed to have fully formed cell walls.

Concentrations up to 256 μM β-escin had no effect on the nutrient induced germination of immature cysts of *Py. aphanidermatum* (Figure 6.5). However, 512 μM β-escin reduced the proportion of cysts that germinated to just 5%, compared with 74% germination in the control. The presence of an immature cyst wall had little effect on the tolerance of cysts of *Py. aphanidermatum* to gramicidin S (Figure 6.6). The 2.0 μM treatment of gramicidin S completely suppressed cyst germination - the same concentration that completely suppressed when added to motile zoospores. Treatments of up to 512 μM β-escin had no effect on the nutrient induced germination of immature cysts of *Ph. parasitica* (Figure 6.7).

The germination of mature cysts of *Py. aphanidermatum* in 2.0 mM β-escin was 20% (Figure 6.8). This concentration of β-escin had no noticeable effect on the nutrient induced germination of mature cysts of *Ph. parasitica* (Figure 6.10). The germination of mature cysts of *Py. aphanidermatum* in 1.5 μM gramicidin S was 3% (Figure 6.9). This level of suppression is comparable to that in experiments that used zoospores and immature cysts of *Py. aphanidermatum* (Figures 6.2 and 6.6).

From Figures 6.1 to 6.10, lines of best fit can be used to determine LD_{50} values (Table 6.2). The effect of up to 2.0 mM β-escin on cysts (immature and mature) of *Ph. parasitica* was not

significant, and a suitable line of best fit could not be found. Consequently the LD_{50} of β-escin for cysts of *Ph. parasitica* cannot be predicted, but was greater than 2.0 mM. All other lines of best fit were statistically significant (correlation of all transformed lines was $F \leq 0.017$ and $R \geq 0.672$).

The LD_{50} values for gramicidin S were in the same order of magnitude for zoospores, immature cysts and mature cysts, with the range of LD_{50} values being between 457 nM and 763 nM. All lines of best fit for assessments with gramicidin S were sigmoid, except for mature cysts of *Py. aphanidermatum* when a straight line returned the best correlation.

The best relationship between zoospore nutrient induced germination and β-escin concentration for both species of fungi was exponential. The LD_{50} for zoospores of *Py. aphanidermatum* was 24.3 μM; the corresponding value for *Ph. parasitica* was higher at 45.5 μM. The relationship between β-escin concentration and germinated immature cysts of *Py. aphanidermatum* was sigmoid, with LD_{50} being 271 μM. The corresponding value for mature cysts was 1.2 mM.

Figures 6.1 and 6.2. The effects of β-escin and gramicidin S concentration on the nutrient induced germination* of zoospores of *Py. aphanidermatum*.

Figure 6.1. β-escin .

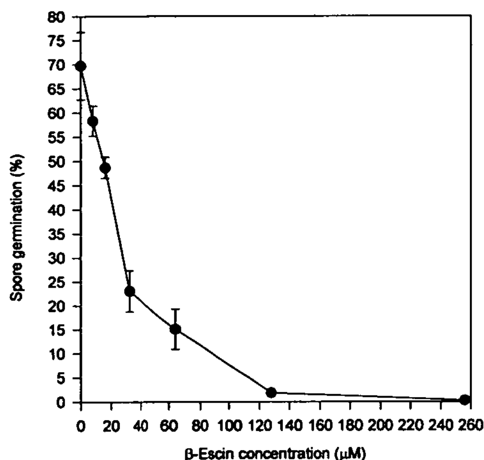
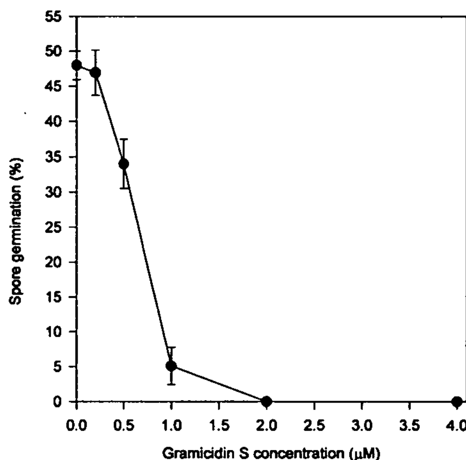


Figure 6.2. Gramicidin S.



* Means ± s.e.m. for 3 replicates, assessed 2h after the addition of concentrated CV8B, based on score of at least 100 spores per replicate. The concentration of DMSO in all treatments for Figure 6.1 was 60 mM and 341 μM for Figure 6.2.

Figures 6.3 and 6.4. The effects of β-escin and gramicidin S concentration on the nutrient induced germination* of zoospores of *Ph. parasitica*.

Figure 6.3. β-escin.

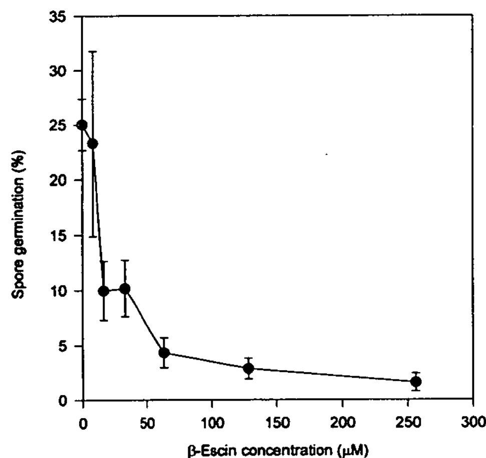
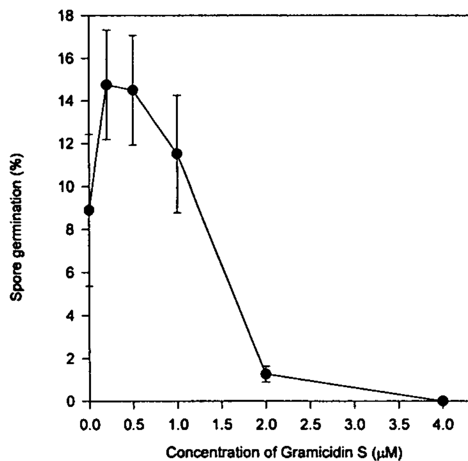


Figure 6.4. Gramicidin S.



* Means ± s.e.m. for 3 replicates, assessed 2h after the addition of concentrated CV8B, based on score of at least 100 spores per replicate. The concentration of DMSO in all treatments for Figure 6.3 was 60 mM and 3.2 mM for Figure 6.4.

Figures 6.5 and 6.6. The effects of β-escin and gramicidin S concentration on the nutrient induced germination* of immature cysts of *Py. aphanidermatum*.

Figure 6.5. β-escin .

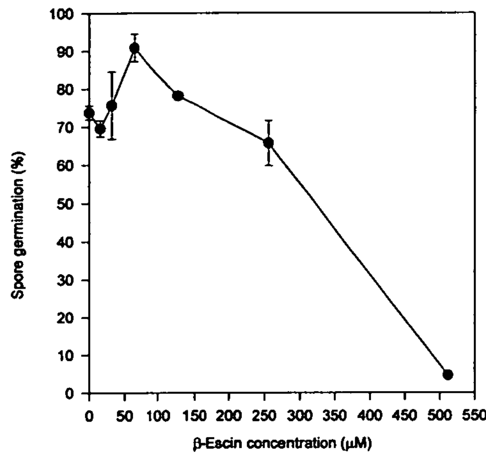
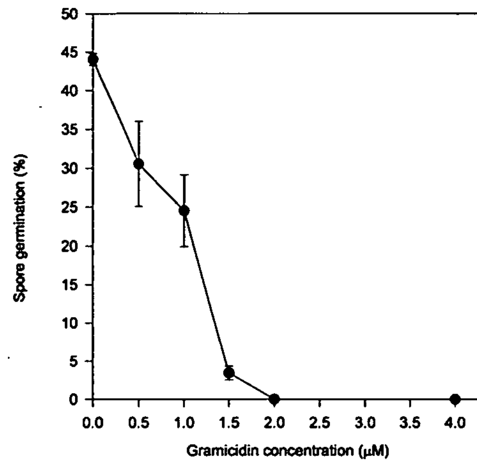
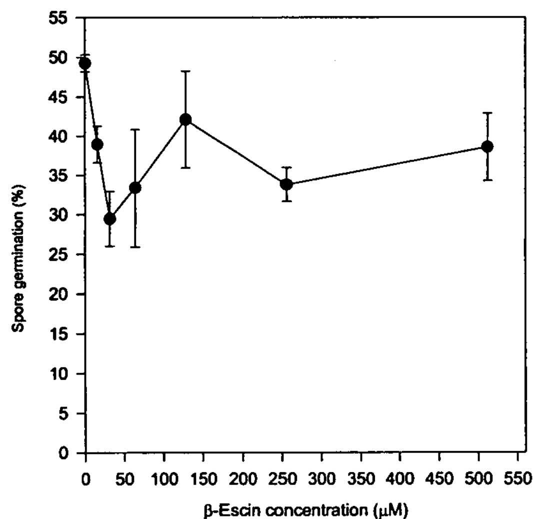


Figure 6.6. Gramicidin S.



* Means ± s.e.m. for 3 replicates, assessed 2h after the addition of concentrated CV8B, based on score of at least 100 spores per replicate. The concentration of DMSO in all treatments for Figure 6.5 was 81 mM and 3.2 mM for Figure 6.6.

Figure 6.7. The effect of β-escin concentration on the nutrient induced germination* of immature cysts of *Ph. parasitica*.



* Means ± s.e.m. for 3 replicates, assessed 2h after the addition of concentrated CV8B, based on score of at least 100 spores per replicate. The concentration of DMSO in all treatments was 81 mM.

Figures 6.8 and 6.9. The effects of β-escin and gramicidin S concentration on the nutrient induced germination* of mature cysts of *Py. aphanidermatum*.

Figure 6.8. β-escin.

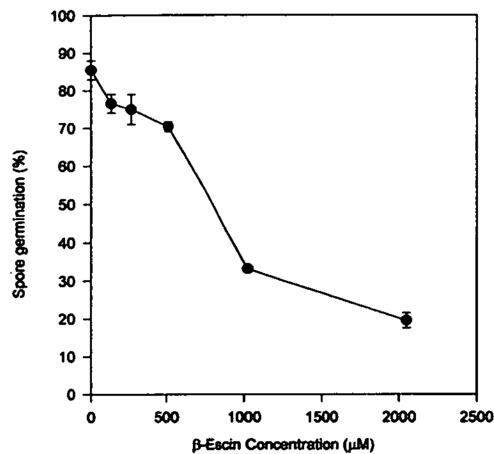
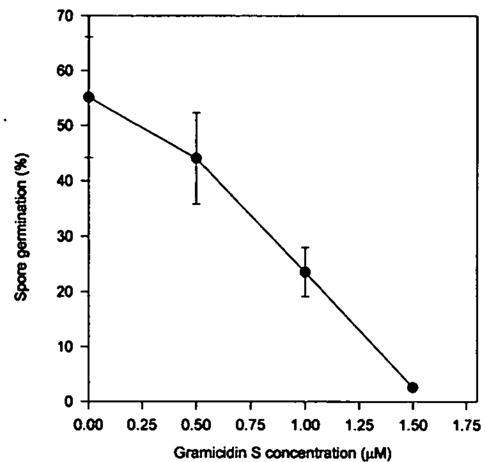
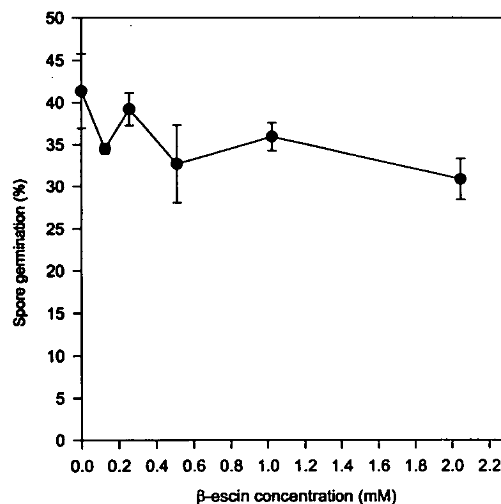


Figure 6.9. Gramicidin S.



* Means ± s.e.m. for 3 replicates, assessed 2h after the addition of concentrated CV8B, based on score of at least 100 spores per replicate. The concentration of DMSO in all treatments for Figure 6.8 was 324 mM and 2.7 mM for Figure 6.9. The high concentration of DMSO the experiment in Figure 6.8 was required so that 2 mM β-escin would be fully dissolved.

Figure 6.10. The effect of β-escin concentration on the nutrient induced germination* of mature cysts of *Ph. parasitica*.



* Means ± s.e.m. for 3 replicates, assessed 2h after the addition of concentrated CV8B, based on score of at least 100 spores per replicate. The concentration of DMSO in all treatments was 324 mM. The high concentration of DMSO was required so that 2 mM β-escin would be fully dissolved.

Table 6.2. LD_{50} values for β-escin and gramicidin S on various forms of spore of *Py. aphanidermatum* and *Ph. parasitica*.

Figure	Fungus	Spore type	Toxin	Line type	LD_{50}[a]	F[b]	R (transformed data)	*df*	R.[c] (observed & fitted)
6.1.	*Py. aphanidermatum*	Zoospores	β-escin	Exponential	24.3 μM	1.3×10^{-10}	0.944	20	0.973
6.3.	*Ph. parasitica*	Zoospores	β-escin	Exponential	45.5 μM	5.62×10^{-5}	0.764	20	0.783
6.5.	*Py. aphanidermatum*	Immature cysts	β-escin	Sigmoid	271 μM	2.08×10^{-6}	0.838	20	0.860
6.7.	*Ph. parasitica*	Immature cysts	β-escin	n.a.	> 2.0 mM	n.a.	n.a.	20	n.a.
6.8.	*Py. aphanidermatum*	Mature cysts	β-escin	Linear	1.2 mM	1.8×10^{-9}	0.950	17	0.950
6.10.	*Ph. parasitica*	Mature cysts	β-escin	n.a.	> 2.0 mM	n.a.	n.a.	17	n.a.
6.4.	*Ph. parasitica*	Zoospores	Gramicidin S	Sigmoid	547 nM	8.57×10^{-9}	0.939	17	0.597
6.2.	*Py. aphanidermatum*	Zoospores	Gramicidin S	Sigmoid	462 nM	8.67×10^{-6}	0.849	17	0.958
6.6.	*Py. aphanidermatum*	Immature cysts	Gramicidin S	Sigmoid	457 nM	3.22×10^{-7}	0.902	17	0.960
6.9.	*Py. aphanidermatum*	Mature cysts	Gramicidin S	Linear	763 nM	1.66×10^{-2}	0.672	11	0.672

[a] Calculated from fitted line.
[b] Significance of regression line.
[c] Correlation between observed points and fitted points.
n.a. Not applicable.

6.4. Visual observations of the effects of β-escin and gramicidin S on zoospores of *Py. aphanidermatum*.

In small Petri dishes, motile zoospores of *Py. aphanidermatum* were added to a range of concentrations of β-escin and gramicidin S. The ratio of zoospore suspension to treatment added was 1 ml to 3 ml. Then after an incubation period (75 min for gramicidin S and 110 min for β-escin) in the dark at 23 °C a representative proportion (at least 100 spores per replicate) of non-motile spores were examined microscopically and scored for their appearance. The non-motile spores included those that might have encysted before the treatment was applied and those that had become immobilised after the treatments were applied. The four classes of appearance were:

1. Visibly healthy cells.
2. Granular cytoplasm (but intact cells).
3. Ruptured cells (cytoplasmic leakage).
4. Lysed cells (non-membrane-bound granular cytoplasm).

In 80 μM β-escin approximately 10% of non-motile spores were healthy (Figure 6.11). Out of the three classes of 'unhealthy' cells most were intact, but 23% of all cells examined in 80 μM β-escin were not intact. In 2.0 μM gramicidin S all cells were unhealthy, but only 8% of spores were not intact (Figure 6.12). In the higher concentration (4.0 μM) of gramicidin S the proportion of unhealthy spores that were not intact was only 9%. The other 91% had granular contents, but the cells were intact.

Figures 6.11 and 6.12. The effects of β-escin and gramicidin S on the appearance* of non-motile cells of *Py. aphanidermatum*.

Figure 6.11 β-escin.

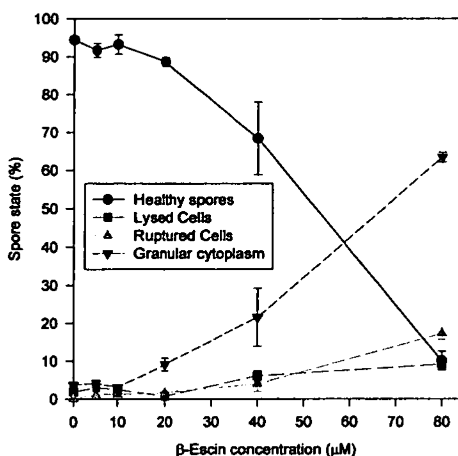
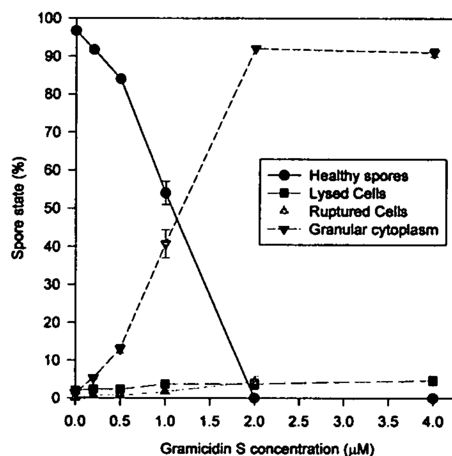


Figure 6.12 Gramicidin S.



* Means ± s.e.m. for 3 replicates, assessed after 110 min. for Figure 6.11 and 75 min. for Figure 6.12, based on score of at least 100 spores per replicate. The concentration of DMSO in all treatments for Figure 6.11 was 8.3 mM and 341 μM for Figure 6.12.

6.5. The effects of β-escin and gramicidin S on sporangiogenesis and sporangial discharge of *Ph. parasitica*.

When mats of *Ph. parasitica* were bathed in mineral salts solution at 20 °C and illuminated (24 $h.d^{-1}$) sporangia production occurred. During the later stages of the incubation period a proportion of sporangia discharge their contents. Therefore, zoospores were observed swimming in the mineral salts solution prior to removal of the mineral salts solution.

To test the effects of β-escin and gramicidin S on sporangiogenesis and sporangial discharge the mineral salts solution was supplemented with various concentrations of either β-escin or gramicidin S. The DMSO concentration was kept constant across all treatments for each experiment. At the end of the incubation in mineral salts solution the density of sporangia, the proportion of sporangia that had discharged and the concentration of motile zoospores in the mineral salts solution was determined (see section 2.3.4). These assessments were classed as pre-shock assessments.

After the supplemented mineral salts solution had been removed with washes of SDW and the plates had been cold shocked, zoospores were liberated into SDW and assessments were made of sporangial density, proportion of sporangia that had discharged their contents and the density of motile zoospores. These assessments were classed as post-shock assessments.

The removal of mineral salts solution and the induction of zoospore release into SDW had no noticeable effect on sporangial density. That is, the density of sporangia pre- and post-shock was identical. Therefore, the data from these two assessments were pooled for each replicate. Supplements of β-escin up to 128 μM and gramicidin S up to 32 μM to mineral salts solution were found to have no significant ($P > 0.05$) effect on sporangial density of *Ph. parasitica* (Figures 6.13 and 6.14).

Treatments of gramicidin S of 8 μM or greater reduced the proportion of sporangia that had discharged their contents to approximately 6%, from 15% in the pre-shock gramicidin S-free control at pre-shock (Figure 6.15). The proportion of sporangia that had discharged after the induction of zoospore release (post-shock) was unaffected by gramicidin S treatments up to 32 μM. Increasing concentrations of β-escin led to a reduction of sporangial discharge (Figure 6.16), from 13% in the β-escin free control and 18% in the post-shock control. The 128 μM treatment of β-escin reduced the proportion of sporangia that had discharged at pre-shock to less than 1% and to about 2% post-shock.

The number of zoospores released into the gramicidin S-free control (pre-shock) was 10.3 zoospores.μl^{-1} (Figure 6.17). In the 32 μM gramicidin S treatment this number was significantly ($P = 0.010$) reduced to 4.6 zoospores.μl^{-1}; 32 μM gramicidin S caused a similar post-shock reduction in zoospore release. For post-shock assessments the gramicidin S-free controls had 41.7 zoospores.μl^{-1}, whereas only 25.8 zoospores.μl^{-1} were found in the 32 μM gramicidin S treatment. No zoospores were seen in the 128 μM β-escin treatment (pre-shock) (Figure 6.18). However, 12.0 zoospores.μl^{-1} were seen in the 128 μM β-escin treatment (post-shock).

Figures 6.13 and 6.14. The effects of β-escin and gramicidin S concentration on sporangial density* of *Ph. parasitica.*

Figure 6.13. Gramicidin S.

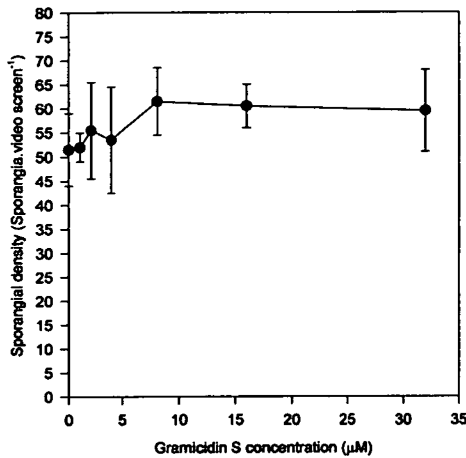
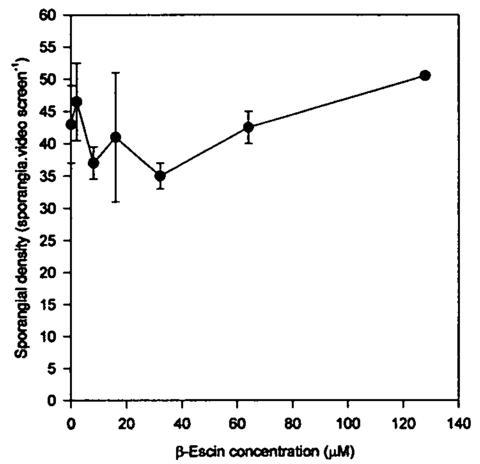


Figure 6.14. β-escin.



* Means ± s.e.m. for 3 replicates, based on 6 random fields of view (3 fields prior to cold-shock and 3 fields after zoospore release) per replicate. The concentration of DMSO in all treatments for Figure 6.13 was 27 mM and 22 mM for Figure 6.14.

Figures 6.15 and 6.16. The effects of β-escin and gramicidin S concentration on the proportion of sporangia of *Ph. parasitica* discharging their contents*.

Figure 6.15. Gramicidin S.

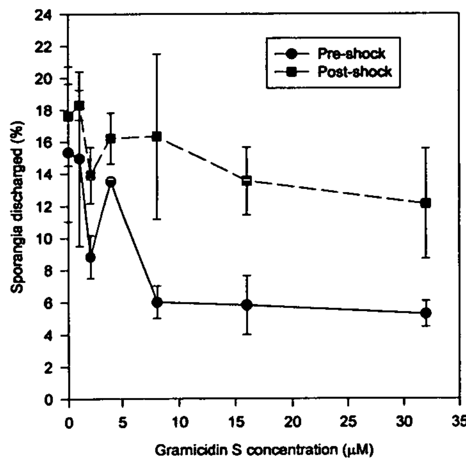
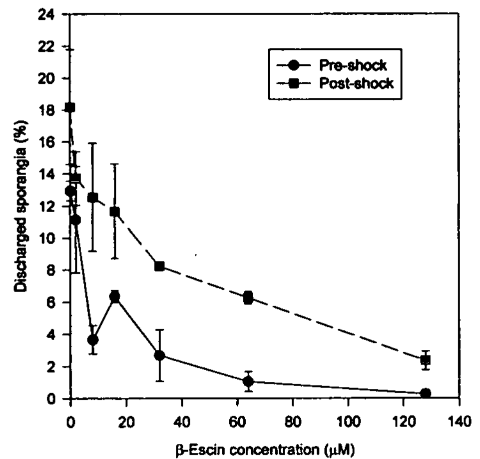


Figure 6.16. β-escin.



* Means ± s.e.m. for 3 replicates, based on 3 random fields of view per replicate. The concentration of DMSO in all treatments for Figure 6.15 was 27 mM and 22 mM for Figure 6.16. See text for explanation of pre-shock and post-shock.

Figures 6.17 and 6.18. The effects of β-escin and gramicidin S concentration on the number of zoospores released from sporangia of *Ph. parasitica* *.

Figure 6.17 Gramicidin S.

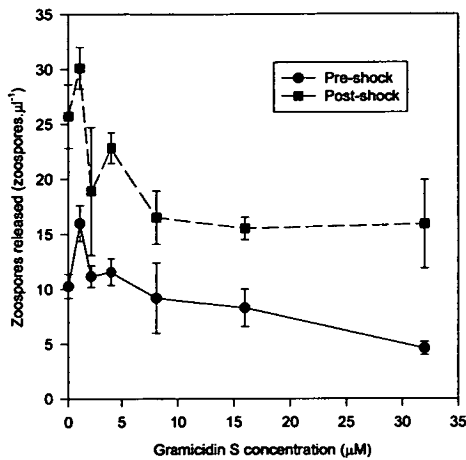
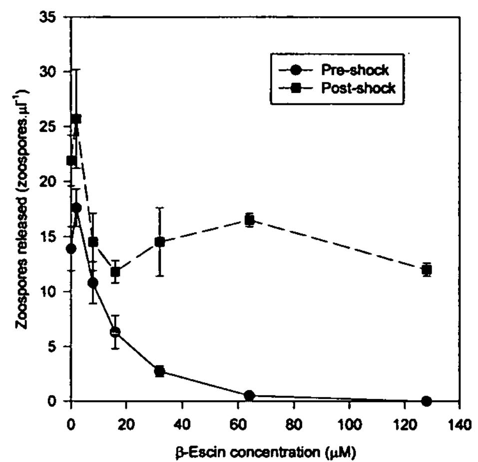


Figure 6.18 β-escin.

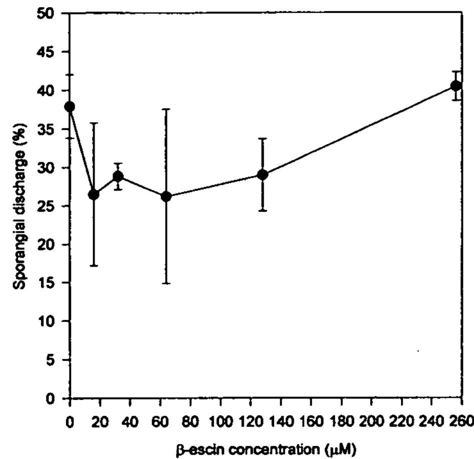


* Means ± s.e.m. for 3 replicates, based on 2 haemocytometer counts per replicate. The concentration of DMSO in all treatments for Figure 6.17 was 27 mM and 22 mM for Figure 6.18. See text for explanation of pre-shock and post-shock.

6.6. Effect of β-escin on zoospore discharge from pre-formed sporangia of *Ph. parasitica.*

This assessment for *Ph. parasitica* differed from above (section 6.5), in that the release solution was supplemented with β-escin instead of mineral salts solution so that sporangiogenesis occurred in unsupplemented mineral salts solution for all treatments. Thus, this experiment determined the effect of β-escin on the ability of pre-formed sporangia of *Ph. parasitica* to discharge their contents. β-escin up to 256 µM had no significant (P = 0.562) effect on sporangial discharge, which ranged from 25% to 41% after 2h (Figure 6.19).

Figure 6.19. The effect of β-escin on the proportion of pre-formed sporangia of *Ph. parasitica* that empty their contents*.



* Means ± s.e.m. for 3 replicates, assessed after 2h, based on score of at least 100 sporangia per replicate. The concentration of DMSO in all treatments was 40 mM.

6.7. The Effect of β-escin and gramicidin S on zoospore motility and subsequent cyst germination of *Py aphanidermatum*.

Zoospores of *Py. aphanidermatum* were incubated for 2h in various concentrations of β-escin and gramicidin S, then the proportion of spores that were motile was determined (see section 2.3.6), along with the proportion of cysts that had subsequently germinated. In the control (no gramicidin S) 30% of zoospores had retained motility (Figure 6.20). A gramicidin S concentration of 200 nM reduced this value to 10% and 4 μM gramicidin S completely suppressed zoospore motility. The concentration of DMSO was proportional to gramicidin S concentration. The maximum concentration of DMSO was 341 μM, but 28 mM DMSO had been shown not significantly to affect zoospore motility (see Table 6.1). Only 9% of zoospores retained motility during the 2h incubation in the β-escin free control (Figure 6.21). However, in the treatment with 16 μM β-escin the proportion was 42%. Concentrations of 64 μM or above caused all zoospores to lose motility.

Figures 6.20 and 6.21. The effects of β-escin and gramicidin S on the proportion of zoospores of *Py. aphanidermatum* that remain motile during the 2h incubation period *.

Figure 6.20 Gramicidin S.

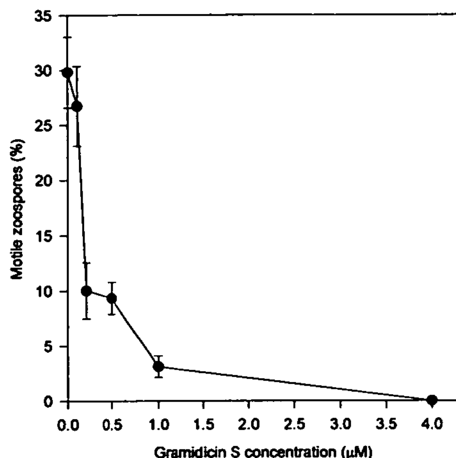
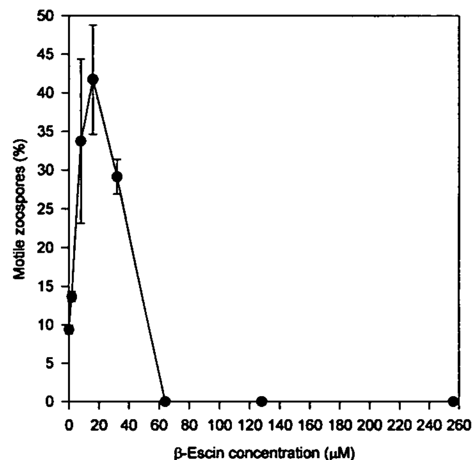


Figure 6.21 β-escin.



* Means ± s.e.m. for 3 replicates, based on 3 random fields of view per replicate. The concentration of DMSO in all treatments for Figure 6.21 was 26 mM and in Figure 6.20 the concentration of DMSO was 85 μM per μM of gramicidin S, so the maximum DMSO concentration was 341 μM.

The immotile cells (presumed to be cysts) were scored for germination after the β-escin and gramicidin S treatments were applied. Only the 4 μM gramicidin S treatment noticeably suppressed subsequent cyst germination of *Py. aphanidermatum*; no cells had germinated after 2h (Figure 6.22). A concentration of 64 μM β-escin increased the proportion of cysts that germinated (Figure 6.23), from 2% in the controls to 18% in the 64 μM treatment. But less than 1% had germinated in the 256 μM β-escin treatment.

Figures 6.22 and 6.23. The effects of β-escin and gramicidin S on the proportion of cysts of *Py. aphanidermatum* that subsequently germinate during the 2h incubation period *.

Figure 6.22 Gramicidin S.

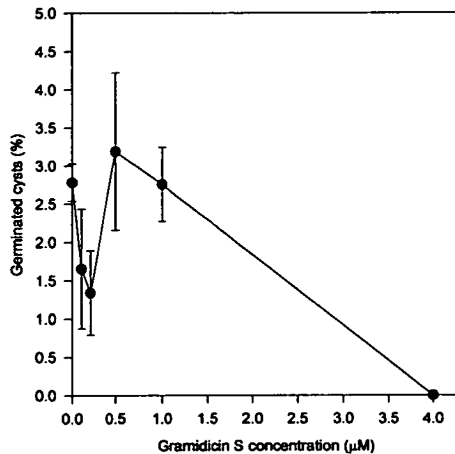
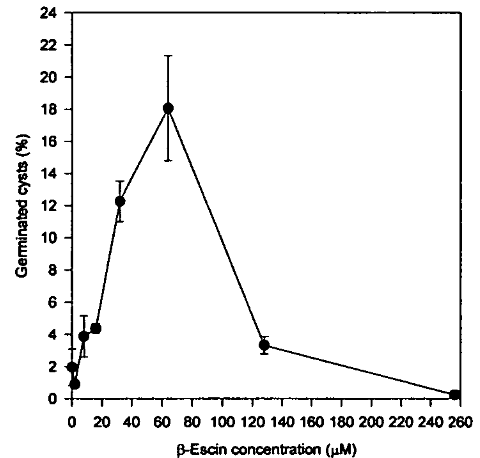


Figure 6.23 β-escin.



* Means ± s.e.m. for 3 replicates, based on 3 random fields of view per replicate. The concentration of DMSO in all treatments for Figure 6.23 was 26 mM and in Figure 6.22 the concentration of DMSO was 85 μM per μM of gramicidin S, so the maximum DMSO concentration was 341 μM.

6.8. Effects of β-escin and gramicidin S on germination of vortex-encysted zoospores of *Py. aphanidermatum*.

Vortex-encysted zoospores of *Py. aphanidermatum* (200 μl) were added to various concentrations of β-escin and gramicidin S (800 μl) immediately after vortexing. After a 2 h incubation in the dark at 23 °C the proportion of cysts that had germinated was determined (see section 2.3.7). Concentrations up to 160 μM of β-escin had no significant (P = 0.412) effect on the germination of vortex-encysted zoospores of *Py. aphanidermatum* (Figure 6.24). However, the 320 μM treatment did significantly (t-test, P = 0.0048) reduce cyst germination to 21% compared with 61% in the β-escin free control. In the gramicidin S free control 59% of cysts had germinated (Figure 6.25). The 500 nM gramicidin S treatment significantly (t-test, P = 0.033) increased germination to 71%, but all higher concentrations reduced the proportion of cysts germinating. No germination occurred in the 4 and 8 μM gramicidin S treatments.

Figures 6.24 and 6.25. The effects of β-escin and gramicidin S on the proportion of vortex-encysted zoospores of *Py. aphanidermatum* that germinate during the 2h incubation period*.

Figure 6.24. β-escin.

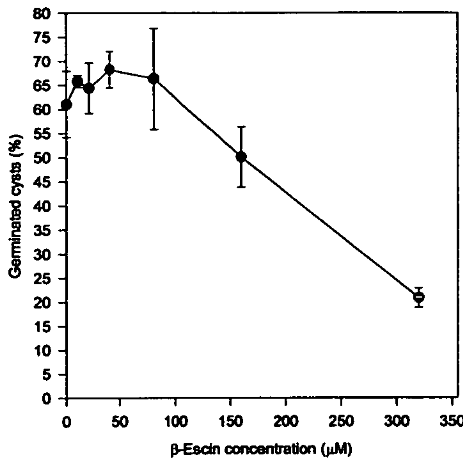
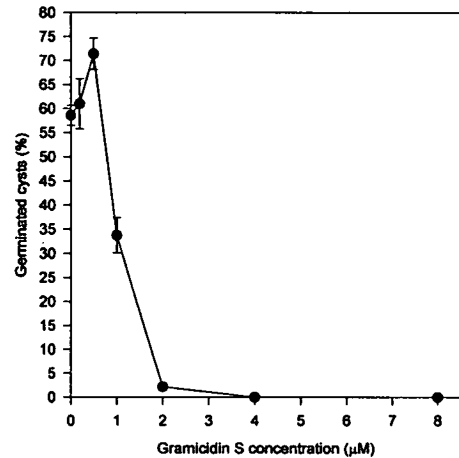


Figure 6.25. Gramicidin S.



* Means ± s.e.m. for 3 replicates, assessed after 2h, based on score of at least 100 cysts per replicate. The concentration of DMSO in all treatments for Figure 6.24 was 33 mM and 682 μM for Figure 6.25.

6.9. The effects of β-escin and gramicidin S with various cationic supplements on germination of zoospores, immature cysts and mature cysts.

The germination of zoospores and mature cysts of *Py. aphanidermatum* were assessed in various 20 mM cationic treatments in either the presence or absence of 3 μM gramicidin S (Tables 6.3 and 6.4). The experiments were done as in Section 6.3. That is, 20 minutes after the treatment was applied to spores concentrated CV8B was added to each Petri dish and germination was assessed after a further 2h. The addition of the broth immediately induced encystment of any motile zoospore and promoted high levels of germination in the gramicidin S-free controls.

Originally motile zoospores of *Py. aphanidermatum* showed 86% germination in SDW controls. The addition of 20 mM Ca^{2+}, Mg^{2+} or Na^{+} alone had marginal effect on zoospore germination (Table 6.3). The presence of 3 μM gramicidin S in the absence of cations reduced zoospore germination to 0.3%. Gramicidin S also reduced zoospore germination to 0.3% in the presence of Na^{+}. However, the presence of 20 mM Ca^{2+} with gramicidin S did not cause a reduction in zoospore germination. Mg^{2+} also counteracted the effect of 3 μM

gramicidin S, but only partly so - to 57% germination. In another experiment (results not shown 20 mM K^+ was as ineffective as Na^+ in counteracting the effect of gramicidin S.

The same experiment was done with 'mature' cysts, pre-vortexed and incubated for 10 min. before the treatments were applied. The germination of mature cysts in SDW was not significantly (P = 0.0874) affected by the addition of 20 mM Ca^{2+}, Mg^{2+}, K^+ or Na^+ supplements (Table 6.4): the mean germination in the absence of 3 μM gramicidin S was 91%. The addition of gramicidin S to the SDW treatment reduced cyst germination to 7%. The presence of either 20 mM Na^+ or K^+ only partially ameliorated the effect of gramicidin S. However, when gramicidin S was added with either 20 mM Ca^{2+} or Mg^{2+} the viability of the cysts was not significantly (t-tests, $P \geq 0.764$) different from that in the equivalent gramicidin S-free treatment.

Table 6.3. The effect of gramicidin S in the presence and absence of cations on germination of originally motile zoospores of *Py. aphanidermatum.**

Cation	No gramicidin S	+ 3 μM Gramicidin S
	% (and Arcsine)	% (and Arcsine)
SDW	86.2 ± 2.6% (68.3 ± 2.1)	0.3 ± 0.3% (1.9 ± 1.9)
Ca^{2+}	87.9 ± 1.6% (69.7 ± 1.4)	84.4 ± 2.3% (66.9 ± 1.8)
Mg^{2+}	68.9 ± 3.7% (56.2 ± 2.3)	56.8 ± 2.1% (48.9 ± 1.2)
Na^+	80.1 ± 1.2% (63.5 ± 0.8)	0.3 ± 0.3% (1.9 ± 1.9)
σ_d	(2.5)	
P (ANOVA)	(2.04×10^{-15})	

* Means ± s.e.m. for 3 replicates, assessed after 2h, based on score of at least 100 cysts per replicate. The concentration of DMSO in all treatments was 1.2 mM. All cations were added as nitrate salts.

Table 6.4. The effect of gramicidin S in the presence and absence of cations on the germination of mature cysts of *Py. aphanidermatum*.*

Cation	No Gramicidin S	+ 3 μM Gramicidin S
	% (and Arcsine)	% (and Arcsine)
SDW	91.3 ± 2.1% (73.1 ± 2.2)	7.3 ± 0.5% (15.6 ± 0.5)
Ca^{2+}	92.5 ± 0.8% (74.1 ± 0.9)	90.3 ± 4.2% (72.7 ± 4.2)
Mg^{2+}	91.6 ± 0.1% (73.1 ± 0.1)	89.7 ± 6.9% (73.3 ± 6.5)
Na^{+}	93.8 ± 1.3% (75.7 ± 1.4)	16.5 ± 4.5% (23.6 ± 3.5)
K^{+}	87.8 ± 1.2% (69.6 ± 1.1)	26.9 ± 4.8% (31.1 ± 3.1)
σ_d	(4.3)	
P (ANOVA)	(7.96×10^{-13})	

* Means ± s.e.m. for 3 replicates, assessed after 2h, based on score of at least 100 cysts per replicate. The concentration of DMSO in all treatments was 3.2 mM. All cations were added as nitrate salts.

Similar experiments to those above were done with *Py. aphanidermatum* and β-escin (Table 6.5). The germination in the SDW control or 20 mM cation treatments was greater than 55%. The addition of β-escin alone (600 μM) reduced germination to only 14%. Supplements of Na^{+} or Mg^{2+} only partly ameliorated this effect, but the addition of either Ca^{2+} or K^{+} at 20 mM concentration completely overcame the inhibitory effect of β-escin.

Table 6.5. The effect of β-escin in the presence and absence of cations on the germination of originally motile zoospores of *Py. aphanidermatum*.*

Cation	No β-Escin	+ 600 µM β-Escin
	% (and Arcsine)	% (and Arcsine)
SDW	82.5 ± 7.9% (66.2 ± 5.8)	14.4 ± 1.6% (22.2 ± 1.3)
Ca^{2+}	68.5 ± 4.6% (56.0 ± 2.9)	61.4 ± 7.9% (51.8 ± 4.8)
Mg^{2+}	59.4 ± 8.0% (50.6 ± 4.8)	16.9 ± 2.5% (24.1 ± 2.0)
Na^{+}	55.8 ± 6.4% (48.4 ± 3.7)	29.2 ± 1.5% (32.7 ± 1.0)
K^{+}	66.8 ± 3.5% (54.9 ± 2.1)	57.3 ± 4.1% (49.2 ± 2.4)
σ_d	(4.9)	
P (ANOVA)	1.12×10^{-7}	

* Means ± s.e.m. for 3 replicates, assessed after 2h, based on score of at least 100 cysts per replicate. The concentration of DMSO in all treatments was 93 mM. All cations were added as nitrate salts.

Further tests examined the effects of different cation concentrations in overcoming the inhibition of gramicidin S and β-escin for *Py. aphanidermatum* (Figures 6.26 and 6.27). In the absence of ions, gramicidin S (3 µM) wholly suppressed zoospore germination (assessed as in Section 6.3) (Figure 6.26). Neither K^{+} nor Na^{+} overcame this suppression at ionic concentrations up to 20 mM. In contrast, Mg^{2+} at 5 mM partly overcame the effect of gramicidin S, but 20 mM Mg^{2+} had no further effect in overcoming suppression. Ca^{2+} had a more pronounced concentration dependent effect in overcoming the suppression; the effect was greatest at 20 mM Ca^{2+} concentration. When the experiment was repeated with 'mature' cysts (Figure 6.27), high concentrations (up to 20 mM) of Na^{+} and K^{+} partly overcame the effect of 3 µM gramicidin S. Supplements of Ca^{2+} and Mg^{2+} were much more effective, such that even 2 mM supplements almost completely overcame the inhibitory effect of gramicidin S.

A similar experiment was done to test the effect of 25 µM β-escin on zoospores of *Ph. parasitica* in the presence and absence of cations (Figure 6.28). The percentage nutrient induced germination of cells was between 30 and 40% in ion-free treatments. Supplements

up to 20 mM Na^+ or Mg^{2+} did not increase the percentage germination, but K^+ or Ca^{2+} supplements of 5 mM or higher significantly increased germination to 60 to 70%.

Figures 6.26 and 6.27. The effects of increasing cation concentration in solutions of 3 μM gramicidin S on the germination of zoospores and mature cysts of *Py. aphanidermatum.*[*]

Figure 6.26 Zoospores.

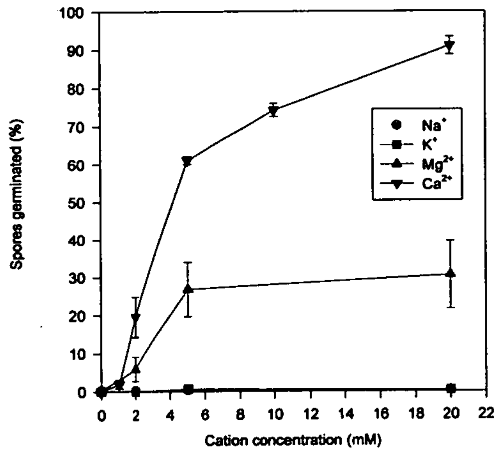
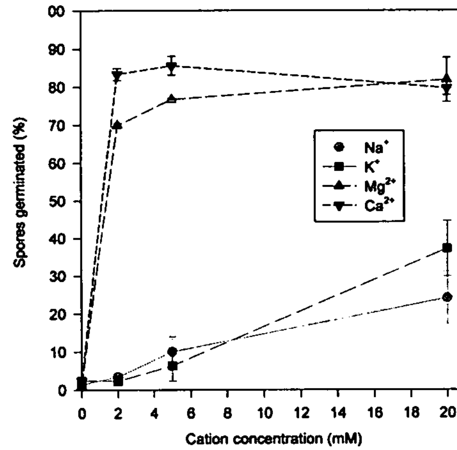
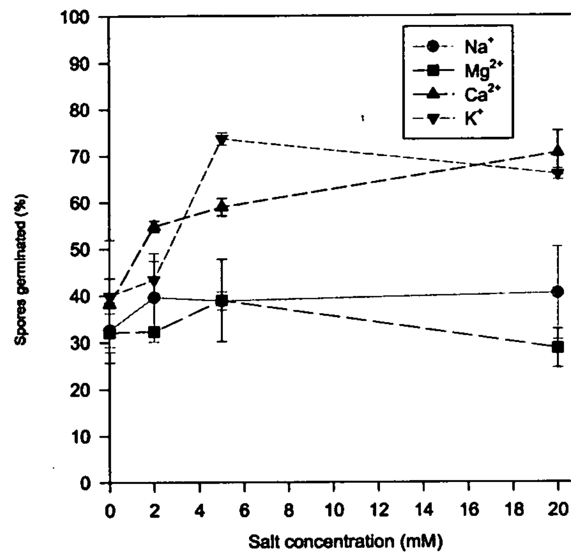


Figure 6.27 Mature cysts.



[*] Means ± s.e.m. for 2 replicates (except for the Ca^{2+} plot in Figure 6.26 when the number of replicates was 3), assessed after 2h, based on score of at least 100 cysts per replicate. The concentration of DMSO in all treatments was 1.2 mM. All cations were added as nitrate salts.

Figure 6.28. The effect of increasing cation concentration in solutions of 25 μM β-escin on the germination of zoospores of *Ph. parasitica.*[•]



[•] Means ± s.e.m. for 2 replicates, assessed after 2h, based on score of at least 100 cysts per replicate. The concentration of DMSO in all treatments was 3.9 mM. All cations were added as nitrate salts.

In two further experiments with zoospores of *Py. aphanidermatum* the effects of Sr^{2+} were compared with those of Ca^{2+}. Both of these ions, at 2 mM concentration or higher, markedly relieved the inhibition caused by 1 μM gramicidin S (Figure 6.29). These ions might have been effective at even lower concentrations, but this was not tested. In contrast, only Ca^{2+} (and not Sr^{2+}) was effective in overcoming the suppression caused by 80 μM β-escin (Figure 6.30). The effect of Ca^{2+} was greatest at the highest tested concentration, but it still only enabled about 25% of the cells to germinate. In the absence of β-escin, but in the presence of 20 mM Ca^{2+} it would be expected that at least 80% of the zoospores would germinate. This value was determined in a separate experiment.

Figures 6.29 and 6.30. The effect of increasing Ca^{2+} or Sr^{2+} concentrations in solutions of 1 μM gramicidin S or 80 μM β-escin on the germination of zoospores of *Py. aphanidermatum*.*

Figure 6.29 Gramicidin S.

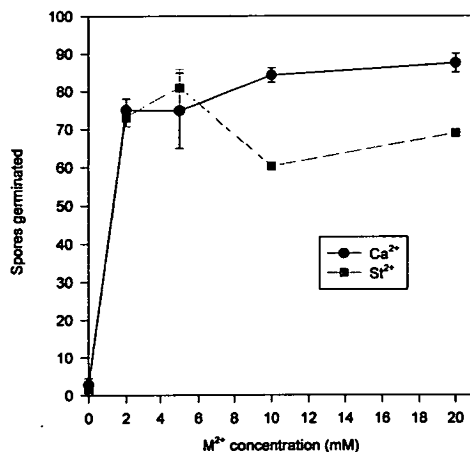
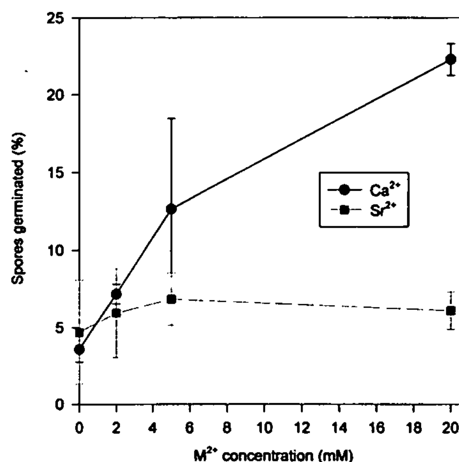


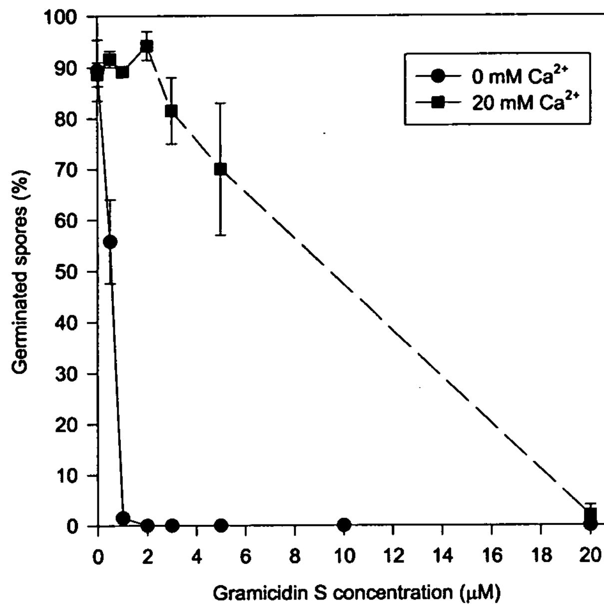
Figure 6.30 β-escin.



* Means ± s.e.m. for 2 replicates in Figure 6.29 and for 3 replicates in Figure 6.30, assessed after 2h, based on score of at least 100 cysts per replicate. The concentration of DMSO in all treatments in Figure 6.29 was 232 μM and 13 mM in Figure 6.30. All cations were added as chloride salts.

In another experiment (Figure 6.31), zoospores of *Py. aphanidermatum* were exposed to either SDW or 20 mM Ca^{2+} mixed with various concentrations of gramicidin S. In the gramicidin S-free solutions with 20 mM Ca^{2+} or without Ca^{2+} the proportion of zoospores that germinated was approximately 91%. Even 2 μM gramicidin S caused an almost total suppression of germination in the absence of Ca^{2+}. In contrast, 5 μM gramicidin S was required to cause an apparent (but not significant) reduction of germination in the presence of 20 mM Ca^{2+}. A concentration of 20 μM gramicidin S was found to completely suppress germination, even in the presence of 20 mM Ca^{2+}.

Figure 6.31. The effect of increasing gramicidin S concentration in solutions of either no Ca^{2+} or 20 mM Ca^{2+} on the nutrient induced germination of zoospores of *Py. aphanidermatum.*[*]



[*] Means ± s.e.m. for 2 replicates, assessed after 2h, based on score of at least 100 cysts per replicate. The concentration of DMSO in all treatments was 11 mM. Calcium was added as a nitrate salt.

6.9.1. The effects of different times of addition of cations on the germination of zoospores of *Py. aphanidermatum* in the presence of β-escin or gramicidin S.

In all the previous experiments (Table 6.3 to 6.5, and Figures 6.26 to 6.31) ions that were tested for their ability to overcome the inhibition of β-escin and gramicidin S were added at the same time as these compounds. Further experiments, described here, were done to determine whether a delayed addition of ions could overcome the effects of the toxic compounds. In each experiment zoospores of *Py. aphanidermatum* were exposed initially to a cation and/or a toxin. Then, after five minutes incubation, the concentration of either the cation or toxin was altered by the addition of an appropriate solution. After a further 20 min incubation concentrated CV8B was added, then after a final 2h incubation the proportion of cells that germinated was determined.

Each treatment initially comprised 1 ml of zoospore suspension to which 3 ml of the treatment (toxin and/or cation solution) was added. After 5 min incubation all treatments

received 200 μl of another solution. After 20 min incubation 466 μl of concentrated CV8B was added to each Petri dish. The determination of germination was as in section 6.3. Throughout the experiment the DMSO concentration in all treatments was constant.

When zoospores were initially exposed to SDW, Ca^{2+} (20 mM) or gramicidin S (1 μM) and Ca^{2+} (20 mM) the proportion of zoospores that germinated was at least 63% (Table 6.6). When cells were initially exposed to 1 μM gramicidin S, with no further treatment, their germination was reduced to 16%. The addition of 20 mM (final concentration) Ca^{2+} to these cells caused no reversal of the effect of gramicidin S. In contrast, gramicidin S (1 μM) caused no toxic effect when added after 5 min. to cells already exposed to 20 mM Ca^{2+}.

Table 6.6. The effect of timing on the ability of calcium to overcome the inhibition of gramicidin S to zoospores of *Py. aphanidermatum.**

Initial treatment	Treatment after 5 min.	Zoospore germination % (and Arcsine)
SDW	No change	83.5 ± 2.4% (66.2 ± 1.9)
1 μM Gramicidin S	No change	16.4 ± 5.4 % (23.4 ± 4.0)
20 mM Ca^{2+}	No change	63.7 ± 3.3% (53.0 ± 2.0)
1 μM Gramicidin S + 20 mM Ca^{2+}	No change	82.9 ± 5.1% (66.0 ± 3.7)
1 μM Gramicidin S	+ 20 mM Ca^{2+}	10.2 ± 3.7% (18.1 ± 3.3)
20 mM Ca^{2+}	+ 1 μM gramicidin S	72.3 ± 3.6% (58.3 ± 2.3)
	σ_d	(4.2)
	P (ANOVA)	(1.19×10^{-7})

* Means ± s.e.m. for 3 replicates, assessed after 2h, based on score of at least 100 cysts per replicate. The concentration of DMSO in all treatments was 803 μM. Calcium was added as a nitrate salt.

The effects of potassium supplements on suppression caused by β-escin were assessed in two experiments (Table 6.7). There was no significant difference between the experiments (2-way ANOVA, $P_{experiment}$ = 0.0582) and there was no significant (2-way ANOVA, $P_{interaction}$ =

0.080) interaction between the data of the two experiments. In both experiments the presence of K^+ alone, when added initially, did not affect the proportion of zoospores that germinated compared to the SDW control. The initial addition of β-escin caused a substantial reduction of zoospore germination. The initial addition of K^+ (20 mM) and β-escin (51 μM) led to some reduction of germination, although not as great as for β-escin alone. The addition of β-escin, after 5 min, to cells exposed to 20 mM K^+ also caused some (low) reduction of germination. The addition of K^+, after 5 min, to cells exposed to β-escin caused some reversal of the toxic effect of β-escin.

Table 6.7. The effect of timing on the ability of potassium to overcome the inhibition of β-escin to zoospores of *Py. aphanidermatum*.*

Initial treatment	Treatment after 5 min	Experiment 1 Zoospore germination % (and Arcsine)	Experiment 2 Zoospore germination % (and Arcsine)
SDW	No change	87.4 ± 1.6% (69.3 ± 1.3)	83.9 ± 3.1% (66.6 ± 2.4)
50.8 μM β-escin	No change	52.2 ± 5.2% (46.3 ± 3.0)	32.9 ± 0.6% (35.0 ± 0.4)
20 mM K^+	No change	90.9 ± 2.2% (72.6 ± 2.1)	88.1 ± 3.9% (70.3 ± 3.4)
50.8 μM β-escin + 20 mM K^+	No change	71.8 ± 4.2% (58.0 ± 2.7)	66.6 ± 2.1% (54.7 ± 1.3)
50.8 μM β-escin	+ 20 mM K^+	62.3 ± 5.0% (52.2 ± 2.9)	64.8 ± 3.6% (53.6 ± 2.1)
20 mM K^+	+ 50.8 μM β-escin	69.6 ± 4.4% (56.7 ± 2.8)	73.8 ± 0.5% (59.2 ± 0.3)
	σ_d	(3.6)	(2.8)
	P (ANOVA)	(7.24×10^{-5})	(4.66×10^{-7})

* Means ± s.e.m. for 3 replicates, assessed after 2h, based on score of at least 100 cysts per replicate. The concentration of DMSO in all treatments was 8 mM. Potassium was added as a nitrate salt.

In a similar experiment (Table 6.8) Ca^{2+} largely overcame the toxic effect of β-escin if Ca^{2+} (20 mM) was added to the zoospore suspension at the same time as β-escin or 5 minutes after β-escin was added.

Table 6.8. The effect of timing on the ability of calcium to overcome the inhibition of β-escin to zoospores of *Py. aphanidermatum*.*

Initial treatment	Treatment after 5 min	Zoospore germination % (and Arcsine)
SDW	No change	83.2 ± 3.6% (66.1 ± 2.8)
50.8 μM β-escin	No change	45.0 ± 5.0% (42.1 ± 2.9)
20 mM Ca^{2+}	No change	77.4 ± 0.9% (61.6 ± 0.6)
50.8 μM β-escin + 20 mM Ca^{2+}	No change	70.1 ± 2.7% (56.9 ± 1.7)
50.8 μM β-escin	+ 20 mM Ca^{2+}	73.4 ± 2.6% (59.0 ± 1.7)
20 mM Ca^{2+}	+ 50.8 μM β-escin	73.8 ± 1.7% (59.2 ± 1.1)
	σ_d	(2.8)
	P (ANOVA)	(4.62×10^{-5})

* Means ± s.e.m. for 3 replicates, assessed after 2h, based on score of at least 100 cysts per replicate. The concentration of DMSO in all treatments was 8 mM. Calcium was added as a nitrate salt.

6.10. The effect of zoospore population density on susceptibility to β-escin and gramicidin S.

Zoospore suspensions of *Py. aphanidermatum* were prepared with various concentrations of zoospores by successive 2-fold dilutions of an originally dense suspension, then a standard concentration of gramicidin S or β-escin was added (Figures 6.32 and 6.33). In the absence of β-escin or gramicidin S dilution of the zoospore suspension had no significant effect on germination assessed as in Section 6.3. In contrast, the degree of inhibition of both β-escin and gramicidin S depended on the zoospore population density. The inhibitive effects were progressively greater at progressively lower zoospore concentrations. In the experiment with

1 μM gramicidin S there was an almost inverse linear relationship with zoospore concentration (Figure 6.32). In the case of β-escin (20 μM), a distinct transition was apparent: a high percentage of cells germinated at zoospore concentrations of 591, 280 and 140 zoospores.μl^{-1}, but further dilution of the zoospore population to 67 or fewer per μl caused a marked increase in cell death caused by β-escin.

Figures 6.32 and 6.33. The effects of gramicidin S and β-escin on the germination* of various population densities of zoospores of *Py. aphanidermatum*.

Figure 6.32 Gramicidin S.

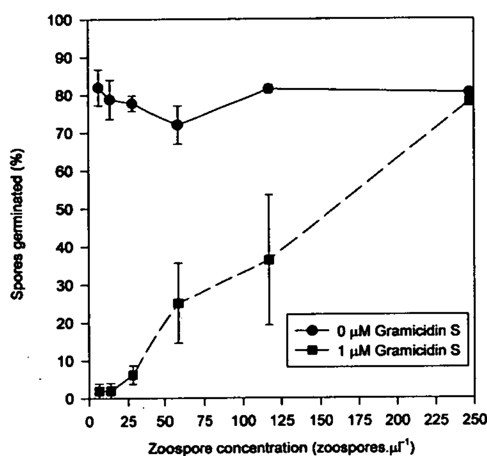
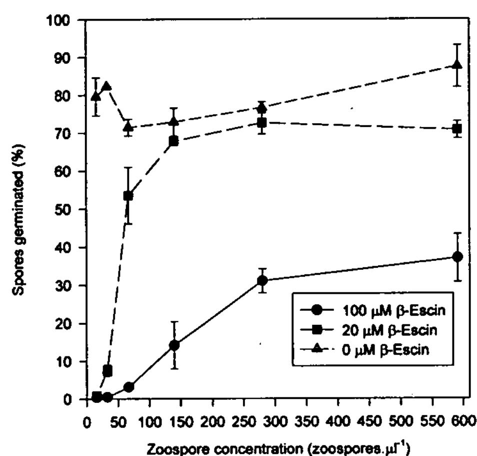


Figure 6.33 β-escin.



* Means ± s.e.m. for 2 replicates, assessed after 2h, based on score of at least 100 cysts per replicate. The concentration of DMSO in all treatments in Figure 6.32was 398 μM and 1.6 mM for Figure 6.33.

6.11. The degradation of β-escin by bacterial isolates from soil.

Four strains of soil bacteria were isolated from single colonies on nutrient agar and used to inoculate 100 ml batches of Mineral Nutrient Solution (see Section 2.1.4) containing 10 mM β-escin. An uninoculated control batch of β-escin was also used. After 9 days at 30 °C in the dark on a rotary shaker (112 rpm) the β-escin solutions were clarified by centrifugation (30 min at 16000 g). Then the solutions at various dilutions were added to suspensions of zoospores of *Py. aphanidermatum*. Only the 10× and 20× dilutions are shown in Table 6.9. In both dilutions the concentration of DMSO was 158 mM, and the concentration of mineral nutrient salts was constant in all treatments and in both dilutions (see Table 6.9). The proportion of cells that germinated was determined after adding concentrated CV8B to each replicate to induce zoospore encystment and germination of viable cells (as in section 6.3).

The β-escin solution that had not been inoculated with bacteria was most inhibitive to zoospores, whereas the β-escin solution pre-incubated in the presence of three bacterial strains was significantly less inhibitive. A fourth bacterial strain, however, did not significantly reduce the inhibition of the original β-escin solution. The four bacteria were not fully characterised but were large, rod-shaped cells typical of *Bacillus* spp.

Table 6.9. The effect of axenic bacterial cultures amended with β-escin at two dilutions on the nutrient induced germination* of zoospores of *Py. aphanidermatum.*

Bacterial strain.	20× dilution % (and Arcsine)	10× dilution % (and Arcsine)
Strain 1 + β-escin.	47.1 ± 1.1 (43.3 ± 0.6 a)	41.5 ± 1.7 (40.1 ± 1.0 a)
Stain 2 + β-escin.	21.0 ± 6.6 (27.0 ± 4.7 b)	14.3 ± 8.5 (21.3 ± 7.3 b)
Strain 8 + β-escin.	46.9 ± 4.4 (43.2 ± 2.5 a)	33.3 ± 0.0 (35.3 ± 0.0 a)
Strain 10 + β-escin.	44.1 ± 2.0 (41.6 ± 1.1 a)	28.8 ± 1.8 (32.5 ± 1.2 a)
Control (no bacteria) + β-escin.	18.7 ± 0.3 (25.6 ± 0.2 b)	11.9 ± 1.6 (20.1 ± 1.4 b)
P (ANOVA)	(6.91×10^{-3})	(2.97×10^{-2})
σ_d	(3.5)	(4.8)
$LSD_{0.05}$	(7.9)	(10.8)

* Means ± s.e.m. for 2 replicates, assessed after 2h, based on score of at least 100 cysts per replicate. Concentration of salts in each treatment were 3.7 mM of Na_2HPO_4, 2.2 mM of KH_2PO_4, 574 μM of K_2SO_4, 1.7 mM of NaCl, 81 μM of $MgSO_4 \cdot 7H_2O$, 680 nM of $CaCl_2 \cdot 2H_2O$ and 36 nM of $FeSO_4 \cdot 7H_2O$.
Values followed by the same letter do not differ significantly at $P = 0.05$.

6.12. The effect of crude saponin extracts from oat seedlings on the motility of zoospores, the germination of cysts and the nutrient induced germination of zoospores.

Crude oat extracts were prepared from the root tips, leaves and seeds with roots, but with the root tips removed, as explained in Section 2.1.5. The extracts were added at either their original strength or at one-tenth strength to suspensions of zoospores of *Py.*

aphanidermatum. After 2 h incubation the proportion of the original zoospores that were motile and the proportion of cysts that had germinated were determined (as in Section 2.3.7) (Table 6.10). After a further 2h incubation an aliquot of each treatment was removed, vortexed and the number of cysts enumerated by haemocytometer counts.

The proportion of spores observed to be motile after two hours of incubation in the SDW control was 27%. In treatments with either 6.10 $mg.ml^{-1}$ of root tip extract or 32.6 $mg.ml^{-1}$ of leaf extract no zoospores were observed to be motile at 2h. However, one-tenth dilutions of these solutions did not completely suppress zoospore motility. A solution containing 29 $mg.ml^{-1}$ of 'seed-root' extract caused no reduction of zoospore motility compared to the control, but a one-tenth dilution of this extract raised the proportion of motile spores compared to the SDW control. The results of the assessment of zoospore numbers at 4h were significantly correlated ($R = 0.90$, $F = 3.46 \times 10^{-8}$, $df = 20$) with the results of the assessment of motility at 2h (based on analysis of the non-transformed data). When cyst germination was measured at 2h only 2.6% of cysts had germinated in the SDW control. All types and concentrations of the oat extracts significantly increased the proportion of cysts that had germinated. The proportion of cysts that germinated in an extract did not necessarily correlate with concentration of the extract. For example, 68% of cysts had germinated in 29 $mg.ml^{-1}$ of seed and root extract, whereas 90% had germinated in 6 $mg.ml^{-1}$ of root tip extract.

Table 6.10. The effect of crude oat extracts on the motility and subsequent germination of zoospores of *Py. aphanidermatum*.

Oat extract	Motility (2h)* % (and Arcsine)	Motility (4h)** zoospores.μl^{-1}	Subsequent germination (2h)* % (and Arcsine)
Control	26.9 ± 0.2 (31.2 ± 0.1)	7.5 ± 0.8	2.6 ± 1.3 (7.6 ± 3.8)
610 $\mu g,ml^{-1}$ root tip	43.0 ± 1.4 (40.9 ± 0.8 a)	21.5 ± 1.8 a	60.4 ± 8.5 (51.2 ± 5.1 b)
6.10 $mg.ml^{-1}$ root tip	0.0 ± 0.0 (0.0 ± 0.0 b)	0.8 ± 0.2 b	89.8 ± 2.0 (71.5 ± 1.9 a)
3.26 $mg.ml^{-1}$ leaves	42.2 ± 1.1 (40.5 ± 0.6 a)	36.9 ± 4.7	62.4 ± 4.5 (52.3 ± 2.7 b)
32.6 $mg.ml^{-1}$ leaves	0.0 ± 0.0 (0.0 ± 0.0 b)	1.7 ± 1.2 b	91.4 ± 0.4 (73.0 ± 0.4 a)
2.90 $mg.ml^{-1}$ seeds and roots	44.5 ± 0.5 (41.8 ± 0.3 a)	29.8 ± 3.1	54.2 ± 4.2 (47.4 ± 2.4 b)
29.0 $mg.ml^{-1}$ seeds and roots	31.2 ± 1.1 (34.0 ± 0.7)	22.4 ± 4.2 a	68.3 ± 3.5 (55.8 ± 2.2 b)
P (ANOVA)	(4.47×10^{-19})	6.48×10^{-10}	(7.77×10^{-9})
σ_d	(0.7)	2.3	(4.2)
$LSD_{0.05}$	(1.5)	4.9	(9.0)

*Means ± s.e.m. for 3 replicates, assessed after 2h, based on three fields of view per replicate.
** Means ± s.e.m. for 3 replicates, assessed after 4h, based on two haemocytometer counts per replicate.
Values followed by the same letter do not differ significantly at $P = 0.05$.

The same treatments as above were applied to suspensions of vortex-encysted zoospores of *Py. aphanidermatum*. Cysts in the SDW control showed only 29% germination after 2h incubation at 23 °C in the dark (Table 6.11). All crude oat extracts significantly increased the proportion of cysts that had germinated when compared to the control, the lowest level of cyst germination in any crude oat extract being 87%. As before, the level of cyst germination was not simply related to the amount ($mg.ml^{-1}$) of crude extract.

Table 6.11. The effect of crude oat extracts on percentage germination of vortex-encysted zoospores of *Py. aphanidermatum*.*

Crude extract	% germination	Arcsine.
Control	29.2 ± 2.3	32.7 ± 1.4
610 $\mu g,ml^{-1}$ root tip	87.0 ± 1.8	68.9 ± 1.6 c
6.10 $mg.ml^{-1}$ root tip	96.7 ± 0.9	79.7 ± 1.4 a
3.26 $mg.ml^{-1}$ leaves	91.9 ± 2.7	73.8 ± 2.8 b,c
32.6 $mg.ml^{-1}$ leaves	97.0 ± 1.3	80.3 ± 2.0 a
2.90 $mg.ml^{-1}$ seeds and roots	91.2 ± 0.5	72.7 ± 0.5 b,c
29.0 $mg.ml^{-1}$ seeds and roots	95.4 ± 0.5	77.6 ± 0.6 a,b
P (ANOVA)		8.41×10^{-11}
σ_d		2.3
$LSD_{0.05}$		4.9

*Means ± s.e.m. for 3 replicates, assessed after 2h, based on 100 cysts per replicate.
Values followed by the same letter do not differ significantly at $P = 0.05$.

Serial dilutions of 'oat root tip' extract and 'root and seed' extract were also tested for their effects on the germination of zoospores of *Py. aphanidermatum* (germination being determined as in Section 6.3). High concentrations of both types of extract caused a marked suppression of cell germination; whereas lower concentrations had little effect (Figures 6.34 and 6.35). The conversion of percent germinated to probits resulted in very highly significant ($F \leq 21.9 \times 10^{-6}$, $R \geq 0.85$, $df = 19$) correlations between probit value of cell germination and oat extract concentration. From lines of best fit, LD_{50} values were determined to be 9.3 $mg.ml^{-1}$ of root tip extract, and 38.0 $mg.ml^{-1}$ of 'seed and root' extract.

Figures 6.34 and 6.35. The effects of crude oat extracts on the nutrient induced germination of zoospores of *Py. aphanidermatum.*[•]

Figure 6.34 Extract from seeds and roots.

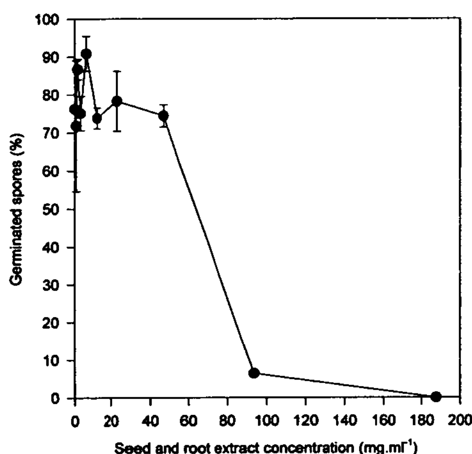
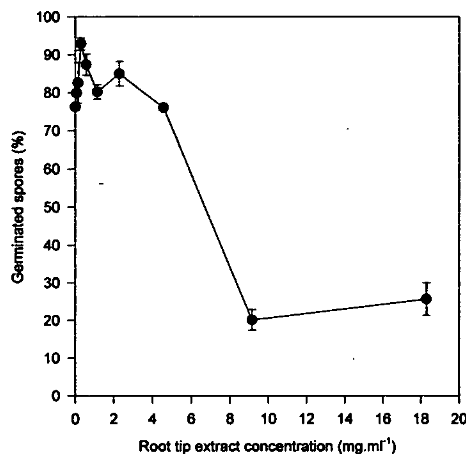


Figure 6.35 Extract from root tips.



[•] Means ± s.e.m. for 3 replicates, assessed after 2h, based on score of at least 100 cysts per replicate.

6.13. Production of zoospore-inhibitive compounds by *Brevibacillus brevis.*

A strain of *B. brevis* (w.t.) known to produce gramicidin S was cultured in various liquid media (Section 2.4), then the crude culture filtrates were assessed for effects on zoospore germination (method as in Section 6.3) (Table 6.12). For comparison, a mutant strain of *B. brevis* known to lack the ability to produce gramicidin S was also used to inoculate some broths and their effects on zoospore germination were also tested.

When *B. brevis* (w.t.) was cultured in CV8B, malt extract broth, a gelatine solution, potato extract broth or nutrient broth, the germination of zoospores was not significantly (t-tests, $P \geq 0.202$) different from that of the corresponding uninoculated broth control. In all uninoculated broths except potato extract broth (which seemed to be inhibitory to zoospores) the proportion of zoospores viable was at least 57%.

In several cases the broths inoculated with the wild-type strain of *B. brevis*, and incubated for 2 days, were highly inhibitory to zoospores, compared with the corresponding uninoculated

broth. This was true for inoculated neutralised bacteriological peptone broth, peptone P broth, bacteriological peptone broth, and yeast extract broth. However, little or no inhibition was observed for inoculated CV8 broth, malt extract broth, gelatine broth and potato extract broth. Only a few types of broth were inoculated with the gramicidin S-negative mutant strain of *B. brevis*, but in all of these cases there was either no evidence of inhibition to zoospores or significantly less inhibition than that of the corresponding broth inoculated with wild-type *B. brevis*.

In one test, 4-day-old yeast extract broths were compared for their effects on zoospores of both *Ph. parasitica* and *Py. aphanidermatum* (Table 6.12). Both fungi were found to be sensitive to the broth inoculated with the wild-type strain of *B. brevis*. But only *Ph. parasitica* was sensitive to the broth inoculated with the mutant strain of *B. brevis* (and even then the degree of inhibition was less than that caused by the broth inoculated with the wild-type strain).

Yeast extract broth was inoculated with *B. brevis* (w.t.), incubated for 4 days, then clarified by centrifugation (15000 g for 30 min) and diluted with uninoculated yeast extract broth. To these dilutions of inoculated broth zoospores of *Py. aphanidermatum* were introduced, and the proportion that germinated was assessed after 2h (as in Section 6.3). In uninoculated broth 96% of zoospores were found to germinated (Figure 6.36). Increasing the ratio of inoculated to uninoculated broth caused the proportion of zoospores that germinated to decrease. In quarter-strength (ratio 1:3) inoculated broth only 1.5% of zoospores were found to be germinated. The relationship between the concentration of inoculated broth (% v/v) and germination was sigmoid (Figure 6.37). The regression of the probit transformed line was very highly significant ($R = 0.96$, $F = 4.89 \times 10^{-11}$, $df = 19$) and gave a probit value of 5.0 (LD_{50}) corresponding to a concentration of 8.52% inoculated yeast broth.

The LD_{50} of gramicidin S with zoospores of *Py. aphanidermatum* was previously (Table 6.2) determined as 462 nM. Assuming that the suppression of zoospore germination was wholly caused by gramicidin S production and that the yeast extract broth does not affect the efficacy of gramicidin S, then production of gramicidin S by *B. brevis* in yeast extract broth is equivalent to 6.6 $mg.l^{-1}$ (5 μM) over four days.

Table 6.12. The effects of different broth cultures of *Brevibacillus brevis* on the germination [arcsine (and %)] of zoospores*. The broths were either tested alone (uninoculated) or after a wild-type (w.t.) strain of *B. brevis* had grown in them. In some cases the broths had been inoculated with a strain of *B. brevis* (E-1) that does not produce gramicidin S.

Broth	Clarified[a]	Fungus	Incubation Length	Volume	Control[b]	*B.brevis* (w.t.)	*B.brevis* (E.1.)	*P* (ANOVA)	$LSD_{0.05}$
Neutralised bacteriological Peptone	No	*Py. aphanidermatum*	2 days	200 ml	66.8 ± 6.7 (84%)	43.8 ± 2.9 (48%)	n.d.	3.43×10^{-2}	n.a.
Neutralised bacteriological Peptone	No	*Py. aphanidermatum*	2 days	200 ml	70.2 ± 1.0 (89%)	21.0 ± 2.6 (13%)	64.0 ± 1.6 (81%)	3.07×10^{-6}	6.5
Peptone P.	No	*Py. aphanidermatum*	2 days	200 ml	68.9 ± 3.2 (87%)	9.0 ± 1.7 (2%)	n.d.	7.62×10^{-5}	n.a.
Bacteriological Peptone	No	*Py. aphanidermatum*	2 days	200 ml	63.8 ± 3.6 (81%)	9.7 ± 1.6 (3%)	n.d.	1.63×10^{-4}	n.a.
CV8B	No	*Py. aphanidermatum*	2 days	200 ml	56.1 ± 0.8 (69%)	53.8 ± 1.6 (65%)	n.d.	2.73×10^{-1}	n.a.
Malt Extract	No	*Py. aphanidermatum*	2 days	200 ml	58.4 ± 3.3 (73%)	62.9 ± 3.4 (79%)	n.d.	4.06×10^{-1}	n.a.
Gelatine	No	*Py. aphanidermatum*	2 days	200 ml	48.9 ± 2.2 (57%)	55.5 ± 2.2 (68%)	n.d.	2.02×10^{-1}	n.a.
Potato extract	No	*Py. aphanidermatum*	2 days	200 ml	8.5 ± 1.4 (2%)	7.3 ± 0.9 (2%)	n.d.	4.80×10^{-1}	n.a.
Nutrient Broth	No	*Py. aphanidermatum*	2 days	200 ml	71.7 ± 2.1 (90%)	70.3 ± 2.0 (89%)	69.1 ± 2.1 (87%)	6.92×10^{-1}	n.a.
Yeast Extract	No	*Py. aphanidermatum*	2 days	200 ml	64.7 ± 0.5 (82%)	0.0 ± 0.0 (0%)	n.d.	1.75×10^{-8}	n.a.
Yeast Extract	No	*Py. aphanidermatum*	2 days	200 ml	80.5 ± 1.7 (97%)	8.5 ± 1.4 (2%)	49.5 ± 3.4 (58%)	2.04×10^{-6}	8.2
Yeast Extract	No	*Py. aphanidermatum*	3 days	500 ml	77.2 ± 1.4 (95%)	4.6 ± 2.4 (1%)	70.9 ± 1.8 (89%)	2.97×10^{-7}	6.6
Yeast Extract	Yes	*Py. aphanidermatum*	4 days	500 ml	75.0 ± 1.5 (93%)	0.0 ± 0.0 (0%)	60.9 ± 1.6 (76%)	2.82×10^{-8}	4.4
Yeast Extract	Yes	*Ph. parasitica*	4 days	500 ml	52.0 ± 2.9 (62%)	1.9 ± 1.9 (0%)	23.9 ± 2.9 (18%)	3.04×10^{-5}	9.0

* Means ± s.e.m. for 3 replicates, assessed after 2h, based on score of at least 100 cysts per replicate.
[a] Clarified by centrifugation (15000 g for 30 min).
[b] Uninoculated broth.
n.d. Not determined.
n.a. Not applicable.

Figures 6.36 and 6.37. The effect of diluting yeast extract broth inoculated with *B. brevis* (wild type) on the viability of zoospores of *Py. aphanidermatum.**

Figure 6.36 Untransformed data.

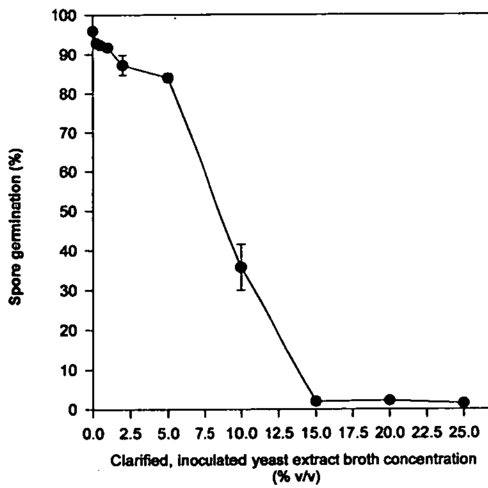
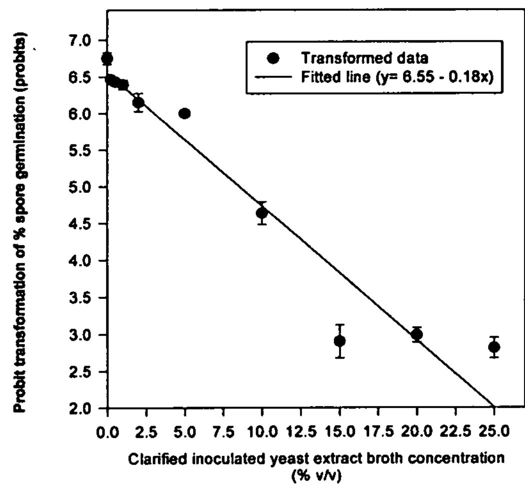


Figure 6.37 Probit transformed data.



* Means ± s.e.m. for 2 replicates, assessed after 2h, based on score of at least 100 cysts per replicate.

6.14. *Brevibacillus brevis* (wild-type) produces a haemolytic compound.

When *B. brevis* (wild-type) was cultured on blood agar (at 23 °C in the dark for 7 days), a zone of haemolysis appeared around the colonies (Figure 6.38), whereas no such zone was observed when the mutant strain (E-1) was cultured under identical conditions (Figure 6.39).

Figure 6.38. *Brevibacillus brevis* (wild-type) cultured on blood agar* for 7 days.

* *B. brevis* (wild-type) cultured for 7 days at 23 °C in the dark on 6% (v/v) horse blood agar. Agar plate was scanned using a flat-bed scanner (Hewlett Packard ScanJet 6100C/T) to produce the image.

Figure 6.39. *Brevibacillus brevis* (E1) cultured on blood agar* for 7 days.

* *B. brevis* (E-1) cultured for 7 days at 23 °C in the dark on 6% (v/v) horse blood agar. Agar plate was scanned using a flat-bed scanner (Hewlett Packard ScanJet 6100C/T) to produce the image.

6.15. Discussion.

In the majority of experiments presented in this chapter DMSO concentration was constant in each treatment of the experiment. Although 28 mM DMSO was shown not to affect significantly sporangiogenesis of *Ph. parasitica* or the motility and subsequent germination of zoospores of *Py. aphanidermatum,* there is considerable evidence that DMSO does affect biological membranes. DMSO up to 40% w/w (5.3 M) is commonly employed in long-term cryopreservation of cells by virtue of its cryoprotectant properties (Lovelock & Bishop, 1959). DMSO also increases solute permeability across cell membranes (Anchordoguy *et al.*, 1992). Increased solute permeability is due, in part, to DMSO reducing the diffusion pathway across the membrane (Yu & Quinn, 1998). DMSO could also enhance the partition coefficient of solutes between the aqueous phase and the membrane. These findings were determined with DMSO at molar concentrations. The maximum concentration of DMSO used in the experiments of this chapter was 324 mM, and this was an exceptional case. In most experiments the DMSO concentration was less than 40 mM. Reports that DMSO increases membrane permeability do not suggest that a consequence of increased permeability is reduced membrane stability (Anchordoguy *et al.*, 1992).

In Table 6.13 are summarised the main findings of this chapter. The LD_{50} of β-escin is three to five orders of magnitude greater than of gramicidin S. The LD_{50} of gramicidin S is relatively unaffected by the maturity of cyst wall, whereas increase of maturity of cyst wall makes the cells less susceptible to β-escin. All three divalent cations tested (Ca^{2+}, Mg^{2+} and Sr^{2+}) antagonised the effect of gramicidin S, whereas the two monovalent cations (Na^{+} and K^{+}) did not alter the effectiveness of gramicidin S. Calcium and potassium cations could antagonise the effect of β-escin, but their effectiveness was not as marked as the antagonism of gramicidin S by divalent cations.

Neither toxin at concentrations tested could suppress the formation of sporangia of *Ph. parasitica*. However, β-escin did noticeably suppress the emptying of sporangia and the number of zoospores released. Both β-escin and gramicidin S suppressed zoospore motility, but at 60 μM β-escin significantly increased the proportion of cysts that subsequently germinated. However, both toxins suppressed the germination of vortex-encysted zoospores.

Table 6.13 Summary of the effects of β-escin and gramicidin S.

	β-escin	Gramicidin S
LD_{50} of zoospores	~35 μM	~ 500 nM
LD_{50} of immature cysts	> 270 μM	~ 500 nM
LD_{50} of mature cysts	> 1.0 mM	~ 750 nM
Antagonised by Ca^{2+}	Yes	Yes
Antagonised by Sr^{2+}	No	Yes
Antagonised by Mg^{2+}	No	Yes
Antagonised by K^{+}	Yes	No
Antagonised by Na^{+}	No	No
Sporangial density	No effect	No effect
Subsequent zoospore release (sporangiogenesis)	Suppressed	No effect
Sporangia discharging their contents (sporangiogenesis)	Suppressed	Slight suppression
Zoospore release from pre-formed sporangia	No effect	Not tested
Zoospore motility	Suppress	Suppressed
Subsequent cyst germination	Peak at 60 μM	Suppressed
Germination of vortex-encysted zoospores	Suppressed	Suppressed
Degraded by soil bacteria	Yes	Not tested

The effect of membrane disruption by either β-escin or gramicidin S is increased leakage of cytoplasmic material and loss of membrane integrity (Katsu *et al.*, 1988). The mechanism by which these two toxins operate is different. It was microscopically observed that, at concentrations above the LD_{50} concentration the non-viable (that is, non-functioning water expulsion vesicle) cells were different for the two toxins. With gramicidin S virtually all unhealthy cells were intact, whereas with β-escin a third of the unhealthy cells were not intact.

β-escin, a triterpeneglycoside saponin from *Castanea sativa*, binds irreversibly to sterols of membranes and forms an insoluble complex. This complexing to membrane-bound sterols causes pore formation, loss of electrochemical concentration gradients and the loss of membrane integrity (Fenwick *et al.*, 1992). So the primary mode of action of saponins is similar to that of the polyene macrolide antibiotics (Osbourn, 1996). β-escin has already been shown to be effective at reducing zoospore viability – as determined by cytological examination (Deacon & Mitchell, 1985). However, Olsen (1971a, 1971b, 1971c) found that the triterpeneglycosides were

unable to inhibit mycelial growth, reduce endogenous respiration, induce leakage of inorganic ions, or inhibit uptake of potassium, magnesium and inorganic phosphate in hyphae of *Py. irregulare*. In other fungi tested, particularly *Gaeumannomyces graminis* var. *tritici,* the triterpeneglycoside was highly inhibitory to mycelial growth. Later work by Olsen (1973) found a correlation between the sterol content of fungi and the inhibitory effect of avenacin, another triterpeneglycoside saponin. A characteristic of the oomycota is the inability to synthesise sterols *de novo* (Elliott, 1983). Arneson and Durbin (1968) found that *Pythium* and *Phytophthora* were resistant to α-tomatine (a steroidal glycoalkaloid). The insensitivity of the oomycetes to α-tomatine was attributed to the absence of membrane sterols. However, reproduction by the Pythiaceae, including the asexual production of zoospores, requires sterols; and so zoospores have sterols in their membranes.

The presence of a cell wall conferred increased tolerance to β-escin on zoospores of *Ph. parasitica* and *Py. aphanidermatum.* Also a noticeable increase in tolerance was observed between cysts with immature and mature cell walls. Furthermore, the production of sporangia of *Ph. parasitica* was not affected by β-escin concentration up to 128 μM. These results suggest that the cell wall acts as a barrier to β-escin. Oomycetes differ from the eumycetes in having β-glucans (cellulose) instead of chitin as the principal cell wall component (Bartnicki-Garcia & Wang, 1983). Therefore, it is possible that the differences in the sensitivity to β-escin between the oomycetes and the eumycetes are not only attributed to the differing quantities of membrane bound sterols, but also to the different components of the cell walls. The observation that 64 μM β-escin increased the proportion of cysts that germinated when encystment had occurred in the presence of β-escin, but failed to affect germination of pre-encysted zoospores, supports the hypothesis that walls of the oomycetes act as a barrier to β-escin. The ability of β-escin to increase the level of cyst germination cannot be explained here, but may possibly be attributed to the glucose moiety of β-escin inducing germination. D(+)glucose was clearly shown to induce germination of three species of *Pythium* by Donaldson (1992).

Gramicidin S is a cyclic decapeptide, (L-Val-L-Orn-L-Leu-D-Phe-L-Pro-)$_2$, produced by *Brevibacillus brevis* (Katsu *et al.*, 1988). The β-sheet structure is well characterised with the two cationic ornithine residues on one side of the molecular plane and the hydrophobic residues on the other side (Katsu *et al.*, 1988). Many hydrophobic antibiotics cannot permeate the compound

structure of the cell envelope of Gram-negative bacteria (Nikaido and Vaara, 1995). Their action is usually limited to disruption of the outer (lipopolysaccharide) membrane. However, gramicidin S increases the permeability of both the outer and the inner cytoplasmic membrane of *Escherichia coli* (Katsu *et al.*, 1986). This adsorbtion of gramicidin S damages and prevents the functioning of the plasmamembrane of both Gram-positive and Gram-negative bacteria (Yonezawa *et al.*, 1981), although the accumulation of gramicidin S is predominantly in the outer leaflet of the phospholipid bilayer (Katsu *et al.*, 1989; Lewis *et al.*, 1998). Gramicidin S forms salt bridges with phosphate groups of two neighbouring phospholipid molecules. The effectiveness of the antibiotic is attributed to the rigidity of the peptide. The result of gramicidin S-phospholipid bridges is a mosaic structure in the membrane, in which phospholipid clusters are surrounded by lipid-gramicidin S complexes. In turn, this facilitates the formation of a collodial solution (Ovchinnikov and Ivanov, 1982). Katsu *et al.* (1988) investigating the action of gramicidin S on human erythrocytes, observed the formation of a collodial solution, and reported that this was facilitated by gramicidin S stimulating the movement of acyl chains of the lipids. Therefore, the mechanism of gramicidin S is debatable, but clearly involves interaction with the phospholipids and not the membrane bound sterols (Mihailescu & Horvath, 1999).

The ability of gramicidin S to interfere with the internal membrane of Gram-negative bacteria is mirrored by the observations that the presence of the cell wall does not affect the inhibition of gramicidin S. That is, gramicidin S passes across the cell wall unaffected. The ability of gramicidin S to move across the cell wall of zoospore cysts suggests that it also will affect the membrane of hyphae and sporangia. It was clear that gramicidin S inhibited the germination of vortex-encysted zoospores, but had negligible effect on sporangiogenesis of *Ph. parasitica* or the subsequent discharging of zoospores from sporangia. This paradox can be resolved by the finding (Figure 6.32) that the inhibition of a given concentration of gramicidin S was inversely proportional to the numbers of fungal zoospores in a suspension. That is, increasing the area of zoospore membrane in a fixed quantity of gramicidin S results in the amount of gramicidin S being too low to cause lethal damage to a membrane. Therefore, it is not surprising that when a whole mycelial mat is subjected to 32 μM gramicidin S there is little effect on sporangiogenesis. Mycelial mats exposed to increasing concentrations of β-escin did show suppressed sporangiogenesis. However, the range of β-escin concentrations used included higher concentrations than were used for gramicidin S. A second way to resolve the paradox is based on

differing wall components in germinating cysts and sporangia. That is, gramicidin S can inhibit the biogenesis of wall material in germ tubes, but not in sporangia.

Zoospores of *Py. aphanidermatum* were more susceptible to both toxins than were the spores of *Ph. parasitica*. Generally the zoospore populations of *Py. aphanidermatum* were approximately fifty times more dense than the populations from *Ph. parasitica*. It has been clearly shown that the lower the zoospore density the greater the sensitivity to either β-escin or gramicidin S. Zoospores of both species are similar in size (approximately 10 μm long), so the observed difference in toxin tolerance between these species cannot be explained by experimental factors. Therefore, it can be concluded that zoospores of *Ph. parasitica* are markedly more tolerant of β-escin and gramicidin S than zoospores of *Py. aphanidermatum*.

For the steroidal saponins, 26-desglucoavenacides A and B, of oat tissues, the pathogen *Septoria avenae* f. sp. *avenae* detoxifies the saponin by enzymatic hydrolysis of L-rhamnose and D-glucose (Wubben *et al.*, 1996). Armah *et al.* (1999) have shown that an intact sugar moiety of avenacin A-1 is required to reorganise membrane cholesterol into pores, and that saponin activity was abolished by the removal of any of the monosaccharide residues. The detoxification of the saponin α-tomatine by *Botrytis cinerea* is also by deglycosylation (Quidde *et al.*, 1998). The enzymic detoxification of the saponins, avenacin A-1 and α-tomatine, is by physicochemically similar, immunologically identical and highly homologous steroidal glycoalkaloid enzymes (Osbourn *et al.*, 1995). The degradation of β-escin by three isolates of soil bacterium is perhaps due to the cleavage of the glucose moiety from β-escin, although there is also a possibility that some β-escin was physically absorbed onto bacterial cell components. The inability of the other strain (strain 2) to detoxify β-escin could be attributed to either inability to remove the glucose moiety or lack of β-escin absorption.

The production of a gramicidin S-like substance by the wild type *B. brevis* was established. However, the quantity of presumptive compound that was calculated to be produced (6.6 $mg.l^{-1}$) is probably a conservative estimate. In the broths where gramicidin S was produced divalent cations were present and would antagonise the effectiveness of gramicidin S. Uninoculated potato broth was the only broth tested that was inhibitive to zoospores of *Py. aphanidermatum,* and was the broth with the highest carbohydrate to total nitrogen ratio. However, this correlation does not explain the observed inhibition. All other broths with the exception of CV8B are high

in protein or protein digests. Dry peptone P, neutralised bacteriological peptone and bacteriological peptone all have a similar concentration (2.4 to 2.7 % w/w) of nitrogen in amino form. The concentration of amino nitrogen in yeast extract before the addition of water is 5.1% w/w; however, this broth contained the lowest concentration of total nitrogen of the 'digest' broths. Although the pH of the broths was not identical, it was not correlated with gramicidin S production. Why gramicidin S production by *B. brevis* in yeast extract was superior to that in the other broths is unknown, but antibiotic production and spoliation are influenced by the composition of growth media (Edwards, 1993).

The sporangia of *Phytophthora* spp. are walled structures with zoospores being liberated when the papillum ruptures. In *Saprolegnia terrestris* high turgor pressure in the sporangial lumen forces the papillum to rupture (Money and Webster, 1989). Both gramicidin S and β-escin destabilise membranes and it would be assumed that they would facilitate increased sporangial discharge. In this work no evidence was found to support this hypothesis. On the contrary, both toxins suppressed sporangial discharge. Increased permeability of the sporangial membrane may explain how the toxins inhibit sporangial discharge. Elevated membrane permeability could dissipate the turgor difference across the membrane. However, Olsen (1971a, 1971b) found that β-escin caused inorganic ions and UV-absorbing material to leak from several genera of fungi, but not from *Py. irregulare* where β-escin-induced leakage was nil.

Both toxins suppressed zoospore motility, with a LD_{50} concentration, based on subsequent cyst germination, being enough to immobilise most zoospores. Low concentrations of β-escin elevated the fraction of spores that were motile. Encystment of zoospores can be induced by many chemicals *in vitro*, including high concentrations of salts or specific amino acids (Jones *et al.*, 1991), lectins (Hardham and Suzaki, 1986) and various polysaccharides (Irving and Grant, 1984; Grant *et al.*, 1985; Estrada-Garcia *et al.*, 1990; Zhang *et al.*, 1990). Donaldson and Deacon (1993c) found that all the monosaccharides and the disaccharides they tested, including 25 mM D(+)glucose, induced encystment in *Pythium* spp. The curtailment of zoospore motility by β-escin could be mediated by the glucose moiety. However, the disruption of transmembrane potentials by either β-escin or gramicidin S would also perturb normal motile behaviour.

β-escin destabilises the membrane by interaction with membrane bound sterols. Calcium and potassium, but not magnesium, strontium or sodium suppressed the inhibition of β-escin.

Calcium and potassium are in the same chemical series (series 4); likewise magnesium and sodium (series 3). It was expected that strontium, a divalent cation in series 5, would act as a surrogate for calcium in this respect, but it did not do so. Therefore, the effects of calcium and potassium may be due to their ionic radii (a steric effect). Toxicity of avenacin A-1 is dependent on the presence of the monosaccharide residues (Armah *et al.,* 1999).

The toxic effect of gramicidin S was irreversible by the delayed addition of calcium. However, the addition of either potassium or calcium up to five minutes post-β-escin addition could almost completely negate β-escin action. This latter observation supports the above hypothesis that calcium and potassium do not compete with β-escin for the same point of interaction with membranes. Multivalent cations, particularly calcium, are known to induce dramatic structural changes in model membranes composed of acidic phospholipids (for example, Hauser & Shipley, 1984). Increasing concentrations of calcium ions increase the packing of phosphatidylcholine-phosphatidic acid mixtures (Kouaouci *et al.*, 1985). This calcium-induced packing will increase the membrane stability, and counteract the effect of β-escin. This might explain how delayed addition of calcium to β-escin treated cells reverses most of the effects of β-escin. Calcium might not reverse the action of gramicidin S because they have similar or identical sites of interaction, and the affinity of gramicidin S for phospholipids is very high, as indicated by the sub-millimolar LD_{50}.

Calcium, strontium and to a lesser degree magnesium were able to reduce the effectiveness of gramicidin S. The mode of action of gramicidin S is by interaction with membrane phospholipids. The intrinsic dissociation constants for calcium and magnesium ion binding to phospholipids are much lower than for monovalent cations (Gennis, 1989). The binding of either monovalent (Cunningham *et al.*, 1986) or divalent (Borle and Seelig, 1985; Akutsu *et al.*, 1986) cations appears to have little effect on the conformation of the lipid polar headgroup. In most cases calcium binding with phospholipid is 1:1 (Altenbach and Seelig, 1984). The inhibition of gramicidin S by divalent cations is therefore probably by cations, particularly divalent cations, occupying the sites of interaction of the cationic ornithine residues of gramicidin S.

With the target site of gramicidin S being the phospholipids of the membrane and β-escin targeting the membrane bound sterols, there is potential for synergism in using these two

naturally occurring toxins in the control of zoosporic fungi in glasshouse irrigation systems. Although both gramicidin S and β-escin have similar molecular weights (M_r = 1.2 kg.mol^{-1} and 1.1 kg.mol^{-1}, respectively) and both are hydrophobic (they do not dissolve in water), only β-escin activity is reduced by the presence of a cell wall. In irrigation systems there are abundant non-fungal sterols and phospholipids to potentially absorb the toxins. Therefore, for disease control, the effective concentration of β-escin or gramicidin S in an irrigation system is yet to be established.

The presence of cations in irrigation systems will suppress the effectiveness of the toxins against zoospores and other stages of the infection sequence. The phytotoxicity of β-escin and gramicidin S is yet to be determined. β-escin and gramicidin S could only be used to control zoosporic fungi in irrigation systems if the tolerance of plants is considerably greater than for the zoosporic fungi. In this chapter it has been shown that the action of gramicidin S is unaffected by the presence of the cellulose cell wall of cysts, and that β-escin is affected by the cell walls of cysts. The cell walls of tomatoes and cucumbers are principally of cellulose, so the prospect of β-escin or gramicidin S being effective and useful in irrigation systems is unknown.

Stanghellini (1998) proposed the addition of bacterial cultures along with mycotoxic secondary metabolites to irrigation systems. Therefore, the addition of *B. brevis* culture to an irrigation system would not be novel. However, the proposition of adding crushed oat seedlings is yet to be considered. The discovery of high concentration of compounds (presumably avenacins) that were inhibitive to zoospores in the root tip confirms that preformed toxic compounds are found in oat root tips. The high levels of germination of cysts in crude saponin extracts can be attributed to known germination triggers, such as, amino acids and sugars (Donaldson & Deacon, 1993c). Similarly, the encystment of zoospores by the crude saponin extracts can also be attributed, partially, to the presence of known encystment triggers. However, the ability of 6.1 mg.ml^{-1} of root tip extract to completely suppress the motility of zoospores of *Py. aphanidermatum* could be due to the relative high concentration of inhibition compounds. Although crude saponin extracts from oats contain compounds that are inhibitive to zoospores, the presence of amino acids and sugars might be detrimental to an irrigation system. These topics merit further work in commercial or model glasshouse irrigation systems.

Chapter 7.

The effect of combining potential control techniques on aspects of the infection sequence of zoosporic fungi.

7.1. Introduction.

In the preceeding chapters several potential methods for disease control were presented. These methods were the manipulation of cation concentrations, the addition of natural toxins (gramicidin S, β-escin and ethanol) and the phenomenon of natural suppression in recirculating irrigation systems. In a couple of these proceeding chapters the interaction between different control methods was partially investigated - for example, the effect of certain cations on the toxicity of β-escin and gramicidin S. The putative control methods that are described in the previous four chapters may interact either synergistically or antagonistically.

Each potential control method targets the infection sequence of zoosporic fungi at different points, and by various mechanisms. For example, ethanol is particularly toxic to sporangiogenesis and zoospore release, but relatively ineffective at suppressing zoospore motility. In contrast, elevated concentrations of K^+ do not affect sporangiogenesis, but markedly suppress zoospore motility. So it can be speculated that this particular pair of control approaches would work synergistically. However, potential antagonism between control approaches has also been exposed in the proceeding chapters. For example, ethanol suppresses sporangiogenesis, whereas Ca^{2+} at a concentration of approximately 5 mM enhances sporangiogenesis. The work presented in this chapter examines *in vitro* antagonism and synergism between the various approaches of controlling zoosporic fungi that have been presented in this thesis.

7.2. Synergism between β-escin and gramicidin S.

β-escin and gramicidin S mediate cell inhibition by interaction with different cell surface membrane components. β-escin interacts with membrane-bound sterols, and gramicidin S interacts with phospholipids. In the previous chapter the LD_{50} values for β-escin and gramicidin S for zoospores of *Py. aphanidermatum* were determined to be 50.8 μM and 740 nM, respectively (Table 6.2).

To determine whether β-escin and gramicidin S would act synergistically, five treatments were prepared to which zoospores of *Py. aphanidermatum* were added, and then zoospore nutrient induced germination was assessed (as in Section 6.3). In the absence of either toxin the nutrient

induced germination of zoospores was 93% (Table 7.1). The addition of 50.8 μM β-escin reduced zoospore nutrient induced germination to 15%, and to 84% in 740 nM gramicidin S. Assuming the effects of gramicidin S and β-escin on nutrient induced germination were additive then the proportion of zoospores expected to be germinable in a mixture of 740 nM gramicidin S and 50.8 μM β-escin would be 13% (that is, 83.6% × 15.4%). However, the actual proportion of zoospores found to be germinable in this mixture was lower than expected at 4%. In a mixture of β-escin and gramicidin S at half LD_{50} concentration (25.4 μM β-escin and 370 nM gramicidin S) the proportion of zoospores germinable was 56%.

Table 7.1. Synergism between β-escin and gramicidin S assessed by the nutrient induced germination of zoospores of *Py. aphanidermatum**.

Treatment	Germination Arcsine (and %)
SDW	74.9 ± 1.8 a (93.0 ± 1.5)
50.8 μM Escin	22.7 ± 3.2 (15.4 ± 3.8)
740 nM Gramicidin	66.8 ± 4.6 a (83.6 ± 5.4)
25.4 Escin + 370 nM Gramicidin	48.3 ± 4.4 (55.7 ± 7.6)
50.8 Escin + 740 nM Gramicidin	12.0 ± 0.4 (4.3 ± 0.3)
σ_d	4.7
$LSD_{0.05}$	10.4
P (ANOVA)	3.19×10^{-7}

* Means ± s.e.m. for 3 replicates, assessed after 2h, based on score of at least 100 spores per replicate. The concentration of DMSO in all treatments was 16.3 mM.
Values followed by the same letter do not differ significantly at $P = 0.05$.

7.3. Synergism between natural suppression and 10 mM K^+ supplement on the motility of zoospores of *Py. aphanidermatum* and the subsequent germination of encysted zoospores.

In each of the following five experiments (Sections 7.3 to 7.7) a poorly suppressive irrigation solution and highly suppressive irrigation solution from Stockbridge House was used. The

designation of an irrigation solution as either poorly or highly suppressive was based on the analyses performed in Chapter 4. The designations refer to the ability of the irrigation solution to suppress (or inability to suppress) the particular aspect of the infection sequence examined in the experiment. Determination of significant effects was by two-way ANOVA, unless the employment of one-way ANOVA was more appropriate.

Zoospores of *Py. aphanidermatum* were incubated for 2h in either inoculated open irrigation solution harvested on the 30th of July (poorly suppressive irrigation solution) or the inoculated closed (pasteurised) irrigation solution harvested on the 9th of September (the highly suppressive irrigation solution) and in the presence or absence of 10 mM K^+ (final concentration). After incubation the proportion of zoospores that had remained motile and the proportion of encysted zoospores that had germinated was determined as in Section 2.3.6.

The proportion of zoospores motile after incubation in the poorly suppressive irrigation solution not supplemented with K^+ was 34% (Table 7.2), whereas only 14% of zoospores were motile in the highly suppressive irrigation solution. The effect of adding 10 mM K^+ to either of these irrigation solutions was very highly significant ($P_{\text{K supplement}} = 1.65 \times 10^{-4}$), but there was also significant ($P_{\text{interaction}} = 0.040$) interaction between the irrigation solution sample and the K^+ supplement. Adding 10 mM K^+ to the poorly suppressive irrigation solution had negligible affect, whereas adding 10 mM K^+ to the highly suppressive irrigation solution caused considerable additional suppression of zoospore motility.

The proportion of zoospores that had encysted and subsequently germinated during the incubation period was very significantly ($P_{\text{irrigation solution}} = 1.95 \times 10^{-3}$) greater in the highly suppressive irrigation solution than the poorly suppressive irrigation solution (Table 7.3). The effect of the 10 mM K^+ supplement was to increase cyst germination very highly significantly ($P_{\text{K supplement}} = 1.48 \times 10^{-4}$) in both irrigation solutions. There was no significant ($P_{\text{interaction}} = 0.355$) interaction between irrigation solution and potassium supplement.

Table 7.2. Synergism between irrigation solutions and a 10 mM K^+ supplement, as assessed by the proportion of zoospores of *Py. aphanidermatum* that remained motile*.

Treatment	Poorly suppressive irrigation solution[a]. Arcsine (and %)	Highly suppressive irrigation solution[b]. Arcsine (and %)	Mean
No K^+ added	35.3 ± 5.7 (34.0 ± 9.6)	21.4 ± 2.5 (13.6 ± 2.9)	(23.8)
10 mM K^+ added	32.0 ± 1.5 (28.2 ± 2.4)	1.7 ± 1.7 (0.3 ± 0.3)	(14.3)
Mean	(31.1)	(7.0)	(19.0)
σ_d	4.7		
$LSD_{0.05}$	10.9		
$P_{\text{irrigation solution}}$	8.84×10^{-3}		
$P_{\text{K supplement}}$	1.65×10^{-4}		
$P_{\text{interation}}$	4.00×10^{-2}		

* Means ± s.e.m. for 3 replicates, based on 3 random fields of view per replicate.
[a] Inoculated open irrigation solution harvested on the 30th of July.
[b] Inoculated closed (pasteurised) irrigation solution harvested on the 9th of September.

Table 7.3. Synergism between irrigation solutions and a 10 mM K^+ supplement, as assessed by the proportion of spontaneously encysted zoospores of *Py. aphanidermatum* that subsequently germinated[*].

Treatment	Poorly suppressive irrigation solution[a]. Arcsine (and %)	Highly suppressive irrigation solution[b]. Arcsine (and %)	Mean
No K^+ added	22.5 ± 0.8 (14.7 ± 1.0)	38.2 ± 0.7 (38.3 ± 1.1)	(26.5)
10 mM K^+ added	33.7 ± 2.0 (30.9 ± 3.2)	45.4 ± 3.4 (50.7 ± 5.9)	(40.8)
Mean	(22.8)	(44.5)	(33.7)
σ_d	2.9		
$LSD_{0.05}$	6.7		
$P_{irrigation\ solution}$	1.95×10^{-3}		
$P_{K\ supplement}$	1.48×10^{-4}		
$P_{interation}$	3.55×10^{-1}		

[*] Means ± s.e.m. for 3 replicates, based on 3 random fields of view per replicate.
[a] Inoculated open irrigation solution harvested on the 30th of July.
[b] Inoculated closed (pasteurised) irrigation solution harvested on the 9th of September.

7.4. Synergism between natural suppression and Ca^{2+} supplement on the release of zoospores from pre-formed sporangia of *Py. aphanidermatum*.

Sporangia-bearing mats of *Py. aphanidermatum* were produced as in Section 2.3.1.2, but the release solution was substituted with either inoculated irrigation solution harvested on the 24th of October (the highly suppressive irrigation solution) or the uninoculated open irrigation solution harvested on the 1st of July (the poorly suppressive irrigation solution) and in the presence or absence of a 20 mM Ca^{2+} supplement. After the standard release period (2h) the numbers of zoospores liberated by the pre-formed sporangia were enumerated as in Section 2.3.5.

The highly suppressive irrigation solution significantly ($P_{irrigation\ solution}$ = 0.0243) suppressed the number of zoospores that were released from pre-formed sporangia compared to the poorly suppressive irrigation solution irrespective of the calcium supplement (Table 7.4). The addition

of 20 mM Ca^{2+} to either irrigation solution did not significantly ($P_{Ca\ supplement}$ = 0.126) affect the number of zoospores liberated.

Table 7.4. Synergism between irrigation solutions and a 20 mM Ca^{2+} supplement, as assessed by the number of zoospores that are released from pre-formed sporangia of *Py. aphanidermatum*[•].

Treatment	Poorly suppressive irrigation solution[a]. Zoospores.μl^{-1}	Highly suppressive irrigation solution[b]. Zoospores.μl^{-1}	Mean
No Ca^{2+} added	147 ± 37	90 ± 15	119
20 mM Ca^{2+} added	198 ± 22	121 ± 17	160
Mean	173	106	139
σ_d	34		
$LSD_{0.05}$	75		
$P_{irrigation\ solution}$	2.43×10^{-2}		
$P_{Ca\ supplement}$	1.26×10^{-1}		
$P_{interation}$	6.87×10^{-1}		

[•] Means ± s.e.m. for 3 replicates, based on 2 haemocytometer counts per replicate.
[a] Uninoculated open irrigation solution harvested on the 1st of July.
[b] Inoculated open irrigation solution harvested on the 24th of October.

7.5. Synergism between natural suppression and Ca^{2+} supplement on the germination of vortex-encysted zoospores of *Py. aphanidermatum*.

To 200 µl of pre-encysted zoospores of *Py. aphanidermatum* was added either 800 µl of open inoculated irrigation solution harvested on the 30th of July (poorly suppressive irrigation solution) or inoculated closed (pasteurised) irrigation solution that was harvested on the 9th of September (the highly suppressive irrigation solution) in the presence or absence of 20 mM Ca^{2+} (final concentration) supplement. After 2h incubation, the proportion of cysts that had germinated was assessed as in Section 2.3.7. Neither the irrigation solution nor the presence or absence of Ca^{2+} affected the proportion of cysts that germinated (Table 7.5). In all treatments the percentage germination was between 55 and 68%.

Table 7.5. Synergism between irrigation solutions and a 20 mM Ca^{2+} supplement, as assessed by the proportion of vortex-encysted zoospores of *Py. aphanidermatum* that germinated[*].

Treatment	Poorly suppressive irrigation solution[a]. Arcsine (and %)	Highly suppressive irrigation solution[b]. Arcsine (and %)	Mean
No Ca^{2+} added	55.6 ± 2.8 (67.9 ± 4.6)	51.3 ± 4.3 (60.7 ± 7.2)	(64.3)
20 mM Ca^{2+} added	54.0 ± 2.2 (65.3 ± 3.6)	48.3 ± 3.2 (55.6 ± 5.4)	(60.5)
Mean	(66.6)	(58.2)	(62.4)
σ_d	4.5		
$P_{\text{irrigation solution}}$	1.56×10^{-1}		
$P_{\text{Ca supplement}}$	4.87×10^{-1}		
$P_{\text{interation}}$	8.35×10^{-1}		

[*] Means ± s.e.m. for 3 replicates, based on 100 cysts per replicate.
[a] Inoculated open irrigation solution harvested on the 30th of July.
[b] Inoculated closed (pasteurised) irrigation solution harvested on the 9th of September.

7.6. Synergism between natural suppression and either 5 mM K^+ or 35 mM ethanol on the infection sequence of *Py. aphanidermatum*.

The experimental protocol for producing zoospores of *Py. aphanidermatum* (see Section 2.3.1.2) was modified in the following way. All stages, except the establishment of mycelial mats in CV8B, the mineral salts solution and the zoospore release solution were substituted with either inoculated open irrigation solution that was harvested on the 24th of October (the poorly suppressive irrigation solution) or inoculated closed (pasteurised) solution harvested on the 24th of October (the highly suppressive irrigation solution). The experiments also tested the effects of presence or absence of either 35 mM ethanol or 5 mM K^+.

The number of zoospores that were liberated into the unsupplemented poorly suppressive irrigation solution was 105 zoospores.μl^{-1} (Table 7.6), whereas only 0.3 zoospores.μl^{-1} were released into the unsupplemented highly suppressive irrigation solution. The effect of

supplementing the irrigation solutions with either 5 mM K^+ or 35 mM ethanol was dependent on the suppressiveness of the irrigation solution, as indicated by the very highly significant ($P_{interaction}$ = 6.66 x 10^{-4}) interaction between the irrigation solution and the supplement. Irrespective of the supplement, there were very few zoospores released into the highly suppressive irrigation solution. However, the 5 mM K^+ supplement significantly ($P < 0.05$) increased the number of zoospores released into the poorly suppressive irrigation solution compared to the unsupplemented control, whereas the 35 mM ethanol supplement significantly ($P < 0.05$) suppressed the number of zoospores released compared to the unsupplemented control.

Table 7.6. Synergism between irrigation solutions and either 5 mM K^+ or 35 mM ethanol, as assessed by the number of zoospores released from sporangia of *Py. aphanidermatum.*[*] The effect was assessed when the treatment was applied to the whole experimental protocol[**].

Treatment	Poorly suppressive irrigation solution[a]. Zoospores.μl^{-1}	Highly suppressive irrigation solution[b]. Zoospores.μl^{-1}	Mean
No K^+ or ethanol added.	105.0 ± 55.4	0.3 ± 0.2	52.7
5 mM K^+ added	252.7 ± 11.8	0.4 ± 0.1	126.6
35 mM ethanol added	8.6 ± 2.5	2.5 ± 1.3	5.6
Mean	122.1	1.1	61.6
$LSD_{0.05}$	69.1		
σ_d	32.8		
$P_{irrigation\ solution}$	3.43 × 10^{-5}		
$P_{supplement}$	7.61 × 10^{-4}		
$P_{interation}$	6.66 × 10^{-4}		

[*] Means ± s.e.m. for 3 replicates, based on 2 haemocytometer counts per replicate.
[**] Except mycelial growth, which occurred in CV8B.
[a] Inoculated open irrigation solution harvested on the 24th of October.
[b] Inoculated closed (pasteurised) irrigation solution harvested on the 24th of October.

After the release period (2h) and the assessment of zoospore numbers (above) the mycelial mats were removed from the Petri dishes and were incubated for a further 2h (that is, 4h in total).

After this the proportion of zoospores motile and the proportion of encysted zoospores that had germinated was assessed (as in Section 2.3.6).

In two out of three treatments employing the highly suppressive irrigation solution, there were too few spores for the assessments of zoospore motility and cyst germination to be worthwhile. However, in the unsupplemented poorly suppressive irrigation solution 89% of zoospores were motile throughout the incubation, whereas 44% were motile when the irrigation solution was supplemented with 5 mM K^+, and 55% were motile when the supplement was 35 mM ethanol (Table 7.7). The proportion of cysts that subsequently germinated in the poorly suppressive irrigation solutions was between 26 and 50% (Table 7.8). Neither the K^+ nor ethanol supplement significantly ($P = 0.0544$) affected the proportion of cysts that germinated.

Table 7.7. Synergism between irrigation solutions and either 5 mM K^+ or 35 mM ethanol, as assessed by the proportion of zoospores of *Py. aphanidermatum* that remain motile.* The effect was assessed when the treatment was applied to the whole experimental protocol**.

Treatment	Poorly suppressive irrigation solution[a]. Arcsine (and %)	Highly suppressive irrigation solution[b]. Arcsine (and %)	Mean
No K^+ or ethanol added.	71.1 ± 2.5 (89.2 ± 2.7)	n.d.	(89.2)
5 mM K^+ added	41.5 ± 8.7 (44.1 ± 14.6)	n.d.	(44.1)
35 mM ethanol added	48.1 ± 7.1 (55.3 ± 12.1)	69.2 ± 10.4 (82.1 ± 9.0)	(68.7)
Mean	(62.9)	(82.1)	(67.8)
σ_d	11.0		
P	6.21×10^{-2}		

* Means ± s.e.m. for 3 replicates, based on 3 fields of view per replicate.
** Except mycelial growth, which occurred in CV8B.
[a] Inoculated open irrigation solution harvested on the 24th of October.
[b] Inoculated closed (pasteurised) irrigation solution harvested on the 24th of October.
n.d. Not done.

Table 7.8. Synergism between natural suppression and either 5 mM K^+ or 35 mM ethanol, assessed by the proportion of spontaneously encysted zoospores of *Py. aphanidermatum* that subsequently germinated.* The effect was assessed when the treatment was applied to the whole experimental protocol**.

Treatment	Poorly suppressive irrigation solution[a]. Arcsine (and %)	Highly suppressive irrigation solution[b]. Arcsine (and %)	Mean
No K^+ and ethanol added.	34.0 ± 4.5 (31.7 ± 7.3)	n.d.	(31.7)
5 mM K^+ added	30.5 ± 3.8 (26.3 ± 5.6)	n.d.	(26.3)
35 mM ethanol added	45.2 ± 1.1 (50.4 ± 1.9)	n.d.	(50.4)
Mean	(36.1)	n.a.	(36.1)
σ_d	4.9		
P	5.44×10^{-2}		

* Means ± s.e.m. for 3 replicates, based on 3 fields of view per replicate.
** Except mycelial growth, which occurred in CV8B.
[a] Inoculated open irrigation solution harvested on the 24th of October.
[b] Inoculated closed (pasteurised) irrigation solution harvested on the 24th of October.
n.d. Not done.
n.a. Not applicable.

Immediately after the mycelial mats were removed an aliquot (500 μl) of zoospores from each Petri dish were removed and mechanically encysted by vortexing (70s). The encysted zoospores were incubated for 2 h and then the proportion of cysts that had germinated was determined as in Section 2.3.7. That is, the incubation of vortex-encysted zoospores was concurrent with the incubation of motile zoospores (Tables 7.7 and 7.8).

The K^+ supplement in the poorly suppressive irrigation solution significantly ($P < 0.05$) suppressed the proportion of cysts that germinated compared to either the unsupplemented control or the ethanol supplemented treatment (Table 7.9). Again, in two out of three treatments employing the highly suppressive irrigation solution, there were too few spores for the assessment of cyst germination to be worthwhile.

Table 7.9. Synergism between irrigation solutions and either 5 mM K^+ or 35 mM ethanol, as assessed by the proportion of vortex-encysted zoospores of *Py. aphanidermatum* that germinated.* The effect was assessed when the treatment was applied to the whole experimental protocol**.

Treatment	Poorly suppressive irrigation solution[a]. Arcsine (and %)	Highly suppressive irrigation solution[b]. Arcsine (and %)	Mean
No K^+ or ethanol added.	54.8 ± 4.8 (66.2 ± 7.6)	n.d.	(66.2)
5 mM K^+ added	38.2 ± 2.8 (38.4 ± 4.7)	n.d.	(38.4)
35 mM ethanol added	52.1 ± 1.8 (62.2 ± 3.1)	50.3 ± 3.1 (59.1 ± 5.4)	(60.7)
Mean	(55.6)	(59.1)	(56.5)
$LSD_{0.05}$	10.3		
σ_d	4.7		
P	3.25×10^{-2}		

* Means ± s.e.m. for 3 replicates, based on 3 fields of view per replicate.
** Except mycelial growth, which occurred in CV8B.
[a] Inoculated open irrigation solution harvested on the 24th of October.
[b] Inoculated closed (pasteurised) irrigation solution harvested on the 24th of October.
n.d. Not done.

7.7. Synergism between natural suppression and 35 mM ethanol on sporangiogenesis of *Ph. parasitica*.

The protocol for the production of zoospores of *Ph. parasitica* (Section 2.3.1.1) was modified in the following way. The mineral salts solution was substituted with either uninoculated open irrigation solution harvested on the 9th of September (the poorly suppressive irrigation solution) or inoculated closed (filtered) irrigation solution harvested on the 24th of October (the highly suppressive irrigation solution) and in the presence or absence of a 35 mM ethanol supplement. So sporangiogenesis occurred in an irrigation solution that was either unsupplemented or supplemented with ethanol.

Prior to the removal of the irrigation solution the number of zoospores in the bathing medium was enumerated by haemocytometer counts (Table 7.10). In the unsupplemented poorly suppressive irrigation solution 39.4 zoospores.μl^{-1} were enumerated, which was significantly ($P < 0.05$) more than in the unsupplemented highly suppressive irrigation solution, where only 3.1 zoospores.μl^{-1} were enumerated. The supplement of either irrigation solution with 35 mM ethanol very significantly ($P_{Ethanol} = 6.45 \times 10^{-3}$) suppressed the number of zoospores that were released.

Table 7.10. Synergism between irrigation solutions and 35 mM ethanol, as assessed by the number of zoospores that were released* prior to cold shock from sporangia of *Ph. parasitica* that had formed in the treatment.

Treatment	Poorly suppressive irrigation solution[a]. Zoospores.μl^{-1}	Highly suppressive irrigation solution[b]. Zoospores.μl^{-1}	Mean
No ethanol added	39.4 ± 11.5	3.1 ± 0.2	21.3
35 mM Ethanol added	0.4 ± 0.1	0.0 ± 0.0	0.2
Mean	19.9	1.6	10.7
σ_d	8.1		
$LSD_{0.05}$	17.9		
$P_{irrigation\ solution}$	1.28×10^{-2}		
$P_{Ethanol\ supplement}$	6.45×10^{-3}		
$P_{interation}$	1.43×10^{-2}		

* Means ± s.e.m. for 3 replicates, based on 2 haemocytometer counts per replicate.
[a] Uninoculated open irrigation solution harvested on the 9th of September.
[b] Inoculated closed (filtered) irrigation solution harvested on the 24th of October.

Similarly, the number of zoospores that were released after the cold shock into SDW was greater when the mineral salts solution had been substituted with the poorly suppressive irrigation solution than the highly suppressive irrigation solution (Table 7.11). The supplement of 35 mM ethanol to either irrigation solution caused the number of zoospores to be released to be very significantly ($P_{Ethanol} = 3.78 \times 10^{-3}$) suppressed.

Table 7.11. Synergism between irrigation solutions and 35 mM ethanol, as assessed by the number of zoospores that were released* after the cold shock from sporangia of *Ph. parasitica* that had formed in the treatment.

Treatment	Poorly suppressive irrigation solution[a]. Zoospores.μl^{-1}	Highly suppressive irrigation solution[b]. Zoospores.μl^{-1}	Mean
No ethanol added	42.3 ± 11.3	18.4 ± 9.5	30.4
35 mM Ethanol added	1.2 ± 0.6	0.1 ± 0.1	0.7
Mean	21.8	9.3	15.5
σ_d	10.4		
$LSD_{0.05}$	22.9		
$P_{\text{irrigation solution}}$	1.28×10^{-1}		
$P_{\text{Ethanol supplement}}$	3.78×10^{-3}		
$P_{\text{interation}}$	1.43×10^{-1}		

* Means ± s.e.m. for 3 replicates, based on 2 haemocytometer counts per replicate.
[a] Uninoculated open irrigation solution harvested on the 9th of September.
[b] Inoculated closed (filtered) irrigation solution harvested on the 24th of October.

The addition of 35 mM ethanol to the irrigation solutions very significantly ($P_{\text{ethanol supplement}} = 2.68 \times 10^{-3}$) suppressed the density of sporangia that had formed during the experiment in both irrigation solutions (Table 7.12.). When the effect of the ethanol supplement is taken into account then the suppressiveness of the irrigation solution does not significantly ($P_{\text{irrigation solution}} = 0.187$) affect the density of sporangia that formed on the mycelial mats.

Table 7.12. Synergism between irrigation solutions and 35 mM ethanol, as assessed by the density of sporangia of *Ph. parasitica* * that had formed in the treatment.

	Poorly suppressive irrigation solution[a]. Sporangia.screen^{-1}	Highly suppressive irrigation solution[b]. Sporangia.screen^{-1}	Mean
No ethanol added	6.8 ± 1.6	4.6 ± 1.0	5.7
35 mM Ethanol added	1.5 ± 0.9	0.6 ± 0.4	1.1
Mean	4.2	2.6	3.4
σ_d	1.5		
$LSD_{0.05}$	3.4		
$P_{\text{irrigation solution}}$	1.87×10^{-1}		
$P_{\text{Ethanol supplement}}$	2.68×10^{-3}		
$P_{\text{interation}}$	5.58×10^{-1}		

* Means ± s.e.m. for 3 replicates, based on 3 fields of view per replicate.
[a] Uninoculated open irrigation solution harvested on the 9th of September.
[b] Inoculated closed (filtered) irrigation solution harvested on the 24th of October.

7.8. Antagonism between 5 mM Ca^{2+} and 35 mM ethanol on the sporangiogenesis of *Ph. parasitica*.

The protocol for the production of zoospores of *Ph. parasitica* (Section 2.3.1.1) was modified in the following way. Mineral salts solution free of $Ca(NO_3)_2$ was either unsupplemented or supplemented with 5 mM Ca^{2+} and/or 35 mM ethanol.

The number of zoospores that were released in calcium and ethanol free MSS (the control treatment) prior to cold shock was 29.3 zoospores.µl^{-1}, while 27.5 zoospores.µl^{-1} were released into SDW after the cold shock (Table 7.13). The supplement of MSS with 5 mM Ca^{2+} significantly ($P < 0.05$) increased the number of zoospores released (both pre- and post-cold shock). The addition of 35 mM ethanol to the calcium-free MSS also significantly ($P < 0.05$) suppressed the number of zoospores to be released (both prior and post-cold shock) compared to the control treatment. However, when the MSS was supplemented with both 5 mM Ca^{2+} and 35 mM ethanol, the number of zoospores released (both pre- and post-cold shock) was not significantly ($P > 0.05$) different from the calcium-free, ethanol supplemented MSS. That is,

there was significant ($P_{interaction} \leq 3.90 \times 10^{-2}$) interaction between the effects of calcium and ethanol on the release of zoospores.

The proportion of sporangia that had discharged their contents (both pre- and post-cold shock) was not significantly ($P \geq 0.705$) affected by the Ca^{2+} concentration in the mineral salts solution. However, the supplement of MSS with 35 mM ethanol, irrespective of calcium concentration, significantly ($P_{Ethanol} \leq 7.84 \times 10^{-5}$) reduced the proportion of sporangia that discharged their contents (both pre- and post-shock) when compared to the calcium-free, ethanol-free control. The density of sporangia in all four treatments was not significantly ($P \geq 0.127$) different.

Table 7.13. Antagonism between 5 mM Ca^{2+} and 35 mM ethanol as assessed by aspects of sporangiogenesis of *Ph. parasitica*.

Ca^{2+}-free MSS supplement	Pre-cold shock results		Post cold-shock results		
	Zoospore release[a] Zoospores.μl^{-1}	Discharged sporangia[b] Arcsine (%)	Zoospore release[a] Zoospores.μl^{-1}	Discharged sporangia[b] Arcsine (%)	Sporangia density[c] Sporangia.screen^{-1}
None	29.3 ± 2.1	25.8 ± 4.6 a (19.7 ± 6.6)	27.5 ± 2.5	38.5 ± 2.1 a (38.7 ± 3.5)	23.3 ± 2.8
Ca^{2+}	50.3 ± 8.2	26.0 ± 2.8 a (19.5 ± 3.7)	55.3 ± 6.6	34.6 ± 3.3 a (32.5 ± 5.5)	32.1 ± 3.2
Ethanol	2.0 ± 1.0 a	2.6 ± 2.6 b (0.6 ± 0.6)	2.0 ± 2.0 a	14.9 ± 3.1 b (7.1 ± 2.6)	25.4 ± 5.6
Ca^{2+} + Ethanol	4.1 ± 2.7 a	0.0 ± 0.0 b (0.0 ± 0.0)	9.2 ± 3.5 a	19.3 ± 1.6 b (11.0 ± 1.7)	16.2 ± 4.0
σ_d	6.2	4.2	5.1	3.7	5.7
$LSD_{0.05}$	13.2	9.3	11.2	8.2	n.a.
$P_{Ca^{2+}}$	3.62×10^{-2}	7.05×10^{-1}	3.85×10^{-3}	9.13×10^{-1}	9.58×10^{-1}
$P_{Ethanol}$	1.37×10^{-5}	3.59×10^{-5}	2.94×10^{-6}	7.84×10^{-5}	1.27×10^{-1}
$P_{Interaction}$	3.90×10^{-2}	6.60×10^{-1}	5.11×10^{-3}	1.57×10^{-1}	5.80×10^{-2}

[a] Means ± s.e.m. for 3 replicates, based on 2 haemocytometer counts per replicate.
[b] Means ± s.e.m. for 3 replicates, based on 3 fields of view per replicate.
[c] Means ± s.e.m. for 3 replicates, based on 6 fields of view per replicate (3 fields prior to cold-shock and 3 fields after cold shock).
n.a. Not applicable.
Values, in the same column, followed by the same letter do not significantly differ significantly at P = 0.05.

7.9. Discussion.

The results in chapter 3 suggested the possibility that modifying the potassium to calcium ratio in favour of potassium while maintaining nitrate concentration would suppress the infection sequence of zoosporic fungi. In a direct comparison of two ratios of potassium and calcium it was found that although there was evidence to support the hypothesis, the extent of suppression was not as a great as expected. This contrasts with the compound effect of gramicidin S and β-escin at LD_{50} concentrations (Table 7.1). The additive suppression of zoospore nutrient induced germination was greater than expected. This mutual enhancement of toxicity is possibly observed because the target sites for the two toxins are different, and so competition for sites of interaction on the membrane is minimal. These two examples, modified potassium to calcium ratio and the compound effect of β-escin and gramicidin S, illustrate that the degree of synergism between two putative disease control methods will not necessarily be as predicted.

Sporangiogenesis of *Ph. parasitica* when measured by zoospore release was enhanced by 5 mM Ca^{2+}, thus confirming the observations presented in Chapter 3. Similarly, ethanol was shown to suppress sporangiogenesis of *Ph. parasitica*, also confirming previous observations (Chapter 5). When 5 mM Ca^{2+} MSS was supplemented with 35 mM ethanol the proportion of sporangia that discharged their contents and the number of zoospores that were liberated were not significantly different from the calcium-free MSS supplemented with ethanol. This demonstrates that 35 mM ethanol can inhibit the promotion of sporangiogenesis induced by 5 mM Ca^{2+}. Although the concentration of calcium is appropriate to commercial irrigation systems the concentration (35 mM) of ethanol is probably greater than what would be commercially acceptable.

The work at Stockbridge House referred to in this thesis did not clearly demonstrate the phenomenon of natural suppression in recirculating irrigation systems. In this chapter the criteria for determining whether an irrigation solution was suppressive was solely based on the *in vitro* assays of Chapter 4. The majority of the experiments presented in this chapter investigated the potential for synergism between theoretical suppressiveness and suppressive supplements. In Chapter 3 potassium ions were found to be particularly effective at suppressing the motility of zoospores of *Py. aphanidermatum*. However, when 10 mM K^+ was added to an irrigation solution that poorly suppressed zoospore motility the effect of the potassium supplement was

negligible, but the effect of adding the same supplement to an irrigation solution that was highly suppressive of zoospore motility was to further reduce zoospore motility. That is, there was significant interaction between the mechanism of theoretical suppressiveness and potassium-mediated suppression of zoospore motility. It would appear that the poorly suppressive irrigation solution had the ability to negate the ability of potassium to suppress zoospore motility.

Fifty-nine percent of zoospores of *Py. aphanidermatum* remained motile in inoculated open irrigation solution harvested on the 30th of July (Table 4.6), whereas the average proportion of zoospores to remain motile in SDW in the same analysis was 47%. This poorly suppressive irrigation solution according to an analysis on the 29th of July had a pH of 7.0, an electrical conductivity of 3.5 mΩ^{-1} and the concentrations of calcium and potassium were 7.4 and 12.5 mM, respectively (appendix 10). It therefore would be expected, based on previous results, that this irrigation solution would significantly suppress motility of zoospores when compared to SDW. Therefore, it has to be concluded that this irrigation solution, along with other solutions, has the ability to negate the ability of cations to suppress the motility of zoospores. However, this ability to negate cation-mediated zoospore suppression was not observed in the highly suppressive irrigation solution. Interaction between natural suppression and suppression mediated by an supplement was not observed in any of the other experiments. That is, when the supplement affected the suppressiveness of a poorly suppressive irrigation solution, a similar affect was observed with the highly suppressive irrigation solution. For example, ethanol suppressed zoospore release (pre- and post-cold shock) and sporangial density when added to either an irrigation solution that was poorly suppressive of sporangiogenesis or highly suppressive of sporangiogenesis.

Increasing calcium concentration has been demonstrated to suppress zoospore release from pre-formed sporangia of *Py. aphanidermatum* (see Figure 3.25). However, when 20 mM Ca^{2+} was supplemented to either a poorly suppressive irrigation solution or a highly suppressive irrigation solution the number of zoospores that were released was ~30% greater than in the unsupplemented irrigation solutions, although these increases were not significant (P = 0.126). Therefore, it appears that both irrigation solutions had the ability to negate the ability of calcium to suppress zoospore release from pre-formed sporangia of *Py. aphanidermatum*.

A similar observation was found when 5 mM K^+ was supplemented to the inoculated open irrigation solution harvested on the 24th of October. When MSS and the release solution was substituted with this irrigation solution the number of zoospores released was 105 zoospores.μl^{-1}, but when this irrigation solution was supplemented with 5 mM K^+ the number of zoospores released was almost two and half times as many (253 zoospores.μl^{-1}). However, when 35 mM ethanol was supplemented to the irrigation solution instead of 5 mM K^+ the number of zoospores released was less than 10 μl^{-1}.

Therefore, in three separate experiments where cations were added to irrigation solutions the effect was contrary to what was expected from the results of experiments testing single factors. These observations do not undermine the suggestion to either supplement the fresh irrigation solution with cations or alter the potassium to calcium ratio, but show there is complexity in the interactions between zoospore biology, irrigation solutions and cationic supplements. However, when ethanol was added to an irrigation solution the result was as expected.

Donaldson and Deacon (1993c) showed that L-aspartic acid and L-glycine at 25 mM induced 100% of zoospores of *Py. aphanidermatum* to encyst. In oat tissue extracts, L-aspartic acid and L-glycine can be assumed to be present, along with other inducers of encystment, such as pectins (Hardham and Suzaki, 1986) and cations (Donaldson, 1992). Therefore, it would be assumed that oat tissue extracts should induce zoospore encystment. However, in full strength oat 'seed and root' extract 22 zoospores.μl^{-1} were found to be motile after four hours of incubation, whereas only 8 zoospores.μl^{-1} were motile in SDW, although in the other two full-strength oat tissue extracts the number of zoospores enumerated was markedly less than in SDW (Table 6.10). Therefore, it would appear that there are substances in oat tissues that not only enhance the motility of zoospores, but also counteract zoospore encystment that is mediated by certain amino acids, cations and pectins.

Chapter 8.

Concluding discussion.

8.1. Discussion.

"....if not a present remedy, at least a patient sufferance." †

Several stages of infection-related biology of *Py. aphanidermatum* and *Ph. parasitica* have been assessed for their sensitivity to various potential control methods.

When tested on individual stages of the infection sequence potassium was superior to calcium and magnesium at suppressing zoospore release and zoospore motility. However, suppression of mycelial growth and enhancement of cyst germination were relatively insensitive to potassium supplements. Overall, of the three cations (Ca^{2+}, Mg^{2+} and K^{+}), magnesium was the least effective at suppressing individual stages of the infection sequence. The suppression of zoospore motility by cations has previously been demonstrated (Donaldson, 1992). However, this project has demonstrated that this phenomenon was also true when the counter-ion was nitrate. Nitrate supplements are commercially more acceptable than chlorides. This project also demonstrated that when the counter-ion was nitrate the cation effect was markedly greater than with chloride. However, the explanation for this observation here and in other work (for example, Bryt *et al.*, 1982; von Broembsen & Deacon, 1997) is unknown.

When the same cationic supplements was made sequentially to each stage of the infection sequence the cumulative effect was significant. Again, potassium, of the three cations, was the most suppressive of the infection sequence. Although it has been demonstrated that calcium supplements markedly suppress infection by *Ph. parasitica* in small-scale trails (von Broembsen & Deacon, 1997), the results of this project suggest that potassium supplements would be more effective than calcium. However, this is yet to be demonstrated in small-scale plant trails. A second potential weakness in calcium supplements has also been highlighted in this project. That is, circa. 5 mM Ca^{2+} is optimal for sporangiogenesis of both *Py. aphanidermatum* and *Ph. parasitica* when assessed by the epidemiologically relevant characteristic of the number of zoospores that are subsequently released from sporangia. Coincidentally, the approximate concentration of calcium in commercial NFT systems is 5 mM. Therefore, the reduction of calcium concentration in NFT systems may also suppress infection by zoospore-releasing pythiaceous pathogens.

---

† From 'Much ado about nothing.' by William Shakespeare.

Finding that not only were *in vitro* potassium supplements superior to calcium at suppressing infection related biology of the two fungi, but also that reducing calcium concentration could also suppress infection, the effect of altering the potassium to calcium ratio was investigated. The results of this investigation found that the infection sequence was suppressed by increasing the ratio of potassium to calcium, while maintaining constant nitrate levels and electrical conductivity. The monetary cost of altering this ratio would be marginal.

The cationic supplements to Mineral Salts Solutions appeared to modify the swimming behaviour of the zoospores that were subsequently liberated, suggesting that the environment for sporangiogenesis affects the biology of the subsequent zoospores. If this suggestion were confirmed then it would have significant implications for future research into the control of zoospore-producing pathogens in irrigation systems.

The trial undertaken by HRI failed to demonstrate natural suppression in a semi-commercial irrigation system, which was the principal trial objective. Consequently the analyses presented in this thesis were unable to determine at which points of the infection sequence the agents of natural suppression might act. However, it was found that in treatments where disease was observed, the associated irrigation solutions promoted the production of sporangia in laboratory experiments; also, that the duration of zoospore motility was greater in some irrigation solutions than in distilled water, even though in the irrigation solutions known suppressers of motility would be found, for example, cations (Bryt *et al.*, 1982; amino acids (Donaldson & Deacon, 1993c; lectins (Hardham & Suzaki, 1986; Longman & Callow, 1987) and phosphatidic acid (a phospholipid) (Zhang *et al.*, 1992). Similarly, certain crude oat root extracts extended the period of zoospore motility compared to the SDW control. Therefore, it is concluded that although certain chemicals suppress zoospore motility, there are also compounds which antagonise this effect. Certain irrigation solutions were shown to antagonise the effect of cations on sporangiogenesis and zoospore release.

It was discovered during this project that ethanol had a noticeable effect on sporangiogenesis of *Ph. parasitica*. Subsequent experiments found that zoospore release from pre-formed sporangia of *Py. aphanidermatum* was also highly sensitive to ethanol, but zoospore motility, germinability and cyst germination were relatively insensitive to ethanol supplements. Ethanol was able to antagonise the effect of 5 mM Ca^{2+} on sporangiogenesis, yet the efficacy of ethanol was not compromised by irrigation solutions. The perceived sensitivity of the

Pythiaceae to ethanol has the potential for the basis of a novel control method in NFT systems.

The saponin, β-escin and the bacterial exopeptide gramicidin S lysed or killed motile, naked zoospores. Gramicidin S was also toxic to zoospore cysts, but the presence of a cyst wall markedly reduced the toxic effect of β-escin. The toxicity of gramicidin S to zoospores or cysts was reduced in the presence of divalent cations (Ca^{2+}, Mg^{2+} or Sr^{2+}), but not with monovalent cations (Na^{+} and K^{+}). The toxicity of β-escin was reduced in the presence of Ca^{2+} or K^{+} (both series 4 cations), but not with Mg^{2+} or Na^{+} (both series 3 cations). The toxicity of β-escin (but not of gramicidin S) on zoospores can be partially relieved by the addition of calcium five minutes after exposure to the toxin. When zoospore suspensions were supplemented with both β-escin and gramicidin S the combined toxicity was greater than expected, suggesting that these toxins act synergistically. Although toxicity of β-escin and gramicidin S in zoospores has been demonstrated, the potential for control agents in irrigation systems is not proven.

An observation made throughout this thesis was the consistent difference between the biology of *Py. aphanidermatum* and *Ph. parasitica*. Although the former grows faster under laboratory conditions and liberates more zoospores, the latter is less sensitive to cationic supplements, natural toxins and suppressive irrigation solutions. These observations may be indicative of their different infection strategies.

8.2. Further work.

I suggest that future work based on the findings of this project would be:

- Small-scale trials to investigate the effectiveness of altering the potassium to calcium ratio (while maintaining constant nitrate levels) on the infection of vinca (*Catharanthus roseus)* seedlings by *Ph. parasitica*. The experimental protocol would be based on the one employed by von Broembsen & Deacon (1997).
- In bench-top experiments, determine the sensitivity of tomato seedlings to β-escin, gramicidin S and ethanol. Then only if the sensitivity to these natural toxins is markedly greater than for *Py. aphanidermatum* should small-scale trials be performed.
- To determine whether ethanol does interfere with 1) the production of vesicles from pre-formed sporangia of *Py. aphanidermatum*, 2) the differentiation of cytoplasm in vesicles into zoospores or 3) the release of zoospore from vesicles.
- To establish whether the environment for sporangiogenesis affects the subsequent behaviour of zoospores. This could be ascertained by supplementing MSS with various treatments (for example, pharmacological agents that interfere with calcium, cations amino acids, or toxins) and then using videomicroscopy record the swimming pattern (speed, amplitude and wavelength) of the released zoospores in SDW. In addition to swimming behaviour the chemotactic abilities of the zoospores should be compared to control populations of zoospores. The procedure for recording zoospore swimming behaviour is established (for example, Warburton, 1997).
- It is clear from the results presented in this thesis that there are chemicals in irrigation solutions and oat extracts that increase zoospore motility (when compared to motility in SDW). The determination of these chemicals would require a large screening experiment. Because simple chemicals (cations, amino acids, monosaccharides and disaccharides) have been shown not to extend the motile period of zoospores the screening should perhaps start with stable, common, soluble peptides of microbial and plant origin.

Appendices.

The results presented in these appendices refer to characteristics of irrigation solutions from the experiments at Horticultural Research International (Stockbridge House). The determination of values was performed at Stockbridge House by HRI staff.

Appendix 1. Number of propagules of *Ph. cryptogea* in hydroponic solutions as determined by the membrane method.

Solution	Date of harvesting hydroponic solution from large glasshouse.					
	1st July	15th July	30th July	9th September	24th October	Mean
Fresh Hydroponic Solution	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	20.8 (4.6)	**4.2 (0.9)**
Uninoculated Open	417.5 (20.4)	50.0 (7.1)	16.8 (4.1)	283.3 (16.8)	1187.5 (34.5)	**391.0 (16.6)**
Uninoculated Closed	75.0 (8.7)	16.8 (4.1)	41.8 (6.5)	341.8 (18.5)	91.7 (9.6)	**113.4 (9.5)**
Inoculated Open	795.0 (28.2)	2.5 (1.6)	29.3 (5.4)	241.8 (15.5)	n.d.	**267.2 (12.7)**
Inoculated Closed	452.5 (21.3)	8.3 (2.9)	8.3 (2.9)	108.3 (10.4)	579.2 (24.1)	**231.3 (12.3)**
Inoculated Closed (Pasteurised)	n.d.	n.d.	n.d.	n.d.	58.3 (7.6)	**58.3 (7.6)**
Inoculated Closed (Filtered)	n.d.	n.d.	n.d.	408.2 (20.2)	362.5 (19.6)	**385.4 (19.6)**
Mean	**348.0 (15.7)**	**15.5 (3.1)**	**19.2 (3.8)**	**230.6 (13.6)**	**383.3 (16.6)**	**207.3 (10.9)**

Values in parentheses are square-root transformed.
n.d. Not done.

Appendix 2. Number of propagules of *Ph. cryptogea* in hydroponic solutions as determined by the dip-stick method.

Solution	Date of harvesting hydroponic solution from large glasshouse.					Mean
	1st July	15th July	30th July	9th September	24th October	
Fresh Hydroponic Solution	1.0	0.0	0.5	1.7	0.0	**0.6**
Uninoculated Open	10.3	0.0	1.3	7.3	9.0	**5.6**
Uninoculated Closed	4.7	0.0	0.7	6.0	62.7	**14.8**
Inoculated Open	5.3	8.0	40.3	23.7	17.3	**18.9**
Inoculated Closed	4.0	0.3	0.3	0.0	17.0	**4.3**
Inoculated Closed (Pasteurised)	n.d.	n.d.	n.d.	2.3	3.0	**2.7**
Inoculated Closed (Filtered)	n.d.	n.d.	n.d.	14.7	10.0	**12.4**
Mean	**5.1**	**1.7**	**8.6**	**8.0**	**17.0**	**8.7**

n.d. Not done.

Appendix 3. Incidence of root infection by *Ph. cryptogea* per 1 cm root in hydroponic solutions.

Solution	Date of harvesting hydroponic solution from large glasshouse.					Mean
	1st July	15th July	30th July	9th September	24th October	
Fresh Hydroponic Solution	0.0	0.0	0.0	0.0	0.5	**0.1**
Uninoculated Open	0.0	0.6	2.1	2.8	2.9	**1.7**
Uninoculated Closed	0.0	0.1	1.7	0.6	2.2	**0.9**
Inoculated Open	1.9	0.2	1.2	1.3	0.8	**1.1**
Inoculated Closed	0.7	0.6	2.2	2.4	2.2	**1.6**
Inoculated Closed (Pasteurised)	n.d.	n.d.	n.d.	0.0	2.3	**1.2**
Inoculated Closed (Filtered)	n.d.	n.d.	n.d.	2.3	1.8	**2.1**
Mean	**0.5**	**0.3**	**1.4**	**1.3**	**1.8**	**1.2**

n.d. Not done.

Appendix 4. Index of root development (0-100) in hydroponic solutions.

Solution	Date of harvesting hydroponic solution from large glasshouse.					Mean
	1st July	15th July	30th July	9th September	24th October	
Fresh Hydroponic Solution	68.2	77.6	68.4	59.2	75.6	**69.8**
Uninoculated Open	53.4	57.2	57.8	56.6	18.9	**48.8**
Uninoculated Closed	79.2	72.2	49.0	58.8	32.2	**58.3**
Inoculated Open	57.6	72.2	60.0	56.6	30.0	**55.3**
Inoculated Closed	41.8	62.2	50.0	55.6	33.3	**48.6**
Inoculated Closed (Pasteurised)	n.d.	n.d.	n.d.	62.2	45.3	**53.8**
Inoculated Closed (Filtered)	n.d.	n.d.	n.d.	58.6	57.8	**58.2**
Mean	**60.0**	**68.3**	**57.0**	**58.2**	**41.9**	**56.1**

n.d. Not done.

Appendix 5. Index of root decolouration (0-100) in hydroponic solutions.

Solution	Date of harvesting hydroponic solution from large glasshouse.					Mean
	1st July	15th July	30th July	9th September	24th October	
Fresh Hydroponic Solution	0.0	0.0	0.0	0.0	0.0	**0.0**
Uninoculated Open	16.7	20.8	35.6	28.4	98.7	**40.0**
Uninoculated Closed	10.0	7.8	42.2	31.2	73.3	**32.9**
Inoculated Open	32.6	21.2	32.2	35.6	79.3	**40.2**
Inoculated Closed	31.8	22.2	58.8	20.0	96.3	**45.8**
Inoculated Closed (Pasteurised)	n.d.	n.d.	n.d.	14.4	50.2	**32.3**
Inoculated Closed (Filtered)	n.d.	n.d.	n.d.	20.0	60.9	**40.5**
Mean	**18.2**	**14.4**	**33.8**	**21.4**	**65.5**	**33.1**

n.d. Not done.

Appendix 6. Lesion number per unit area of root in hydroponic solutions.

Solution	Date of harvesting hydroponic solution from large glasshouse.					Mean
	1st July	15th July	30th July	9th September	24th October	
Fresh Hydroponic Solution	0.0	0.0	0.0	0.0	0.0	**0.0**
Uninoculated Open	1.3	3.6	2.0	2.7	28.4	**7.6**
Uninoculated Closed	0.6	0.2	3.0	3.1	12.4	**3.9**
Inoculated Open	4.5	3.8	2.4	4.3	16.3	**6.3**
Inoculated Closed	3.5	2.7	4.2	0.9	27.9	**7.8**
Inoculated Closed (Pasteurised)	n.d.	n.d.	n.d.	1.5	9.5	**5.5**
Inoculated Closed (Filtered)	n.d.	n.d.	n.d.	1.3	7.7	**4.5**
Mean	**2.0**	**2.1**	**2.3**	**2.0**	**14.6**	**5.1**

n.d. Not done.

Appendix 7. Agronomic factors for tomatoes grown in hydroponic solutions harvested on the 24th October.

Solution	Height (cm)	Stem diameter (mm)	Leaf Area (cm^2)	Leaf Length (mm)	Root weight (g)
Fresh Irrigation Solution	91.7	10.0	415.2	407.3	6.56
Uninoculated Open	57.0	7.7	153.6	272.0	1.33
Uninoculated Closed	61.9	8.0	206.5	291.0	2.52
Inoculated Open	61.9	7.3	199.9	316.2	2.49
Inoculated Closed	67.8	6.9	212.7	301.4	2.51
Inoculated Closed (Pasteurised)	71.4	7.4	223.1	323.4	3.27
Inoculated Closed (Filtered)	84.3	9.2	376.3	401.2	4.50
Mean	**70.9**	**8.1**	**255.3**	**330.4**	**3.31**

Appendix 8. Analysis of inorganic chemical composition of the uninoculated open irrigation solution throughout the trial.

Element	3rd June	17th June	1st July	15th July	29th July	12th August	26th August	16th September	23rd September
Ammonium as N (ppm)	0.5	0.8	0.4	0.1	0.1	0.3	0.3	0.3	0.2
Boron (ppm)	0.3	0.15	0.3	0.19	0.34	0.73	0.34	0.34	0.36
Calcium (ppm)	288	274	196	290	333	305	209	219	265
Carbonate (ppm)	93	32	47	24	32	109	41	46	42
Chloride (ppm)	57	43	38	37	50	80	38	51	48
Conductivity (mS)	3243	2833	2590	2112	3501	4049	3066	3010	3598
Copper (ppm)	0.05	0.03	0.05	0.05	0.11	0.26	0.11	0.22	0.24
Iron (ppm)	2.01	3	1.52	2.1	3.43	7.45	5.5	4.36	5.66
Magnesium (ppm)	89	77	82	69	93	149	88	93	89
Manganese (ppm)	0.07	0.01	0.05	0.04	0.12	0.02	0.06	0.05	0.03
Molybdenum (ppm)	0	0	0	0	0	0	0.04	0	0
Nitrate as N (ppm)	311	296	245	301	452	412	368	341	412
pH	6.3	6.6	6.7	7	7.1	7.8	7.2	7.3	7.1
Phosphorus (ppm)	104	32	46	11	15	18	19	10	9
Potassuim (ppm)	679	437	384	351	485	804	578	516	587
Sodium (ppm)	38	34	43	34	43	64	37	39	45
Sulphate (ppm)	83	44	64	45	44	140	55	27	64
Zinc (ppm)	0.45	0.27	0.26	0.24	0.34	0.84	0.48	0.43	0.45

Appendix 9. Analysis of inorganic chemical composition of uninoculated closed the irrigation solution throughout the trial.

Element	3rd June	17th June	1st July	15th July	29th July	12th August	26th August	16th September	23rd September
Ammonium as N (ppm)	0.5	0.2	0.4	0.2	0.1	0.3	0.3	0.2	0.2
Boron (ppm)	0.39	0.12	0.33	0.39	0.31	0.58	0.35	0.32	0.29
Calcium (ppm)	209	246	189	314	403	641	348	337	359
Carbonate (ppm)	112	27	48	39	29	65	43	78	70
Chloride (ppm)	77	52	65	84	80	132	72	73	67
Conductivity (mS)	2840	2833	2680	3018	3043	4287	3388	3349	3466
Copper (ppm)	0.17	0.05	0.11	0.14	0.1	0.18	0.12	0.16	0.17
Iron (ppm)	1.79	2.7	1.97	2.32	2.14	5.85	3.32	2.79	3.46
Magnesium (ppm)	98	70	107	155	132	205	124	141	136
Manganese (ppm)	0.06	0.03	0.04	0.01	0.09	0.12	0.06	0.04	0.02
Molybdenum (ppm)	0	0	0	0	0	0	0.05	0	0
Nitrate as N (ppm)	206	311	199	266	364	481	404	388	382
pH	6.4	6.4	6.2	6.5	6.9	7.6	7.4	7.5	7.5
Phosphorus (ppm)	142	41	74	48	17	3	6	5	3
Potassuim (ppm)	643	465	326	280	182	240	264	210	166
Sodium (ppm)	53	35	74	106	81	132	88	103	103
Sulphate (ppm)	109	48	105	131	87	146	87	45	89
Zinc (ppm)	0.75	0.34	1.03	0.9	0.8	1.3	0.7	0.84	0.87

Appendix 10. Analysis of inorganic chemical composition of the inoculated open irrigation solution throughout the trial.

Element	3rd June	17th June	1st July	15th July	29th July	12th August	26th August	16th September	23rd September
Ammonium as N (ppm)	0.8	0.1	0.2	0.2	0.3	0.3	0.2	0.1	0.2
Boron (ppm)	0.31	0.22	0.28	0.36	0.41	0.51	0.33	0.34	0.27
Calcium (ppm)	234	260	170	328	295	288	189	217	209
Carbonate (ppm)	80	38	42	59	35	37	39	40	37
Chloride (ppm)	57	138	32	24	44	45	45	48	47
Conductivity (mS)	2925	3187	2412	3363	3501	3572	2864	2946	3247
Copper (ppm)	0.06	0.14	0.05	0.02	0.14	0.28	0.11	0.2	0.21
Iron (ppm)	1.79	2.47	1.3	2.15	2.97	6.06	4.33	4.42	3.89
Magnesium (ppm)	86	97	78	126	81	117	72	75	69
Manganese (ppm)	0.11	0.12	0.07	0.03	0.25	0.31	0.15	0.16	0.18
Molybdenum (ppm)	0	0	0	0	0	0	0.04	0	0
Nitrate as N (ppm)	262	282	221	339	466	433	326	320	359
pH	6.4	6.3	7	7.5	7	6.9	7.4	7.1	7
Phosphorus (ppm)	87	70	30	7	23	26	16	17	15
Potassuim (ppm)	590	570	344	435	487	680	543	493	527
Sodium (ppm)	35	50	36	65	37	49	33	34	38
Sulphate (ppm)	87	64	65	97	61	87	57	59	59
Zinc (ppm)	0.34	0.75	0.16	0.27	0.5	1.05	0.46	0.43	0.49

Appendix 11. Analysis of inorganic chemical composition of the inoculated closed irrigation solution throughout the trial.

Element	3rd June	17th June	1st July	15th July	29th July	12th August	26th August	16th September	23rd September
Ammonium as N (ppm)	0.7	0.4	0.4	0.4	0.2	0.2	0.3	0.2	0.1
Boron (ppm)	0.34	0.27	0.3	0.45	0.33	0.3	0.29	0.23	0.18
Calcium (ppm)	271	262	231	445	403	405	350	301	327
Carbonate (ppm)	85	40	27	29	29	21	22	22	26
Chloride (ppm)	88	106	52	68	52	61	60	52	56
Conductivity (mS)	3031	3276	2590	3449	2939	2977	2904	2501	2764
Copper (ppm)	0.14	0.14	0.11	0.14	0.11	0.11	0.1	0.16	0.15
Iron (ppm)	1.98	1.99	1.92	3.26	2.1	3.12	3.2	2.99	3.29
Magnesium (ppm)	92	118	86	158	113	113	102	80	93
Manganese (ppm)	0.3	0.12	0.21	0.05	0.14	0.17	0.16	0.27	0.22
Molybdenum (ppm)	0	0	0	0	0	0	0.01	0	0
Nitrate as N (ppm)	248	281	222	321	296	323	351	282	300
pH	6.2	5.9	5.9	5.8	6.4	6.7	6.8	6.3	6.3
Phosphorus (ppm)	151	123	88	107	39	19	14	20	15
Potassuim (ppm)	636	711	332	274	157	160	230	220	230
Sodium (ppm)	49	75	58	100	73	80	70	50	54
Sulphate (ppm)	99	79	69	125	86	88	69	57	56
Zinc (ppm)	0.75	1.02	0.82	1.44	0.79	0.72	0.7	0.64	0.6

Appendix 12. Analysis of inorganic chemical composition of the inoculated closed (pasteurised) irrigation solution throughout the trial.

Element	3rd June	17th June	1st July	15th July	29th July	12th August	26th August	16th September	23rd September
Ammonium as N (ppm)	0.7	0.6	0.7	0.5	0.4	0.5	0.6	0.4	0.6
Boron (ppm)	0.49	0.25	0.33	0.59	0.39	0.64	0.5	0.35	0.34
Calcium (ppm)	261	224	208	440	404	618	395	385	339
Carbonate (ppm)	82	63	43	45	38	48	40	37	40
Chloride (ppm)	105	114	62	84	86	129	111	85	91
Conductivity (mS)	3497	3099	2680	3535	3064	3612	3429	2671	3423
Copper (ppm)	0.3	0.22	0.16	0.34	0.27	0.54	0.35	0.37	0.37
Iron (ppm)	1.08	1.8	2.13	4.05	3.09	8.52	6.12	4.74	5.12
Magnesium (ppm)	122	115	104	226	141	218	172	143	134
Manganese (ppm)	0.08	0.14	0.08	0.03	0.13	0.2	0.11	0.13	0.56
Molybdenum (ppm)	0	0	0	0	0	0	0	0	0
Nitrate as N (ppm)	262	177	189	276	294	444	335	240	322
pH	6.1	6.3	6	5.6	5.8	5.7	5.8	5.8	5.7
Phosphorus (ppm)	200	113	119	205	137	156	114	104	122
Potassuim (ppm)	788	583	356	342	152	344	176	43	264
Sodium (ppm)	66	80	75	150	98	158	141	107	115
Sulphate (ppm)	136	81	82	192	121	178	138	102	113
Zinc (ppm)	0.72	0.89	0.82	1.57	1.1	1.97	1.68	1.27	1.3

Appendix 13. Analysis of inorganic chemical composition of the inoculated closed (filtered) irrigation solution throughout the trial.

Element	3rd June	17th June	1st July	15th July	29th July	12th August	26th August	16th September	23rd September
Ammonium as N (ppm)	0.6	0.3	0.5	0.4	0.2	0.2	0.4	0.2	0.2
Boron (ppm)	0.35	0.32	0.35	0.44	0.4	0.36	0.33	0.29	0.16
Calcium (ppm)	233	241	254	393	406	514	476	447	366
Carbonate (ppm)	40	55	31	29	33	49	40	27	37
Chloride (ppm)	81	155	111	81	102	130	107	82	104
Conductivity (mS)	3052	3718	3037	3406	3501	3612	2622	3073	3510
Copper (ppm)	0.17	0.09	0.09	0.15	0.07	0.08	0.04	0.05	0.03
Iron (ppm)	1.68	2	1.52	1.6	1.83	3.56	2.86	1.89	2.95
Magnesium (ppm)	101	149	121	205	119	181	180	154	140
Manganese (ppm)	0.19	0.03	0.04	0.05	0.05	0.12	0.03	0.2	0.32
Molybdenum (ppm)	0	0	0	0	0	0	0.03	0	0
Nitrate as N (ppm)	245	213	221	250	410	370	382	342	436
pH	5.8	6.2	6	5.8	6.4	7.3	7.2	7	7
Phosphorus (ppm)	144	109	99	110	40	10	8	7	8
Potassuim (ppm)	607	672	380	233	58	102	35	55	282
Sodium (ppm)	56	110	98	143	138	169	156	121	147
Sulphate (ppm)	108	159	92	187	152	161	144	113	103
Zinc (ppm)	0.64	0.96	0.73	1.08	0.94	1.04	0.75	0.64	0.61

References.

Adams, P. & Ho, L.C. (1993). Effects of environment on the uptake and distribution of calcium in tomato and on the incidence of blossom-end rot. *Plant and Soil* 154:127-132.

Agudo, L.D. (1992). Lipid content of *Saccharomyces cerevisae* strains with different degrees of ethanol tolerance. *Applied Microbiology and Biotechnology* 37:647-651.

Akutsu, H., Suezaki, Y., Yoshikawa, W. & Kyogoku, Y. (1986). Influence of metal ions and a local anesthetic on the conformation of the choline group of phosphatidycholine bilayers studied by Raman spectroscopy. *Biochimica et Biophysica Acta* 854:213-218.

Alexpoulos, C.J., Lassalles, J.P. & Kado, R.T. (1996). Indroductory Mycology. *Wiley, Chichester.*

Allen, R.N. & Harvey, J.D. (1974). Negative chemotaxis of zoospores of *Phytophthora cinnamomi. Journal of Microbiology* 84:28-38.

Allen, R.N. & Newhook, F.J. (1973). Chemotaxis of zoospores of *Phytophthora cinnamomi* to ethanol in capillaries of soil pore dimensions. *Transactions of the British Mycological Society* 61:287-302.

Allen, R.N. & Newhook, F.J. (1974). Suppression by ethanol of spontaneous turning activity in zoospores of *Phytophthora cinnamomi. Transactions of the British Mycological Society* 63:383-384.

Altenbach, C. & Seelig, J. (1984). Ca^{2+} binding to phosphatidylcholine bilayers as studied by deuterium magnetic resonance. Evidence for the formation of a Ca^{2+} complex with two phospholipid molecules.*Biochemistry* 23:3913-3920.

Anchordoguy, T.J., Carpenter, J.F., Crowe, J.H. & Crowe, L.M. (1992). Temperature-dependent perturbation of phospholipid-bilayers by dimethylsulfoxide. *Biochimica et Biophysica Acta* 1104:117-122.

Anon. (1997). Nutritive composition of Campbell Soup Company Ltd. Products (May 1997). Campbell's U.K. Ltd., Kings Lynn, U.K.

Anon. (2000a). Fertilizer prices. Hydro-Gardens, HGI Worldwide, Inc., Colorado Springs, Colorado, USA. (http://www.hydro-gardens.com/fertcomp.htm)

Anon. (2000b). Fertilizer mixes. Hydro-Gardens, HGI Worldwide, Inc., Colorado Springs, Colorado, USA. (http://www.hydro-gardens.com/fertilizers.html)

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

Armah, C.N., Mackie, A.R., Roy, C., Price, K., Osbourn, A.E., Bowyer, P. & Ladha S. (1999). The membrane-permeabilizing effect of avenacin A-1 involves the reorganization of bilayer cholesterol. *Biophysical Journal* 76:281-290

Arneson, P.A. & Durbin, R.D. (1968). The sensitivity of fungi to α-tomatine. *Phytopathology* 58:536-537.

Ayers, W.A. (1971). Induction of sporangia in *Phytophthora cinnamomi* by a substance from bacteria and soil. *Canadian Journal of Microbiology* 17:1517-1523.

Ayres, W.A., & Zentmyer, G.A. (1971). Effect of soil solution and two soil Pseudomonads on sporangium production by *Phytophthora cinnamomi. Phytopathology.* 61:1188-1193.

Barach, I., Klisiewicz, J.M., & Kosuge, T. (1965). Utilization of carbon compounds by zoospores of *Phytophthora drechsleri* and their effect on motility and germination. *Phytopathology* 55:1257-1261.

Barry, J.A. & Gawrisch, K. (1994). Direct NMR evidence for ethanol binding to the lipid-water interface of phospholipid bilayers. *Biochemistry* 33:8082-8088.

Bartnicki-Garcia, S., & Sing, V.O. (1987). Adhesion of zoospores of *Phytophthora* to solid surfaces. In M.S. Fuller & A. Jaworski (Eds.), *Zoosporic Fungi in Teaching and Research.* (pp. 279-283). Athens, GA, USA: Southeastern Publishing Corporation.

Bartnicki-Garcia, S. & Wang, M. (1983). Biochemical aspects of morphogenesis in Phytophthora. In, *Phytophthora: Its Biology, Taxonomy, Ecology and Pathology.* Eds D.C.Erwin, S.Bartnicki-Garcia and P.H.Tsao. American Phytopathological Society, St.Paul, Minn., USA.

Barton, R. (1958). Occurence and establishment of *Pythium* in soils. *Transactions of the British Mycological Society* 41:207-222.

Beakes, G.W., Burr, A.W., Wood, S.E. & Hardham, A.R. (1995). The application of spore surface features in defining taxonomic versus ecological groupings in oomycete fungi. *Canadian Journal of Botany* 73:S701-S711.

Beaulieu, J.C. & Saltveit, M.E. (1997). Inhibition or promotion of tomato fruit ripening by acetaldehyde and ethanol is concentration dependent and varies with initial fruit maturity. *Journal of the American Society for Horticultural Science* 122:392-398.

Bender, C.G. & Shoemaker, P.B. (1984). Prevalence of verticillium wilt of tomato and *Verticillium dahliae* race-1 and race-2 isolates in Westen North Caralina. *Plant Disease* 68:305-309.

Benhamou, N. & Côté, F. (1992). Ultrastructure and cytochemistry of pectin and cellulose degradation in tobacco roots infected by *Phytophthora parasitica* var. *nicotianae. Phytopathology.* 82:468-478.

Benjamin, M., & Newhook, F.J. (1982). Effect of glass microbeads on *Phytophthora* zoospore motility. *Transactions of the British Mycological Society* 78:43-46.

Berkelmann, B. & Wahanka, W. (1993). Characterization of the bacterial flora in circulating nutrient solutions of a hydroponic system with rockwool. *Summaries International Society for Horticultural Science Symposium Cagliary, Italy, 26-30 April 1993.*

Bernhardt, E.A., & Grogan, R.G. (1982). Effect of soil matric potential on the formation and indirect germination of sporangia of *Phytophthora-parasitica, Phytophthora capsici,* and *Phytophthora* cryptogea. *Phytopathology* 72:507-511.

Bhattacharya, D. & Medlin, L. (1992). Algae containing chlorophylls A+C are paraphyletic: molecular evolutionary analysis of the Chromophyta. *Evolution* 46:1801-1817.

Bimpong, C.E. (1975). Changes in metabolic reserves and enzyme activities during zoospore motility and cyst germination in *Phytophthora palmivora. Canadian Journal of Botany* 53:1411-1416.

Bimpong, C.E., & Clerk, G.C. (1970). Motility and chemotaxis in zoospores of *Phytophthora* palmivora (Butl.) Butl. *Annual Review of Botany* 34:617-624.

Bimpong, C.E. & Hickman, C.J. (1975). Ultrastructural and cytochemical studies of zoospores, cysts and germinating cysts of *Phytophthora palmivora. Canadian Journal of Botany* 53:1310-1327.

Blaker, N.S., & MacDonald, J.D. (1982). Formation and indirect germination of sporangia of *Phytophthora parasitica* and *Phytophthora cryptogea* in saline soils. *Phytopathology* 72:995.

Blaker, N.S., & MacDonald, J.D. (1983). Influence of container medium pH on sporangium formation, zoospore release, and infection of rhododendron by *Phytophthora cinnamomi*. *Plant Disease* 67:259-263.

Blein, N.S., Milat, M.L. & Ricci, P. (1991). Responses of cultured tobacco cells to cryptogein, a proteinaceous elicitor from *Phytophthora cryptogea* - possible plasmalemma involvement. *Plant Physiology* 95:486-491.

Bloodgood, R.A. (1991). Transmembrane signalling in cilia and flagella. *Protoplasma* 164:12-22.

Bogdanova, A.Y., Sherstobitov, A.O. & Gusev, G.P. (1998). Chloride transport in red blood cells of lamprey *Lampetra fluviatilis*: evidence for a novel anion-exchange system. *The Journal of Experimental Biology* 201:693-700.

Bonde, R. & Murphy, E.F. (1952). Resistance of certain tomato varieties and crosses to late blight. *Maine Agricultural Experimental Station Bullettin* 497.

Borle, F. & Seelig, J. (1985). Ca^{2+} binding to phosphatidylglycerol bilayers as studied by differential scanning calorimetry and ^{2}H- and ^{31}P-nuclear magnetic resonance. *Chemistry and Physics of Lipids* 36:263-283.

Botha, S.J., Grobler, A. & Senekal, R. (1997). *In vitro* ethanol tolerance of *Candida albicans*. *Journal of Dental Research* 76:1206.

Bouchibi, N., van Bruggen, A.H.C. & MacDonald, J.D. (1990). Effects of ion concentration and sodium:calcium ratio of a nutrient solution on *Phytophthora* root rot of tomato and zoospore motility and viability of *Phytophthora parasitica*. *Phytopathology* 80:1323-1329.

Brasier, C.M. (1969). The effects of light and temperature on reproduction in vitro in two tropical species of *Phytophthora*. *Transactions of the British Mycological Society* 52:105-113.

Bres, W. & Weston, L.A. (1992). Nutrient accumulation and tipburn in NFT-grown lettuce at several potassium and pH levels. *Hortscience* 27:790-792.

Bruin, G. C. A. & Edgington, L.V. (1983). The chemical control of diseases caused by zoosporic fungi. In *Zoosporic Plant Pathogens: a Modern Perspective*. S.T. Buczacki, ed. Academic Press, London.

Bruneteau, M., Fournol, F., Gandon, C., Becchi, M. & Pivot V. (1997). Isolation and characterization of inositol sphingophospholipids from *Phytophthora parasitica* Dastur. *Lipids* 32:359-362.

Bryt, P.N., Irving, H.R. & Grant, B.R. (1982). The effect of cations on zoospores of the fungus *Phytophthora cinnamomi*. *Journal of General Microbiology* 128:1189-1198.

Budde, A.D., & Helgeson, J.P. (1981). Chronology of phytoalexin production and histological-changes in tobacco callus infected with *Phytophthora parasitica* var. *nicotianae*. *Phytopathology* 71:864.

Burnett, J.H. (1975). Mycogenetics: An Introduction to the General Genetics of Fungi. John Wiley & Sons, London.

Buysens, S., Heungens, K., Poppe, J. & Höfte, M. (1996). Involvement of pyochelin and pyoverdin in suppression of *Pythium*-induce damping-off of tomato by *Pseudomonas aeruginosa* 7NSK2. *Applied and Environmental Microbiology* 62:865-871.

Byrt, P.N., Irving, H.R. & Grant, B.R. (1982). The effect of organic-compounds on the encystment, viability and germination of zoospores of *Phytophthora cinnamomi*. *Journal of General Microbiology* 128:2343-2351.

Cahill, D.M. & Hardham, A.R. (1994). Exploitation of zoospore taxis in the development of a novel dipstick immunoassay for the specific detection of *Phytophthora cinnamomi*. *Phytopathology* 84:193-200.

Cameron, J.N. & Carlile, M.J. (1977). Negative geotaxis of the fungus *Phytophthora*. *Journal of General Microbiology* 98:599-602.

Cameron, J.N. & Carlile, M.J. (1978). Fatty acids, aldehydes and alcohols as attractants for zoospores of *Phytophthora palmivora*. *Nature* 271:448-449.

Cameron, J.N. & Carlile, M.J. (1980). Negative chemotaxis of zoospores of the fungus *Phytophthora palmivora*. *Journal of Microbiology* 120:347-353.

Capasso, R., Cristinzio, G., Evidente, A., Visca, C., Ferranti, P., Blanco, F.D. & Parente, A. (1999). Elicitin 172 from an isolate of *Phytophthora nicotianae* pathogenic to tomato. *Phytochemistry* 50:703-709.

Carlile, M.J. (1983). Motility, taxism and tropism in *Phytophthora*. In S. Bartnicki-Garcia & D.C. Erwin (Eds.), *Phytophthora: Its Biology, Taxonomy, Ecology and Pathology*. (pp. 95-108). St. Paul, Minnesota, USA. American Phytopathological Society.

Cassinelli, C., Noris, E. & Tolentino, D. (1993). *In vitro* inhibition of *Pythium ultimum* by *Pseudomonas* spp. strains. *Med. Fac. Landbouww Univ. Gent.* 58:1287-1296.

Cavalier-Smith, T. (1986). The kingdom Chromista: Origin and systematics, in: *Progress in Physiological Research*, Vol 4. (eds. I. Round and D.J. Chapman). Biopress, Bristol, U.K.

Cerenius, L. & Söderhäll K. (1985). Repeated zoospore emergance as a possible adaptation to parasitism in *Aphanomyces*. *Experimental Mycology* 9:259-263.

Chang, R.J., Ries, S.M. & Patajy, J.K. (1992). Effects of temperature, plant age, inoculum concentration, and cultivar on incubation period and severity of bacterial canker of tomato. *Plant Disease* 76:1150-1155.

Chen, C.Q., Bélanger, R.R., Benhamou, N. & Paulitz, T.C. (1998). Induced systemic resistance (ISR) by *Pseudomonas* spp. impaires pre- and post-infection development of *Pythium aphanidermatum* on cucumber roots. *European Journal of Plant Pathology* 104:877-886.

Chérif, M. & Bélanger, R.R. (1992). Use of potassium silicate amendments in recirculating nutrient solutions to suppress *Pythium ultimum* on long English cucumber. *Plant Disease.* 76:1008-1011.

Chi, C.C. & Sabo, F.E. (1978), Chemotaxis of zoospores of *Phytophthora megasperma* to primary roots of alfalfa seedlings. *Canadian Journal of Botany* 56:795-800.

Chi, Z., Kohlwein, S.D. & Paultaul, F. (1999). Role of phosphatidylinositol (PI) in ethanol production and ethanol tolerance by a high ethanol producing yeast. *Journal of Inducstrial Microbiology and Biotechnology* 22:58-63.

CLERK, G.C. (1972). Germination of sporangia of *Phytopththora palmivora* (Butl.) Butl. *Annals of Botany* 36:801-807.

CONOVER, R.A. & WALTER, J.M. (1953). The occurence of a virulent race of *Phytophthora infestans* on late blight resistant tomato stocks. *Phytopathology* 43:344-345.

COOPER, A. (1979). *The ABC of NFT: Nutrient Film Technique: the World's First Method of Crop Production without a Solid Rooting Medium*. Grower Books, London.

COVARRUBIAS, M. & RUBIN, E. (1993). Ethanol selectivity blocks a noninactivating K^+ current expressed in *Xenopus* oocytes. *Proceedings of the National Academy of Science* 90:6957-6960.

CUNNINGHAM, B.A., SHIMOTAKE, J.E., TAMURA-LIS, W., MASTRAN, W., KWOK, W.M., KAUFFMAN, J.W. & LIS, L.J. (1986). The influence of ion species on phosphatidylcholine bilayer structure and packing. *Chemistry and Physics of Lipids* 39:135-143.

DAVISON, E.M. & TAY, F.C.S. (1986). The effect of aeration on colony diameter, sporangium production and zoospore germination of *Phytophthora cinnamomi*. *New Phytologist* 103:735-744.

DEACON, J.W. & DONALDSON, S.P. (1993). Molecular recognition in the homing responses of zoosporic fungi, with special reference to *Pythium* and *Phytophthora*. *Mycological Research* 97:1153-1171.

DEACON, J.W. & MITCHELL, R.T. (1985). Toxicity of oat roots, oat root extracts, and saponins to zoospores of *Pythium* spp. and other fungi. *Transactions of the British Mycological Society* 84:479-487.

DEACON, J.W. & SAXENA, G. (1998). Germination triggers of zoospore cysts of *Aphanomyces euteiches* and *Phytophthora parasitica*. *Mycological Research* 102:33-41.

DE ZOETEN, G.A., GAARD, G., HABERLACH, G.T., & HELGESON, J.P. (1982). Infection of tobacco callus by *Phytophthora parasitica* var. *nicotianae*. *Phytopathology* 72:743-746.

DICK, M.W. (1995). The straminipilous fungi. A new classification for the biflagellate fungi and their uniflagellate relatives with particualar reference to lagenidiaceous fungi. *C.A.B. International Mycological Paper* No. 168.

DICK, M.W. & WIN-TIN. (1973). The development of cytological theory in the Oomycetes. *Biological Reviews* 48:133-158.

DONALDSON, S.P. (1992). Behaviour of zoospores of *Pythium* species. PhD thesis, University of Edinburgh.

DONALDSON, S.P. & DEACON, J.W. (1992). Role of calcium in adhesion and germination of zoospore cysts of *Pythium* - a model to explain infection of host plants. *Journal of General Microbiology* 138:2051-2059.

DONALDSON, S.P. & DEACON, J.W. (1993a). Changes in motility of *Pythium* zoospores induced by calcium and calcium-modulating drugs. *Mycological Research* 97:877-883.

DONALDSON, S.P. & DEACON, J.W. (1993b). Differential encystment of zoospores of *Pythium* species by saccharides in relation to establishment on roots. *Physiological and and Molecular Plant Pathology* 42:177-184.

DONALDSON, S.P., & DEACON, J.W. (1993c). Effects of amino-acids and sugars on zoospore taxis, encystment and cyst germination in *Pythium aphanidermatum* (Edson) Fitzp, *P. catenulatum* Matthews and *P. dissotocum* Drechs. *New Phytologist* 123:289-295.

ECKERT, J.W. & TSAO, P.H. (1962). A selective antibiotic medium for isolation of Phytophthora and Pythium from plant roots. *Phytopathology* 52:771-777.

EDWARDS, S.G. (1993). Biological control of *Botrytis cinerea* by *Bacillus brevis* on protected Chinese cabbage. PhD thesis, University of Aberdeen.

ELLIOTT, C.G. (1983). Physiology of sexual reproduction in Phytophthora. In, *Phytophthora: Its Biology, Taxonomy, Ecology and Pathology*. Eds D.C.Erwin, S.Bartnicki-Garcia and P.H.Tsao. American Phytopathological Society, St.Paul, Minn., USA.

ENGLANDER, L. & ROTH, L.F. (1980). Interaction of light and sterol on sporangium and chlamydospore production by *Phytophthora lateralis*. *Phytopathology* 70:650-654.

ENGLISH, J.T. & MITCHELL, D.J. (1988). Relationships between the development of root systems of tobacco and infection by *Phytophthora parasitica* var. *nicotianae*. *Phytopathology* 78:1478-1483.

ENGLISH, J.T. & MITCHELL, D.J. (1989). Use of morphometric analysis for characterization of tobacco root- growth in relation to infection by *Phytophthora parasitica* var. *nicotianae*. *Plant and Soil* 113:243-249.

ERWIN, D.C. & RIBEIRO, O.K. (1996). *Phytophthora Diseases Worldwide*. American Phytopathological Society, St.Paul, Minn., USA.

ESTRADA-GARCIA, M.T., CALLOW, J.A. & GREEN, J.R. (1990a). Monoclonal-antibodies to the adhesive cell coat secreted by *Pythium aphanidermatum* zoospores recognize 200 & 103 M_r glycoproteins stored within large peripheral vesicles. *Journal of Cell Science* 95:199-206.

ESTRADA-GARCIA, M.T., RAY, T.C., GREEN, J.R., CALLOW, J.A. & KENNEDY, J.F. (1990b). Encystment of *Pythium aphanidermatum* zoospores is induced by root mucilage polysaccharides, pectin and a a monoclonal antibody to a surface antigen. *Journal of Experimental Botany* 41:693-699.

EVANS, S.G. (1977). Disease risks affect entire crop in NFT systems. *Grower* 88:1233-1239.

EVANS, S.G. (1979). Susceptibility of plants to fungal pathogens when grown by the nutrient-film technique (NFT). *Plant Pathology* 28:45-48.

FAGG, J. & FLETCHER, J.T. (1987). Studies of the epidemiology and control of tomato stem rot caused by *Didymella lycopersici*. *Plant Pathology* 36:361-366.

FAWE, A., ABOU-ZAID, M., MENZIES, J.G. & BELANGER, R.R. (1998). Silicon-mediated accumulation of flavonoid phytoalexins in cucumber. *Phytopathology* 88:396-401.

FENWICK, G.R., PRICE, K.R., TSUKAMOTA, C. & OKUBO, K. (1992). Saponins. *In: Toxic Substances in Crop Plants*. Eds J.P. D'Mello, C.M. Duffus & J.H. Duffus. Cambridge, UK: Royal Society of Chemistry.

FINCHAM, J.R.S., DAY, P.R. & RADFORD, A. (1979). *Fungal genetics*. 4th edn Botanical Monograph No. 4. Blackwell Scientific Publications, Oxford.

FORDHAM, R. & BIGGS, A.G. (1985). *Principles of Vegetable Crop Production*. Collins, Great Britain.

FUSCONI, A., GNAVI, E., TROTTA, A. & BERTA, G. (1999). Apical meristems of tomato roots and their modifications induced by arbuscular mycorrhizal and soilborne pathogenic fungi. *New Phytologist* 142:505-516.

Garibaldi, A. & Gullino, M.L. (1993). Influence of soilless cultivation on soilbourne diseases. *Summaries International Society for Horticultural Science Symposium Cagliary, Italy, 26-30 April 1993.*

Gennis, R.B. (1989). *Biomembranes: Molecular Structure and Function*. Springer-Verlag, London.

Germain, V., Ricard, B., Raymond, P. & Saglio, P. (1997). The role of sugars, hexokinase, and sucrose synthase in the determination of hypoxically induced tolerance to anoxia in tomato roots. *Plant Physiology* 114:167-175.

Ghaouth, A.E., Arul, J., Grenier, J., Benhamou, N., Asselin, A. & Bélanger, R. (1994). Effect of chitosan on cucumber plants: suppression of *Pythium aphanidermatum* and induction of defense reactions. *Phytopathology* 84:313-320.

Gilbertson, T.A., Scobey, R. & Wilson, M. (1991). Permeation of calcium ions through non-NMDA glutamate channels in retinal bi-polar cells. *Science* 251:1613-1615.

Gisi, U. (1983). Methods and theoretical aspects of studying discharge mechanisms in *Phytophthora* nad *Pythium* species. In D.C. Erwin, S. Bartnicki-Garcia, & P.H. Tsao (Eds.), *Phytophthora: Its Biology, Taxonomy, Ecology and Pathology*. (pp. 303-313). St. Paul, Minnesota: The American Phytopathological Society.

Goldberg, N.P., Hawes, M.C. & Stanghellini, M.E. (1989). Specific attraction to and infection of cotton root cap cells by zoospores of *Pythium dissotocum*. *Canadian Journal of Botany-Journal Canadian de Botanique* 67:1760-1767.

Goldberg, N.P., Stanghellini, M.E. & Rasmussen, S.L. (1992). Filtration as a method for controlling pythium root rot of hydroponically grown cucumbers. *Plant Disease* 76:777-779.

Goodman, R.N. & Novacky, A.J. (1994). The hypersensitive reaction to plants to pathogens: a resistance phenomenon. American Phytopathological Society, St. Paul, Minn., USA.

Gow, N.A.R. (1994). Tip growth and polarity, pp428 in *The Growing Fungus* (eds N.A.R. Gow and M. J. Gadd) Chapman and Hall, London.

Gow, N.A.R., Morris, B.M. & Reid, B. (1992). The electrophysiology of root-zoospore interactions. In J.A. Callow & J.R. Green (Eds.), Society for Experimental Biology Seminar Series 48: *Prospectives in Plant Cell Recognition*. (pp. 173-192). Cambridge University Press, UK.

Grant, B.R., Griffith, J.M. & Irving, H.R. (1986). A model to explain ion-induced differentiation in zoospores of *Phytophthora palmivora*. *Experimental Mycology* 10:89-98.

Grant, B.R., Irving, H.R. & Radda, M. (1985). The effect of pectin and related-compounds on encystment and germination of *Phytophthora palmivora* zoospores. *Journal of General Microbiology* 131:669-676.

Gray, W.D. (1941). Studies on the alcohol tolerance of yeasts. *Journal of Bacteriology*. 42:561-574.

Grayson, D.E. (1999). Suppression of zoospore cyst and sporangial germination of *Phytophthora infestans* by treatments that might interfere with calcium-mediated functions. PhD Thesis, University of Edinburgh.

Gregory, A.R. (1983). Some major epidemics caused by *Phytophthora*. In D.C. Erwin, S. Bartruiki-Garcia, & P.H. Tsao (Eds.), *Phytophthora: Its Biology, Taxonomy, Ecology and Pathology*. (pp. 271-278). St. Paul, Minnesota: American Phytopathological Society.

GROVE, S.N. (1970). Fine structure of zoospore encystment and germination in *Pythium aphanidermatum*. *American Journal of Botany* 57:745-746.

GROVE, S.N. & BRACKER C.E. (1978). Protoplasmic changes during zoospore encystment and cyst germinaiton in *Pythium aphanidermatum*. *Experimental Mycology* 2:51-98.

GUBLER, F. & HARDHAM, A.R. (1988). Secretion of adhesive material during encystment of *Phytophthora cinnamomi* zoospores, characterized by immunogold labelling with monoclonal-antibodies to components of peripheral vesicles. *Journal of Cell Science* 90:225-235.

GUBLER, F. & HARDHAM, A.R. (1990). Protein storage in large peripheral vesicles in *Phytophthora* zoospores and its breakdown after cyst germination. *Experimental Mycology* 14:393-404.

GUBLER, F., HARDHAM, A.R. & DUNIEC, J. (1989). Characterizing adhesiveness of *Phytophthora cinnamomi* zoospores during encystment. *Protoplasma* 149:24-30.

GUBLER, F., JABLONSKY, P.P., DUNIEC, J. & HARDHAM, A.R. (1990). Localization of calmodulin in flagella of zoospores of *Phytophthora cinnamomi*. *Protoplasma* 155:233-238.

HALL G. (1993). An integrated approach to the analysis of variation in *Phytophthora nicotianae* and a redescription of the species. *Mycological Research* 97:559-574.

HALSALL, D.M. (1976). Zoospore chemotaxis in Australian isolates of *Phytophthora* species. *Canadian Journal of Microbiology* 22:409-422.

HALSALL, D.M. (1977). Effects of certain cations on the formation and infectivity of *Phytophthora* zoospores. 2. Effects of copper, boron, cobalt, manganese, molybdenum and zinc ions. *Canadian Journal of Microbiology* 23:1002-1010.

HALSALL, D.M. & FORRESTER, R.I. (1977). Effects of certain cations on the formation and infectivity of *Phytophthora* zoospores. 1. Effects of calcium, magnesium, potassium and iron ions. *Canadian Journal of Microbiology* 23:994-1001.

HAMPTON, R.O. & BUCHOLTZ, W.F. (1962). Host specialization in *Pythium graminicola*. *Iowa State College Journal of Science* 37:43-66.

HARDHAM, A.R. (1995). Polarity of vesicle distribution in Oomycete zoospores: development of polarity and importance for infection. *Canadian Journal of Botany* 73(Suppl. 1):S400-S407.

HARDHAM, A.R. & SUZAKI, E. (1986). Encystment of zoospores of the fungus, *Phytophthora cinnamomi*, is induced by specific lectin and monoclonal-antibody binding to the cell-surface. *Protoplasma* 133:165-173.

HARDHAM, A.R., GUBLER, F. & DUNIEC, J. (1991). Ultrastructural and immunological studies of zoospores of *Phytophthora*. In *Phytophthora*, pp 50-69. University Press, Cambridge.

HARDHAM, A.R., SUZAKI, E. & PERKIN, J.L. (1986). Monoclonal antibodies to isolate-specific, species-specific, and genus-specific components on the surface of zoospores and cysts of the fungus *Phytophthora cinnamomi*. *Canadian Journal of Botany* 64:311-321.

HAROLD, F. M. (1997). How hyphae grow: morphogenesis explained. *Protoplasma* 197:137-147.

HARRISH, W.N. (1965). Effect of light on production of oospores and sporangia in species of *Phytophthora*. *Mycologia* 57:85-90.

HAUSER, H. & SHIPLEY, G.G. (1984). Interactions of divalent cations with phosphatidylserine bilayer membranes. *Biochemistry* 23:34-41.

Hawes, M.C., Goldberg, N.P. & Stanghellini, M.E. (1987). Specific chemotaxis of *Pythium dissotocum* zoospores to root cap cells of *Gossypium* species. *Phytopathology* 77:1736.

Heath, I. B. (1995). Integration and regulation of hyphal tip growth. *Canadian Journal of Botany* 73:S131-S139.

Held, A.A. (1972). Host-parasite relations between *Allomyces* and *Rozella*: parasite penetration depends on growth response of host cell-wall. *Arch. Mikrobiol* 82:128-139.

Hemmes, D.E. & Hohl, H.R. (1971). Ultrastructural aspects of encystment and cyst germination in *Phytophthora parasitica*. *Journal of Cell Science* 9:175-191.

Hemmes, D.E. & Pinto da Silva, P. (1980). Localisation of secretion-related, calcium-binding substrates in encysting zoospores of *Phytophthora parasitica*. *Biologie Cellulaire* 37:235-240.

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

Hendrix, J.W. (1967). Light-cholesterol relationships in morphogenesis of *Phytophthora palmivora* and *P. capsici* sporangia. *Mycologia* 59:1107-1111.

Hickman, C.J. (1970). Biology of *Phytophthora* zoospores. *Phytopathology* 60:1128-1135.

Hickman, C.J. & Ho, H.H. (1966). Behaviour of zoospores in plant-pathogenic Phycomycetes. *Annual Review of Phytopathology* 4:195-220.

Hill, A.E., Grayson, D.E. & Deacon, J.W. (1998). Suppressed germination and early death of *Phytophthora infestans* sporangia caused by pectin, inorganic phosphate, ion chelators and calcium-modulating treatments. *European Journal of Plant Pathology* 104:367-376.

Hinch, J.M. & Clarke, A.E. (1980). Adhesion of fungal zoospores to root surfaces is mediated by carbohydrate determinants of the root surface slime. *Phsiological Plant Pathology* 16:303-307.

Ho, H.H. & Hickman, C.J. (1967). Factors governing zoospore responses of *Phytophthora megasperma* var. *sojae*. *Canadian Journal of Botany* 45:1983-1994.

Hockey, A.G. & Jeves, T.M. (1984). Isolation and identification of *Pyrenochaeta lycopersici* causal agent of tomato brown root-rot. *Transactions of the British Mycological Society* 82:151-152.

Holderness, M. & Pegg, G.F. (1986). Interaction of host stress and pathogen ecology on *Phytophthora* infection and symptom expression in nutrient film-grown tomatoes. In *Water, Fungi and Plants*. (Eds P.G.Ayres & L.Boddy).Cambridge University Press, Cambridge.

Hollmann, M., Hartley, M. & Heinemann, S. (1991). Ca^{2+} permeability of KA-AMPA gated glutamate receptor channels depends on subunit composition. *Science* 252:851-853.

Holwill, M.E.J. (1982). Dynamics of eukaryotic flagella movement. In E.B. Amos & J.G. Duckett (Eds.), *Eukaryotic and Prokaryotic Flagella*: Symposium of the Society for Experimental Biology 35. (pp. 289-312). Cambridge: CUP.

Hooley, P., Fyfe, A.M., Maltese, C.E. & Shaw, D.S. (1982). Duplication cycle in nuclei of germinating zoospores of *Phytophthora drechsleri* as revealed by DAPI staining. *Transactions of the British Mycological Society* 79:563-566.

Hutchinson, S.A. & Kamel, M. (1956). The effect of earthworms on the dispersal of soil fungi. *Journal of Soil Science* 7:213-218.

HYDE, G.J. & HARDHAM, A.R. (1992). Confocal microscopy of microtubule arrays in cryosectioned sporangia of *Phytophthora cinnamomi*. *Experimental Mycology* 16:207-218.

IRVING, H.R. & GRANT, B.R. (1984). The effects of pectin and plant-root surface carbohydrates on encystment and development of *Phytophthora cinnamomi* zoospores. *Journal of General Microbiology* 130:1015-1018.

IRVING, H.R., GRIFFITH, J.M. & GRANT, B.R. (1984). Calcium efflux associated with encystment of *Phytophthora palmivora* zoospores. *Cell Calcium* 5:487-500.

ISER, J.R., GRIFFITH, J.M., BALSON, A. & GRANT, B.R. (1989). Accelerated ion fluxes during differentiation in zoospores of *Phytophthora palmivora*. *Cell Differentiation and Development* 26:28-38.

JONES, S.W., DONALDSON, S.P. & DEACON, J.W. (1991). Behaviour of zoospores and zoospore cysts in relation to root infection by *Pythium aphanidermatum*. *New Phytologist* 117:289-301.

JUDELSON, H.S. (1997). The genetics and biology of *Phytophthora infestans*: modern approaches to historical challenge. *Fungal Genetics and Biology* 22:65-76.

KAMOUN, S., VAN WEST, P., DE JONG, A.J., DE GROOT, K.E., VIEESHOUWERS, V.G.A.A. & GOVERS, F. (1997). A gene encoding a protein elicitor of *Phytophthora infestans* is down-regulated during infection of potato. *Molecular Plant-Microbe Interactions* 10:13-20.

KAMOUN, S., VAN WEST, P., VIEESHOUWERS, V.G.A.A. & DE GROOT, K.E. (1998). Resistance of *Nicotiana benthamiana* to *Phytophthora infestans* is mediated by the recognition of the ilicitor protein INF1. *The Plant Cell* 10:1413-1425.

KANNWISCHER, M.E. & MITCHELL, D.J. (1981). Relationships of numbers of spores of *Phytophthora parasitica* var. *nicotianae* to infection and mortality of tobacco. *Phytopathology* 71:69-73.

KATSU, T., KOBAYASHI, H. & FUJITA, Y. (1986). Mode of action of gramicidin S on *escherichia coli* membrane. *Biochemica et Biophysica Acta* 860:608-619.

KATSU, T., KUROKO, M., MORIKAWA, T., SANCHIKA, K., FUJITA, Y., YAMAMURA, H. & UDA, M. (1989). Mechanism of membrane damage induced by the amphipathic peptides gramicidin S and melittin. *Biochemica et Biophysica Acta* 983:135-141.

KATSU, T., NINOMIYA, C., KUROKO, M., KOBAYASHI, H., HIROTA, T. & FUJITA, Y. (1988). Action mechanisms of amphipathic gramicidin S and melittin on erythrocyte membrane. *Biochemica et Biophysica Acta* 939:57-63.

KATSURA, K. & MIYATA, Y. (1971). Swimming behaviour of *Phytophthora capsici* zoospores. In S. Akai & S. Ouchi (Eds.), *Morphological and Biochemical Events in Plant-Parasite Interactions*. (pp. 107-128). Tokyo: Phytopathological Society of Japan.

KERWIN, J.L., JOHNSON, L.M., WHISLER, H.C. & TUININGA, A.R. (1992). Infection and morphogenesis of *Pythium marinum* in species of *Porphyra* and other red algae. *Canadian Journal of Botany* 70:1017-1024.

KHAKI, I.A. & SHAW, D.S. (1974). The inheritance of drug resistance and compatibility types in *Phytophthora drechslei*. *Genetical Research* 23:75-86.

KHEW, K.L., & ZENTMYER, G.A. (1974). Electrotactic response of zoospores of seven species of *Phytophthora*. *Phytopathology* 64:500-507.

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

KIM, Y.J. & HWANG, B.K. (2000). Pepper gene encoding a basic pathogenesis-related 1 protein is pathogen and ethylene inducible. *Physiologia Plantarum* 108:51-60.

KLEMM, A.R., PELL, K.L., ANDERSON, L.M., ANDREW, C.L. & LLOYD, J.B. (1998). Lysosome membrane permeability to anions. *Biochemica et Biophysica Acta -Biomembranes* 1373:17-26.

KOMMEDAHL, T. & WINDELS, C.E. (1979). Pathogen host dominance in disease. In *Ecology of Root Pathogens*. pp1-13. S.V.Krupa & Y.R.Dommergues (ed). Elsevier Scientific Publishing, New York.

KOUAOUCI, R., SILVIUS, J.R., GRAHAM, I. & PÉZOLET, M. (1985). Calcium-induced lateral phase separations in phosphatidylcholine-phosphatidic acid mixtures. A Ramon spectroscopic study. *Biochemistry* 24:7132-7140.

LÉVESQUE, C.A., DE COCK, W.A.M., QUAIL, A. & O'GORMAN, D. (1999). Molecular phylogeny, evolution and groupings of *Pythium* and *Phytophthora* species based on nuclear ribosomal DNA. Poster presented at the Joint American Phytopathological Society and Canadian Phytopathological Society Annual Meeting (7-11th August), Montreal, Canada.

LEWIS, R.N.A.H., PRENNER, E.J., KONDEJEWSKI, L.H., HODGES, R.S., FARMER, S.W., HANCOCK, R.E. & MCELHANEY, R.N. (1998). Induction of nonlamellar lipid phases by gramicidin S and related cyclic peptides and their correlation with cytolytic activities. *Biophysical Journal* 74:A305.

LONGMAN, D. & CALLOW, J.A. (1987). Specific saccharide residues are involved in the recoginiton of plant surfaces by zoospores of *Pythium aphanidermatum*. *Physiological and Molecular Plant Pathology* 30:139-150

LOVELOCK, J.E. & BISHOP, M.W.H (1959). Preservation of freezing damage to living cells by dimethyl sulphoxide. *Nature* 183:1394-1395.

LUNNY, C.Z. & BLAND, C.E. (1976). Ultrastructural observations of mature and encysting zoospores of *Pythium proliferum* de Bary. *Protoplasma* 90:119-137.

MACDONALD, J.D. & DUNIWAY, J.M. (1978). Influence of the matric and osmotic components of water potential on zoospore discharge in *Phytophthora*. *Phytopathology* 86:751-757.

MACKENZIE, D.R., ELLIOTT, V.J., KIDNEY, B.A., KING, E.D. & ROYER, M.H. (1983). Application of modern approaches to the study of the epidemiology of diseases caused by *Phytophthora*. In D.C. Erwin, S. Bartnicki-Garcia, & P.H. Tsao (Eds.), *Phytophthora: Its Biology, Taxonomy, Ecology and Pathology*. (pp. 303-313). St. Paul, Minnesota: The American Phytopathological Society.

MARX, D.H. & BRYAN, W.C. (1969). Effect of soil bacteria on the mode of infection of pine roots by *Phytophthora cinnamomi*. *Phytopathology* 59:614-619.

MARX, D.H. & HAASIS, F.A. (1965). The induction of aspetic sporangial formation in *Phytophthora cinnamomi* by metabolic diffusates of soil microorganisms. *Nature* 206:673-674.

MASAGO, H., YOSHIKAWA, M., FUKADA, M. & NAKANISHI, N. (1977). Selective inhibition of *Pythium* spp. on a medium for direct isolation of *Phytophthora* spp. from soils and plants. *Phytopathology* 67:425-428.

MCPHERSON, G.M., HARRIMAN, M.R. & PATTISON, D. (1995). The potential for spread of root diseases in recirculating hydroponc systems and their control with disinfection. *Med. Fac. Landbouww Univ. Gent.* 60:371-379.

MCPHERSON, G.M. (1998). Project 120: The development of a sustainable system for the prevention of root disease in recirculating hydroponic crops. *Horticultural Research International Report.*

MEINS, F. & AHL, P. (1989). Induction of chitinase and beta-1,3-glucanase in tobacco plants infected with *Pseudomonas tabaci* and *Phytophthora parasitica* var. *nicotianae*. *Plant Science* 61:155-161.

MENZIES, J.G., EHRET, D.L. & STAN, S. (1996). Effect of inoculum density of *Pythium aphanidermatum* on the growth and yield of cucumber plants grown in recirculating nutrient film culture. *Canadian Journal of Plant Pathology* 18:50-54.

MIHAILESCU, D. & HORVATH, L.I. (1999). Molecular dynamics of lipid association at the hydrophobic interface of gramicidin S. *European Biophysical Journal* 28:216-221.

MILLER, A.L., SHAND, E. & GOW, N.A.R. (1988). Ion currents associated with root-tips, emerging laterals and induced wound sites in *Nicotiana tabacum* - spatial relationship proposed between resulting electrical fields and *Phytophthora* zoospore infection. *Plant Cell and Environment* 11:21-25.

MITCHELL, D.J. & ZENTMYER, G.A. (1971). Effects of oxygen and carbon dioxide tensions on sporangium and oospore formation by *Phytophthora* spp. *Phytopathology* 61:807-811.

MITCHELL, H.J. & HARDHAM, A.R. (1999). Characterisation of the water expulsion vacuole in *Phytophthora nicotinae* zoospores. *Protoplasma* 206:118-130.

MITCHELL, R.T. & DEACON, J.W. (1986). Selective accumulation of zoospores of chytridiomycetes and oomycetes on cellulose and chitin. *Transactions of the British Mycological Society* 86:219-223.

MITCHELL, R.T. & DEACON, J.W. (1987). Differential adhesion of zoospore cysts of *Pythium* on roots of graminaceous and non-graminaceous plants. *Transactions of the British Mycological Society* 88:401-403.

MONEY, N. P. & HAROLD, F. M. (1992). Extension growth of the water mould Achyla: interplay of turgor and wall strength. *Proceedings of the National Academy of Sciences U.SA* 81:4245-4249.

MONEY, N.P. & HAROLD, F.M. (1993). Two water molds can grow without measurable turgor pressure. *Planta* 190:426-430.

MONEY, N.P. & WEBSTER, J. (1989). Mechanism of sporangial emptying in *Saprolegnia*. *Mycological Research* 92:45-49.

MOREAU, P., THOQUET, P., OLIVIER, J., LATERROT, H. & GRIMSLEY, N. (1998). Genetic mapping of *Ph-2*, a single locus controlling partial resistance to *Phytophthora infestans* in tomato. *Molecular Plant-Microbe Interactions* 11:259-269.

MOREAU, R.A., YOUNG, D.H., DANIS, P.O., POWELL, M.J., QUINN, C.J., BESHAH, K., SLAWECKI, R.A. & DILLIPLANE, R.L. (1998). Identification of ceramide-phosphorylethanolamine in oomycete plant pathogens: *Pythium ultimum*, *Phytophthora infestans*, and *Phytophthora capsici*. *Lipids* 33:308-317.

MORRIS, B.M. & GOW, N.A.R. (1993). Mechanism of electrotaxis of zoospores of phytopathogenic fungi. *Phytopathology*. 83:877-882.

MORRIS, B.M., REID, B. & GOW, N.A.R. (1992). Electrotaxis of zoospores of *Phytophthora palmivora* at physiologically relevant field strengths. *Plant Cell And Environment* 15:645-653.

MORTIMER, A.M. & SHAW, D.S. (1975). Cytofluormetric evidence for meiosis in gametangial nuclei of *Phytophthora drechsleri*. *Genetical Research* 25:201-205

MOULIN, F., LEMANCEAU, P. & ALABOUVETTE, C. (1994). Pathogencity of *Pythium* species on cucumber in peat-sand, rockwool and hydroponics. *European Journal of Plant Pathology* 100:3-17.

NIKAIDO, H. & VAARA, M. (1995). Molecular basis of bacterial outer membrane permeability. *Microbiological Reviews* 49:1-32

NOVOTNY, C., DOLEZALOVA, L., FLIEGER, M., PANOS, J. & KARST, F. (1992). Ethanol-induced death and lipid-composition of *Saccaromyces cerevisiae* - a comparative study of the role of sterols. *Folia Microbiologica* 37:286-288

OLMSTEAD, R.G. & SWEERE, J.A. (1994). Combining data in phylogenetic systematics - an empirical-approach using 3 molecular-DNA sets in the Solanaceae. *Systematic Biology* 43:467-481.

OLSEN, R.A. (1971a). Triterpeneglycosides as inhibitors of fungal growth and metabolism. 1 Effect on growth, endogenous respiration and leakage of UV-absorbing material from various fungi. *Physiologia Plantarum* 24:524-543.

OLSEN, R.A. (1971b). Triterpeneglycosides as inhibitors of fungal growth and metabolism. 2 Effect on leakage of inorganic ions. *Physiologia Plantarum* 25:204-212.

OLSEN, R.A. (1971c). Triterpeneglycosides as inhibitors of fungal growth and metabolism. 3 Effect on uptake of potassium, magnesium and inorganic phosphate. *Physiologia Plantarum* 25:503-508.

OLSEN, R.A. (1973). Triterpeneglycosides as inhibitors of fungal growth and metabolism. 5 The effect of aescin on fungi with reduced sterol contents. *Physiologia Plantarum* 29:145-149.

OSBOURN, A. (1996). Saponins and plant defence - a soap story. *Trends in Plant Science* 1:4-9.

OSBOURN, A., BOWYER, P., LUNNESS, P., CLARKE, B. & DANIELS, M. (1995). Fungal pathogens of oat roots and tomato leaves employ closely related enzymes to detoxify differen host plant saponins. *Molecular Plant-Pathogen Interactions* 8:971-978.

OVCHINNIKOV, Y.A. & IVANOV, V.T. (1982). The cyclic peptides: Structure, conformation, and function. In: *The Proteins* 3rd Edition. H. Neurath & R.L.Hill (eds).

PANABIERES, F., MARAIS, A., LEBERRE, J.Y., PENOT, I., FOURNIER, D. & RICCI, P. (1995). Charactorization of a gene-cluster of *Phytophthora cryptogea* which codes for elicitins , proteins inducing a hypersensitive-like response in tobacco. *Molecular Plant-Microbe Interactions* 8:996-1003.

PARQUIN, B., ROEWAR, I., WONG, Z. & LONG, B.F. (1995). A robust fungal phylogeny using the mitochonrially encoded NAD_5 protein sequence. *Canadian Journal of Botany* 73:S180-S185.

PAULITZ, T.C. (1997). Biological control of root pathogens in soilless and hydroponic systems. *Hortscience* 32:193-196

PENINGTON, C.J., ISER, J.R., GRANT, B.R. & GAYLER, K.R. (1989). Role of RNA and protein synthesis in stimulated germination of zoospores of the pathogenic fungus *Phytophthora palmivora*. *Experimental Mycology* 13:158-168.

PFENDER, W.F., HINE, R.B. & STANGHELLINI, M.E. (1977). Production of sporangia and release of zoospores by *Phytophthora megasperma* in soil. *Phytopathology* 67:657-663.

PIETERSE, C.M.J., VAN WEST, P., VERBAKEL, H.M., BRASSE, P.W.H.M., VAN DEN BERG-VELTHUIS, G.C.M. & GOVERS, F. (1994). Structure and genomic organization of the *ipiB* and *ipiO* gene clusters of *Phytophthora infestans*. *Gene* 138:67-77.

PLAATS-NITERINK, A.J. VAN DER (1975). Species of *Pythium* in the Netherlands. *Netherlands Journal of Plant Pathology* 8:22-37.

PLAATS-NITERINK, A.J. VAN DER (1981). Monograph of the genus *Pythium*. Baarn, The Netherlands. Centraalbureau voor Schimmelcultures.

POSTMA, J., WILLEMSEN-DE KLEIN, M.J.E.I.M. & VAN ELSAS, J.D. (2000). Effect of the indigenous microflora on the development of root and crown rot caused by *Pythium aphanidermatum* in cucumber grown on Rockwool. *Phytopathology* 90:125-133.

QUIDDE, T., OSBOURN, A.E. & TUDZYNSKI, P. (1998). Detoxification of a-tomatine by *Botrytis cinerea*. *Physiological and Molecular Plant Pathology* 52:151-165.

RANI, K.S. & SEENAYYA, G. (1999). High ethanol tolerance of new isolates of *Clostridium thermocellum* strains SS21 and SS22. *World Journal of Microbiology and Biotechnology* 15:155-159.

RANKIN, L. & PAULITZ, T.C. (1994). Evaluation of rhizosphere bacteria for biological control of pythium root-rot of greenhouse cucumbers in hydroponic culture. *Plant Disease* 78:447-451.

REICHLE, R.E. (1969). Fine structure of *Phytophthora parasitica* zoospores. *Mycologia* 61:30-51.

REY, P., BENHAMOU, N. & TIRILLY, Y. (1998). Ultrastructural and cytochemical investigation of asymptomatic infection by *Pythium* spp. *Phytopathology* 88:234-244.

RIBEIRO, O.K. (1983). Physiology of asexual sporulation and spore germination in *Phytophthora*. In D.C. Erwin & S. Bartnicki-Garcia (Eds.), *Phytophthora: its Biology, Taxonomy, Ecology and Pathology*. (pp. 55-70). St. Paul, Minnesota, USA. American Phytopathological Society.

RIBEIRO, O.K., ZENTMYER, G.A. & ERWIN, D.C. (1976). The influence of qualitative and quantitative radiation on reproduction and spore germination of four *Phytophthora* species. *Mycologia* 68:1162-1173.

RICHARDS, M.D. & BARRATT, R.W. (1946). A partial survey of the genus *Lycopersicon* for resistance to *Phytophthora infestans*. *Plant Disease Reports* 30:16-20.

ROBERTSON, N. F. (1958). Observations on the effect of water on the hyphal apicies of *Fusarium oxysporium*. *Annals of Botany* 22:159-173.

ROBERTSON, N. F. (1959). Experimental control of hyphal branching forms in hyphomycetous fungi. *Journal of the Linnaean Society London* 56:207-211.

ROYLE, D.J. & HICKMAN, C.J. (1964). Analysis of factors governing in vitro accumulation of zoospores of *Pythium aphanidermatum* on roots. II Substances causing response. Canadian Journal Of *Microbiology* 10:201-219.

SALISBURY, F.B. & ROSS, F.B. (1991). *Plant Physiology*. 4th ed, Belmont, California: Wadsworth Pub. Co.

SHAW, D.S. (1983). The cytogenetics and genetics of Phytophthora. In *Pythophthora: Its Biology, Taxonomy, Ecology and Pathology*. Eds D.C.Erwin, S.Bartnicki-Garcia and P.H.Tsao. American Phytopathological Society, St.Paul, Minn., USA.

SHAW, D.S. & KHAKI, I.A. (1971). Genetical evidence for diploidy in Phytophthora. *Genetical Research* 17:167-167.

SHEW, H.D. (1982). Influence of soil matric potential on infection of tobacco by *Phytophthora parasitica* var. *nicotianae. Phytopathology* 72:995.

SHEW, H.D. (1983). Effects of soil matric potential on infection of tobacco by *Phytophthora parasitica* var. *nicotianae. Phytopathology* 73:1160-1163.

SHIDO, O., TAKAGI, H., KADOWAKI, K. & KOMAGATA, K. (1996). Proposal of two new genera, *Brevibacillus* gen nov and *Aneurinibacillus* gen nov. *International Journal of Systemic Bacteriology* 46:939-946.

SHIPTON, W.A. (1985). Zoospore induction and release in a *Pythium* causing equine phycomycosis. *Transactions of the British Mycological Society* 84:147-155.

SHIPTON, W.A. (1987). Regulation by ions of zoospore release in *Pythium. Australian Journal of Botany* 35:79-89.

SHISHKOFF, N. (1989). Zoospore encystment pattern and germination on onion roots, and the colonization of hypodermal cells by *Pythium coloratum. Canadian Journal of Botany* 67:258-262.

SIDEBOTTOM, J.R. & SHEW, H.D. (1984). The effects of soil matric potential and soil texture on sporangial formation by *Phytophthora parasitica* var. *nicotianae. Phytopathology* 74:814.

SIDEBOTTOM, J.R. & SHEW, H.D. (1985). Effects of soil texture and matric potential on sporangium production by *Phytophthora parasitica* var. *nicotianae. Phytopathology* 75:1435-1438.

SING, V.O. & BARTNICKI-GARCIA, S. (1975a). Adhesion of *Phytophthora palmivora* zoospores: Electron microscopy of cell attachment and cyst wall fibril formation. *Journal of Cell Science* 18:123-132.

SING, V.O. & BARTNICKI-GARCIA, S. (1975b). Adhesion of *Phytophthora palmivora* zoospores: detection and ultrastructural visualisation of concanavalin A receptor sites appearing during encystment. *Journal of Cell Science* 19:11-20.

SINGH, N. (1973). Effect of nutrition on growth and sporangial production of two west African isolates of *Phytophthora palmivora* Butl. *Annual Botany* 39:129-136.

SOLL, D.R. & SONNEBORN, D.R. (1972). Zoospore germination in *Blastocladiella emersonii*. IV. Ion control of cell differentiation. *Journal of Cell Science* 10:315-333.

STANGHELLINI, M.E. (1998). Epidemiology of zoosporic pathogens in hydroponic systems - the role of biosurfactants in disease suppression. *Internation Congress of Plant Pathology. 9th - 16th August, 1998. Edinburgh, UK.*

STANGHELLINI, M.E. & RASMUSSEN, S.L. (1994). Identification and origin of plant pathogenic microorganisms in recirculating nutrient solutions. *Advances in Space Research* 14(11):349-355.

STANGHELLINI, M.E., KIM, D.H., RASMUSSEN, S.L. & RORABAUGH, P.A. (1996a). Control of root rot of peppers caused by *Phytophthora capsici* with nonionic surfactant. *Plant Disease* 80:1113-1116.

STANGHELLINI, M.E., RASMUSSEN, S.L., KIM, D.H. & RORABAUGH, P.A. (1996b). Efficacy of nonionic surfactants in the control of zoospore spread of *Pythium aphanidermatum* in a recirculating hydroponic system. *Plant Disease* 80:422-428

STAUB, T. (1991). Fungicide resistance: Practical experience with antiresistance strategies and the role of intergrated use. *Annual Review of Phytopathology* 29:421-442.

STAUNTON, W.P. (1978). Rapid disease spread fears ungrounded. *Grower* 89:201-203.

TAMM, S.L. (1989). Control of reactivation of microtubule sliding by calcium, strontium and barium in detergent-extracted macrocilia of *Beroe*. *Cell motility and cytoskeleton* 12:104-112.

TAY, F.C.S., NANDAPALAN, K. & DAVISON, E.M. (1983). Growth and zoospore germination of *Phytophthora* spp. on P_{10}VP agar with hymexazol. *Phytopathology* 73:234-240.

TEMPLETON, M.D., RIKKERINK, E.H.A. AND BEEVER, R.E. (1994). Small, cysteine-rich proteins and recognition in fungal-plant interactions. *Molecular Plant-Microbe Interactions* 7:320-325.

TERCELAFORGUE, T., HUET, J.C. & PERNOLLET, J.C. (1992). Biosynthesis and secretion of cryptogein, a protein elicitor secreated by *Phytophthora cryptogea*. *Plant Physiology* 98:936-941.

THOMAS, D.D. & BUTLER, D.L. (1989). Cationic interactions regulate the termination of zoospore activity in the water mould *Achlya heterosexualis*. *Journal of General Microbiology* 135:1917-1922.

THOMAS, D.S., HOSSACK, J.A. & ROSE, A.H. (1978). Plasma-membrane lipid composition and ethanol tolerance in *Saccharomyces cerevisiae*. *Archives in Microbiology* 117:239-245.

THORNTON, M.L. (1970). Transport of soil-dwelling aquatic Phycomycetes by earthworms. *Transactions Of The British Mycological Society* 55:391-397.

TROUTMAN, J.L. & WILLS, W.H. (1964). Electrotaxis of *Phytophthora parasitica* zoospores and its possible role in infection of tobacco by the fungus. *Phytopathology* 54:225-228.

TRUESDELL, L.C. & CANTINO, E.C. (1971). The induction and early germination in the zoospore of *Blastocladiella emersonii*. In *Current Topics in Development Biology*, (eds. A.A. Moscana & A. Monroy), pp1-44. New York: Academic Press.

VALENZUELAURETA, J.G., LAWN, D.A., HEISEY, R.F. & ZAMUDIOGUZMAN, V. (1996). First report of fusarium wilt race-3, caused by *Fusarium oxysporum* f.sp. *lycopersici*, of tomato in Mexico. *Plant Disease* 80:105.

VAN ETTEN, J.L., DAHLBERG, K.R., & RUSSA, G.M. (1983). Fungal spore germination. In J. Smith (Ed.), *Fungal Differentiation: A Contemporary Synthesis*. (pp. 235-266). Dekker, NY.

VAN OS, E.A. (1999). Design of sustainable hydroponic systems in relation to environment- friendly disinfection methods. *International Symposium on Growing Media and Hydroponics, August 31 - September 6, 1999, Halkidiki, Greece.*

VAN VOORST, G., VAN OS, E.A. & ZADOKS, J.C. (1987). Dispersal of *Phytophthora nicotianae* on tomatoes grown by nutrient film technique in a greenhouse. *Netherlands Journal of Plant Pathology* 93:195-199.

VON BROEMBSEN, S.L. & DEACON, J.W. (1996). Effects of calcium on germination and further zoospore release from zoospore cysts of *Phytophthora parasitica*. *Mycological Research* 100:1498-1504.

VON BROEMBSEN, S.L. & DEACON, J.W. (1997). Interference with zoospore biology and infectivity of *Phytophthora parasitica* by calcium amendments in nutrient irrigation solutions. *Phytopathology* 87:522-528.

WAECHER-KRISTENSEN, B., GERTSSON, U. & SUNDIN, P. (1993). Prospects for microbial stabilization in the hydroponic culture of tomato using circulating nutrient solution. *Summaries International Society for Horticultural Science Symposium Cagliary, Italy, 26-30 April 1993.*

WANG, M.C. & BARTNICKI-GARCIA, S. (1973). Novel phosphoglucans from the cytoplasm of Phytophthora palmivora and their selective occurrence in certain life cycle stages. *Journal of Biological Chemistry* 248:4112-4118.

WARBURTON, A.J. (1993). Role of calcium in motility and germination of zoospores of *Pythium aphanidermatum*. Honours Thesis, Institute of Cell and Molecular Biology, University of Edinburgh;

WARBURTON, A.J. (1997). Role of calcium in zoospore biology of *Phytophthora* and *Pythium* species. PhD thesis, University of Edinburgh.

WARBURTON, A.J. & DEACON, J.W. (1998). Transmembrane Ca^{2+} fluxes associated with zoospore encystment and cyst germination by the phytopathogen *Phytophthora parasitica*. *Fungal Genetics and Biology*. 25:54-62.

WATERHOUSE, G.M. (1956). *The genus Phytophthora: diagnosis (or descriptions) and figures from original papers*. Kew, Surrey: The Commonwealth Mycological Institute.

WATERHOUSE, G.M. & WATERSTON, J.M. (1964a). *CMI descriptions of pathogenic fungi and bacteria*, No. 35: *Phytophthora nicotianae* var. *parasitica*. Commonwealth Mycological Institute, Surrey.

WATERHOUSE, G.M. & WATERSTON, J.M. (1964b). *CMI descriptions of pathogenic fungi and bacteria*, No. 36: *Pythium aphanidermatum*. Commonwealth Mycological Institute, Surrey.

WEBSTER, J. (1980). *Introduction to Fungi*. 2nd ed, Cambridge: Cambridge University Press.

WESSELS, J.G.H. (1993). Wall growth and protein excretion and morphogenesis in fungi. *New Phytologist* 123: 397-413

WUBBEN, J.P., PRICE, K.R., DANIELS, M.J. & OSBOURN, A.E. (1996). Detoxification of oat leaf saponins by *Septoraia avenae*. *Phytopathology* 86:986-992.

YAMASHITA, K., KASAI, M., YAMAMOTO, Y. & MATSUMOTO, H. (1997). Enhancement of nitrate efflux and the plasma membrane hydrogen ion transport activity of barely roots by nitrate treatment. *Journal of Plant Nutrition* 20:657-668.

YAMASHITA, K., YAMAMOTO, Y. & MATSUMOTO, H. (1996). Characterization of an anion transporter in the plasma membrane of barely roots. *Plant and Cell Physiology* 37:949-956.

YONEZAWA, H., KANEDA, M., TOMINAGA, N., HIGASHI, S. & IZUMIYA, N. (1981). Adsorbtion of ^{14}C-labeled gramicidin S on cell of bacteria. *Journal of Biochemistry* 90:1087-1091.

YU, L.M. (1995). Elicitins from *Phytophthora* and basic resistance in tobacco. *Proceedings of the National Academy of Sciences of the United States of America* 92:4088-4094.

YU, Z. & QUINN, P. (1998). Solvation effects of dimethyl sulphoxide on the structure of phospholipid bilayers. *Biophysical Chemistry* 70:35-39.

ZENTMYER, G.A. (1961). Chemotaxis of zoospores for root exudates. *Science* 133:1595-1596.

ZHANG, Q., GRIFFITH, J.M. & GRANT, B.R. (1992). Role of phosphatidic acid during differentiation of *Phytophthora palmivora* zoospores. *Journal of General Microbiology* 138:451-459.

Zhang, Q., Griffith, J.M., Moore, J.G., Iser, J.R. & Grant, B.R. (1990). The effect of modified pectin, pectin fragments and cations on *Phytophthora palmivora* zoospores. *Phytochemistry* 29:695-700.

Zhou, T. & Paulitz, T.C. (1993). *In vitro* and *in vivo* effects of *Pseudomonas* spp. on *Pythium aphanidermatum*: zoospore behaviour in exudates and on the rhizoplane of bacteria-treated cucumber roots. *Phytopathology* 83:872-876.

Zoquier, L. & Young, R.J. (1985). *In vitro* effect of metalaxyl on radial growth, release and germination of zoospores of 17 *Phytophthora infestans* isolates. *Phytopathology* 75:630.