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**DEVELOPMENT OF A FUNGAL BIOLOGICAL CONTROL
AGENT FOR POTATO CYST NEMATODES IN JERSEY**

by

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A thesis submitted for degree of Doctor of Philosophy

for the University of Luton

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ABSTRACT

The production of Jersey Royal potatoes is an important industry for the island of Jersey. The crop is grown annually, and sometimes biannually, so there is no opportunity to practise crop rotation in order to control potato cyst nematodes (PCN), *Globodera pallida* and *G. rostochiensis*. Control of these pests in Jersey has traditionally relied on the use of nematicides, but with increased public pressure to reduce the use of pesticides and the intention of the Government of Jersey to eventually ban them, there is a desire for an alternative methods of control to be developed.

Three nematophagous fungi, *Plectosphaerella cucumerina*, *Paecilomyces lilacinus* and *Verticillium chlamydosporium*, were isolated from PCN cysts taken from potato fields in Jersey. The efficacy of these fungi for the control of PCN was studied to determine their suitability for use in an integrated pest management programme.

The radial growth rates of the nematophagous fungi were reduced when grown on media amended with the fungicides Gambit and Rizolex, commonly used for the control of *Rhizoctonia solani*, another major pathogen of potatoes. Radial growth of *V. chlamydosporium* was also inhibited by Monceren and the nematicide Vydate. Growth of *R. solani* was inhibited by *P. lilacinus* at 20°C and 10°C in

vitro and by *V. chlamydosporium* at 20°C, but the strain of *V. chlamydosporium* used did not grow at 10°C. *Plectosphaerella cucumerina* was a poor saprophytic competitor when grown against *R. solani*, *P. lilacinus* and *V. chlamydosporium*, therefore it may not be a suitable soil applied agent as it is out grown by other fungi in the soil. *Paecilomyces lilacinus* in a pelleted support matrix made from an alginate, gave better control of *R. solani* than non-formulated *P. lilacinus* alone.

Of the different formulations of nematophagous fungi tested in pots, *P. lilacinus* incorporated into alginate pellets reduced the numbers of PCN by the most (79.5%) and when applied in a field trial, reduced PCN population increase by approximately 60%. *Plectosphaerella cucumerina*, when incorporated into alginate pellets, also reduced field population increase by approximately 60%. A combination of these two formulated fungi tested in a plunge trial gave a poorer level of control than the fungi added individually. The fungi remained viable in alginate pellets for at least 18 months.

The population composition of PCN in Jersey was previously unknown. Using an ELISA technique, this study has shown both PCN species are present, but the proportions were not determined. To ascertain whether the early lifting of Jersey potatoes was selectively reducing levels of one of the species of PCN, DNA was extracted from nematodes stained *in situ* in the roots of potatoes. The results were inconclusive and further work is required.

CONTENTS

Abstract.....	.ii
Contents	iv
List of figures.....	xi
List of tables	xv
Acknowledgements	xvii
Abbreviations	xviii
1 Introduction.....	1
1.1 Economic importance of PCN in Jersey	1
1.2 PCN life cycle	4
1.3 Current control measures	6
1.3.1 Crop rotation.....	6
1.3.2 Resistant cultivars.....	7
1.3.3 Nematicides	7
1.3.4 Removal of volunteers.....	9
1.3.5 Trap cropping	10
1.4 Biological control.....	10
1.5 Nematophagous fungi	11
1.5.1 <i>Plectosphaerella cucumerina</i>	14
1.5.2 <i>Paecilomyces lilacinus</i>	14
1.5.3 <i>Verticillium chlamydosporium</i>	15
1.6 Registration	16
1.7 Aim and objectives.....	17

2	Materials and methods	19
2.1	Isolation of nematophagous fungi.....	19
2.2	Isolate and growth media	19
2.3	Preservation of cultures.....	20
2.3.1	Under mineral oil.....	20
2.3.2	Liquid nitrogen	21
2.4	Large scale production of fungal biomass	21
2.4.1	Preparation and inoculum.....	21
2.4.2	Validation of methodology.....	22
2.4.2.1	Dry weight determination	22
2.4.2.2	Optical densities of <i>Paecilomyces lilacinus</i> and <i>Plectosphaerella cucumerina</i> grown in batch culture	24
2.5	Quantitative assessment of PCN eggs from soil samples	26
2.6	Statistical analysis	27
3	Culturing and selection of nematophagous fungal isolates.....	28
3.1	Introduction.....	28
3.2	Selection of a suitable medium for sporulation and conidiation of nematophagous fungi	30
3.2.1	Introduction	30
3.2.2	Number of spores produced by nematophagous fungi on different growth media.....	30
3.2.2.1	Material and methods	30
3.2.2.2	Results.....	31
3.2.3	Effects of different growth media on the infectivity of <i>P. cucumerina</i>	36
3.2.3.1	Introduction.....	36
3.2.3.2	Materials and methods	36
3.2.3.3	Results.....	39
3.2.4	Discussion.....	42
3.3	Dose requirement for infecting PCN females in a bioassay	43
3.3.1	Introduction	43

3.3.2 Materials and methods.....	43
3.3.2.1 Growing monoconidial isolates	43
3.3.2.2 Separation of <i>Verticillium chlamydosporium</i> conidia from chlamydo spores.....	44
3.3.2.3 Effect of inoculum dose.....	44
3.3.3 Results	45
3.3.4 Discussion.....	47
3.4 Variation in pathogenicity between monoconidial isolates and pathogenicity of isolates when passaged through PCN	48
3.4.1 Introduction	48
3.4.2 Materials and methods.....	49
3.4.3 Results	49
3.4.4 Discussion.....	54
3.5 Effect of antibiotics on pathogenicity	55
3.5.1 Introduction	55
3.5.2 Effects of antibiotics on <i>V. chlamydosporium</i>	56
3.5.2.1 Materials and methods.....	56
3.5.2.2 Results.....	56
3.5.3 Effects of antibiotics on <i>P. lilacinus</i>	58
3.5.3.1 Materials and methods.....	58
3.5.3.2 Results.....	58
3.5.4 Discussion.....	59
3.6 General discussion	60
4 The effect of agrochemicals and other soil fungi on nematophagous fungi.....	63
4.1 Introduction	63
4.2 The effect of agrochemicals on nematophagous fungi	64
4.2.1 Introduction	64
4.2.2 Materials and methods.....	65
4.2.3 Results	66

4.3	Interactions between nematophagous fungi, plant pathogenic fungi and soil saprophytic fungi <i>in vitro</i>	72
4.3.1	Introduction	72
4.3.2	Materials and methods.....	74
4.3.2.1	A standard method for studying interactions between fungi <i>in vivo</i>	74
4.3.2.2	Hyphal interactions between nematophagous fungi and <i>Rhizoctonia solani</i>	76
4.3.3	Results	77
4.3.3.1	Interaction between nematophagous fungi	77
4.3.3.2	Hyphal interactions.....	81
4.4	Bioassay of the effectiveness of <i>P. lilacinus</i> for the control of <i>Rhizoctonia</i> disease	85
4.4.1	Introduction	85
4.4.2	Materials and methods.....	85
4.4.2.1	Production of <i>P. lilacinus</i> biomass	85
4.4.2.2	Production of alginate pellets	86
4.4.2.3	Production of <i>Rhizoctonia solani</i> inoculum	86
4.4.2.4	Bioassay.....	86
4.4.2.5	Disease assessment.....	87
4.4.3	Results	87
4.5	Discussion	89
5	Formulations of nematophagous fungi	95
5.1	Introduction.....	95
5.2	Experiment comparing different formulations of nematophagous fungi	97
5.2.1	Introduction	97
5.2.2	Materials and methods.....	98
5.2.2.1	Production of fungal conidia for application around the tuber	98
5.2.2.2	Production of inoculated Terra-Green®	98
5.2.2.3	Production of alginate pellets	99

5.2.2.4 Glasshouse experiment to test the efficacy of the different formulation methods	100
5.2.2.5 Egg counts	101
5.2.3 Results	104
5.3 Further development of alginate pellets	106
5.3.1 Introduction	106
5.3.2 Material and methods	106
5.3.2.1 Different formulations	106
5.3.2.2 Survival of nematophagous fungi in alginate pellets.....	107
5.3.2.3 Effect of alginate pellet pre-treatment on plant and nematode development.....	108
5.3.3 Results	109
5.3.3.1 Performance of different formulation mixtures.....	109
5.3.3.2 Survival of nematophagous fungi in alginate pellets.....	109
5.3.3.3 Effect of alginate pellet pre-treatment on plant and nematode development.....	109
5.4 Discussion	113
6 The efficacy of formulated nematophagous fungi tested in a plunge and field trial.....	117
6.1 Introduction	117
6.2 Plunge trial to determine the effectiveness of combined use of <i>P.</i> <i>cucumerina</i> and <i>P. lilacinus</i> as control agents of PCN over two seasons	119
6.2.1 Introduction	119
6.2.2 Experimental design	119
6.2.3 Nematode counts	121
6.2.4 Assessment of PCN multiplication.....	121
6.2.5 Results	122
6.2.5.1 The effects of alginate formulation on PCN multiplication ..	122
6.2.6 The effects of alginate formulation on potato yields.....	122
6.3 Plunge trial investigating the longevity of nematophagous fungi in soil	125
6.3.1 Introduction	125

6.3.2 Materials and methods.....	125
6.3.2.1 Results.....	126
6.4 Plunge trial to determine the effects of the rate of application of alginate pellets for control of PCN	128
6.4.1 Introduction	128
6.4.2 Materials and methods.....	128
6.4.3 Results	129
6.5 Field trial	131
6.5.1 Introduction	131
6.5.2 Methods	131
6.5.2.1 Preparation of alginate pellet inoculum.....	131
6.5.2.2 Field trial.....	131
6.5.3 Results	132
6.5.3.1 Soil temperature.....	132
6.5.3.2 Effect of the addition of alginate pellets containing <i>P. lilacinus</i> or <i>P. cucumerina</i> on the multiplication of PCN in soil in the field.....	134
6.6 Post-harvest application of <i>Verticillium chlamydosporium</i>	137
6.6.1 Introduction	137
6.6.2 Methods	137
6.6.2.1 Production of chlamydo spores	137
6.6.2.2 Post-harvest application of <i>Verticillium chlamydosporium</i> to soil previously used in the plunge trial	138
6.6.2.3 Post-harvest application of <i>Verticillium chlamydosporium</i> to soil previously used in the field trial.....	139
6.6.3 Results	139
6.7 Discussion	143
7 Determination of the composition of Jersey PCN populations using biochemical techniques	148
7.1 Introduction.....	148
7.2 Materials and methods	152
7.2.1 Staining nematodes.....	152

7.2.2 Enzyme-linked immunosorbent assay	154
7.2.2.1 General description of the indirect enzyme-linked immunosorbent assay	154
7.2.2.2 Antibodies used in these studies	154
7.2.2.3 Protocol for indirect ELISA.....	156
7.2.3 RAPD-PCR	158
7.2.3.1 Outline of RAPD-PCR	158
7.2.3.2 DNA extraction.....	159
7.2.3.3 Polymerase chain reaction	159
7.2.3.4 Agarose gel electrophoresis.....	162
7.3 Results.....	162
7.3.1 Life stages of PCN in roots	162
7.3.2 ELISA.....	164
7.3.3 Results from RAPD-PCR.....	164
7.4 Discussion	168
8 General discussion	171
8.1 Factors governing the development of PCN populations in Jersey	171
8.1.1 Economic and commercial requirements	171
8.1.2 Effects of nematicides, natural control and timing of harvest.....	171
8.2 Application of nematophagous fungi for control of PCN in Jersey.....	174
8.2.1 Formulation	174
8.2.2 Antagonism and agrochemicals.....	175
8.2.3 Fungal species	176
8.2.4 Recommendation for changes in crop management practices	178
8.3 Recommendations for future work.....	179
References.....	182
Appendices.....	204
Appendix 1 Publications.....	204
Appendix 2 Supplementary data.....	212
Appendix 2.1 Effect of pre-treatment of alginate pellets.....	212
Appendix 2.2 Nematode survey.....	213

LIST OF FIGURES

Figure 1.1 Steep south facing slopes, called ctils in Jersey, covered in polythene to warm the soil and protect early crops from frost.....	3
Figure 1.2 Life cycle of <i>Globodera spp.</i>	5
Figure 1.3 Hatching patterns for <i>G. rostochiensis</i> and <i>G. pallida</i> under a potato crop and decay curves for oxamyl preparations with two or three week half-lives.	9
Figure 2.1 Twenty litre fermenter used for the production of fungal biomass.....	23
Figure 2.2 Lid of twenty litre fermenter showing inlet and outlet ports.....	23
Figure 2.3 Biomass dry weight and optical density (520 nm) for <i>P. lilacinus</i> and <i>P. cucumerina</i>	25
Figure 3.1 The effect of the growth medium on the numbers of spores produced by <i>Plectosphaerella cucumerina</i>	33
Figure 3.2 The effect of the growth medium on the numbers of spores produced by <i>Paecilomyces lilacinus</i>	34
Figure 3.3 The effect of the growth medium on the numbers of spores produced by <i>Verticillium chlamydosporium</i>	35
Figure 3.4 Female PCN infected with <i>V. chlamydosporium</i> and <i>P. lilacinus</i>	40
Figure 3.5 Female PCN infected with <i>P. cucumerina</i>	41
Figure 3.6 Variation in pathogenicity between 20 monoconidial isolates obtained from a single field isolate of each <i>Plectosphaerella cucumerina</i> and <i>Paecilomyces lilacinus</i>	51
Figure 3.7 Variation in pathogenicity between 20 monoconidial isolates obtained from a single field isolate of <i>Verticillium chlamydosporium</i>	52
Figure 3.8 Infectivity of <i>Plectosphaerella cucumerina</i> , <i>Paecilomyces lilacinus</i> and <i>Verticillium chlamydosporium</i> against female PCN after passaging through female PCN.....	52

Figure 3.9 Infectivity of <i>V. chlamydosporium</i> against PCN females grown on MPM and MPAM.....	57
Figure 4.1 Colony radial growth rate (K_r) of nematophagous fungi <i>V. chlamydosporium</i> , <i>P. lilacinus</i> and <i>P. cucumerina</i> on PDA containing agrochemicals.....	68
Figure 4.2 Colony radius of <i>V. chlamydosporium</i> grown at 20°C on Vydate amended medium compared to that on unamended PDA.	69
Figure 4.3 Effect of fungicides on the morphology of <i>Verticillium chlamydosporium</i> and <i>Paecilomyces lilacinus</i>	70
Figure 4.4 a) Effect of fungicides on the morphology of <i>Plectosphaerella cucumerina</i> . b) Sectors produced by <i>P. cucumerina</i> when grown on Rizolex, were subcultured onto new agar plates.	71
Figure 4.5 Standard method for measuring interactions between two opposing fungi.....	75
Figure 4.6 Antagonism between nematophagous fungi and <i>R. solani</i> on nutrient medium at 20°C over a 13 day period. a) <i>R. solani</i> against <i>R. solani</i> . b) <i>P. cucumerina</i> . (left) against <i>R. solani</i> (right), c) <i>V. chlamydosporium</i> (left) against <i>R. solani</i> (right). d) <i>P. lilacinus</i> (left) against <i>R. solani</i> (right).....	80
Figure 4.7 <i>Rhizoctonia solani</i> mycelium showing observed swelling when grown in the presence of <i>Paecilomyces lilacinus</i> on agar.....	83
Figure 4.8 <i>Verticillium chlamydosporium</i> hyphae observed coiling around a <i>Rhizoctonia solani</i> hypha..	84
Figure 5.1 Equipment used for the production of alginate pellets.....	102
Figure 5.2 Dried alginate pellets, approximately 3 mm in diameter and freshly produced alginate pellets	103
Figure 5.3 Comparison of biological control formulations, incorporating the nematophagous fungi <i>P. cucumerina</i> and <i>P. lilacinus</i> , for the control of PCN eggs. The formulations were: fungus incorporated into alginate pellets mixed into the soil; Terra-Green® inoculated with nematophagous fungi placed under the tuber; and a tuber dip containing	

biomass suspended. The controls for each formulation were as the treated groups with the fungi omitted.....	105
Figure 5.4 Effect of the pre-treatment of alginate pellets on plant emergence. Alginate pellets were either untreated, crushed or soaked..	111
Figure 5.5 The effect of pre-treatment of alginate pellets on final numbers of <i>G. rostochiensis</i> eggs.....	112
Figure 6.1 Plunge trial. Pots containing Jersey field soil were sunk into sand, 40 cm deep, at IACR-Rothamsted.....	120
Figure 6.2 Ratios between final and initial egg counts (Pf/Pi) for PCN after one season's growth of potatoes in untreated Jersey soil, and in Jersey soil treated with alginate only, alginated <i>P. cucumerina</i> , alginated <i>P. lilacinus</i> , or both <i>P. cucumerina</i> and <i>P. lilacinus</i> ..	124
Figure 6.3 Potato yields from plants grown in untreated Jersey soil, and Jersey soil treated with uninoculated alginate pellets, alginated <i>P. cucumerina</i> , alginated <i>P. lilacinus</i> , or both <i>P. cucumerina</i> and <i>P. lilacinus</i> ..	124
Figure 6.4 Pf/Pi values for PCN grown for a second season in untreated Jersey field soil, or in Jersey soil treated previously with <i>P. cucumerina</i> (Pc) or <i>P. lilacinus</i> (Pl) alginate pellets, with and without a second application of <i>P. cucumerina</i> and <i>P. lilacinus</i> alginate pellets..	127
Figure 6.5 Yields of potatoes grown for a second season in untreated Jersey field soil, or in Jersey soil treated previously with <i>P. cucumerina</i> or <i>P. lilacinus</i> alginate pellets, with and without a second application of <i>P. cucumerina</i> and <i>P. lilacinus</i> alginate pellets.....	127
Figure 6.6 Effect of the addition of different amounts of alginate pellets containing <i>P. lilacinus</i> or <i>P. cucumerina</i> on the multiplication of PCN in a plunge trial.....	130
Figure 6.7 Effect of the addition of different amounts of alginate pellets containing <i>P. lilacinus</i> or <i>P. cucumerina</i> on the yields of Jersey Royal potatoes in a plunge trial.	130
Figure 6.8 Harvesting tubers at the end of the field trial in Jersey..	133
Figure 6.9 Soil temperature during the growing season in Jersey. Recordings were taken every 2 h from the base of a ridge in the centre of the field.	135

Figure 6.10 Initial PCN egg counts along the ridge used for the field trial. Plots were subsequently treated with <i>Paecilomyces lilacinus</i> , control, and <i>P. cucumerina</i>	135
Figure 6.11 Effect of the addition of alginate pellets containing <i>P. lilacinus</i> or <i>P. cucumerina</i> on the multiplication of PCN in soil in the field.....	136
Figure 6.12 Effect of the addition of alginate pellets containing <i>P. lilacinus</i> or <i>P. cucumerina</i> on the yield of Jersey Royal potatoes in the field..	136
Figure 6.13 Effect of post-harvest application of <i>V. chlamydosporium</i> on PCN egg counts in soil taken from the plunge trial.....	141
Figure 6.14 Effect of post-harvest application of <i>V. chlamydosporium</i> on PCN eggs in soil taken from the field trial.....	142
Figure 7.1 IEF gel showing <i>G. pallida</i> and <i>G. rostochiensis</i> controls with nematodes from Jersey..	150
Figure 7.2 Different life stages of PCN. The nematodes split into four categories, a) juveniles, b) small females, c) large females and d) males.....	153
Figure 7.3 Example of an antigen/antibody complex in an indirect ELISA.....	155
Figure 7.4 Layout of ELISA plate used to determine the number of <i>G. pallida</i> and <i>G. rostochiensis</i> from Jersey field soil.	157
Figure 7.5 Proportion of PCN at different life stages in stained roots taken from the plunge experiment. PCN populations from untreated controls were compared to those on plants treated with 100g of <i>P. lilacinus</i> amended alginate pellets.....	163
Figure 7.6 RAPD-PCR showing DNA products extracted from stained PCN at different life stages within potato roots using different primers a) K16 and b) K4.....	167
Figure A2.1 Nematophagous fungi recovered from nematodes and soil in fields in Jersey.....	213

LIST OF TABLES

Table 1.1 Number of PCN eggs per gram of soil in fields in Jersey in 1996 as categorised by the Department of Agriculture and Fisheries.....	2
Table 1.2 Examples of nematophagous fungi and their modes of action.....	13
Table 3.1 Pathogenicity of <i>Plectosphaerella cucumerina</i> grown on potato dextrose agar (PDA), (SNA), corn meal agar (CMA), straw agar and modified Pachlewska medium (MPM) against PCN.....	39
Table 3.2 Dose requirement of <i>Plectosphaerella cucumerina</i> , <i>Paecilomyces lilacinus</i> and <i>Verticillium chlamydosporium</i> conidia to infect female PCN	46
Table 3.3 Percentage of PCN females infected with either <i>P. cucumerina</i> , <i>P. lilacinus</i> or <i>V. chlamydosporium</i> after the fungus has been passaged through PCN females	53
Table 3.4 Infectivity of an original field isolate of <i>P. lilacinus</i> compared to the same isolate grown on MPM and MPAM.....	59
Table 4.1 Inhibition of colony radial growth of <i>R. solani</i> and three species of nematophagous fungi when paired against themselves and each other.....	78
Table 4.2 Inhibition of colony radial growth of the saprophytic fungi <i>F. oxysporum</i> , <i>P. bilaii</i> , <i>C. globosum</i> and <i>T. harzianum</i> and three species of nematophagous fungi when paired against each other.....	82
Table 4.3 Efficacy of various formulations of <i>P. lilacinus</i> for the control of potato stem canker caused by <i>R. solani</i>	88
Table 5.1 Different formulation mixtures for alginate pellets.....	107

Table 7.1 Program of cycles for RAPD-PCR.	160
Table 7.2 Outline of samples processed with the RAPD-PCR techniques using the primers K16 and K4. Plants were previously treated with 100 g of <i>P. lilacinus</i> amended alginate pellets or left untreated	160
Table 7.3 Outline of samples processed with the RAPD-PCR techniques using the primer E19. Plants were previously treated with 15 g of <i>P. lilacinus</i> amended alginate pellets, 100 g of <i>P. lilacinus</i> amended alginate pellets or left untreated	161
Table 7.4 Determination of the proportion of <i>G. rostochiensis</i> and <i>G. pallida</i> in selected pots from the plunge trial, using indirect ELISA.	166
Table A2.1 The effect of pre-treatment of alginate pellets on numbers of <i>G. rostochiensis</i> eggs	212

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ABBREVIATIONS

Nematodes

PCN	Potato cyst nematode
CCN	Cereal cyst nematode
J2	Second stage juvenile
J3	Third stage juvenile
J4	Fourth stage juvenile

Fungi

PL	<i>Paecilomyces lilacinus</i>
PC	<i>Plectosphaerella cucumerina</i>
VC	<i>Verticillium chlamydosporium</i>

Nematicides

DD	1,3 and 1,2-dichloropropane
DBCP	1,2 dibromo-3-chloropropane

Fungal measurements

Kr	Colony radial growth rate
DI	Disease index

Media

PDA	Potato dextrose agar
CMA	Corn meal agar
MPM	Modified Pachlewska medium
MPAM	Modified Pachlewska antibiotic medium
SNA	Synthetischer Nährboden agar
MEB	Malt extract broth
WAA	Water antibiotic agar

Biochemical techniques

IEF	Isoelectric focusing
RAPD-PCR	Random amplified polymorphic DNA – polymerase chain reaction
ELISA	Enzyme-linked immunosorbent assay

Chemical reagents

PBS	Phosphate buffer solution
PBST	Phosphate buffer solution with Tween
PBSTM	Phosphate buffer solution with Tween and Marvel™
DMSO	Dimethyl sulfoxide
dNTP	Deoxynucleosides triphosphate
EDTA	Ethylenediaminetetraacetic acid
TBE	Tris-borate EDTA

Statistical

SE Standard error of the mean

SD Standard deviation

N Number of replicates

Other

IPM Integrated pest management

dw dry weight

a.i. active ingredients

1 INTRODUCTION

1.1 ECONOMIC IMPORTANCE OF PCN IN JERSEY

The potato cyst nematode (PCN) is a serious pest of potatoes in the UK and is among the most highly specialised and successful plant parasitic nematodes. There are two species of PCN, *Globodera pallida* Stone and *G. rostochiensis* Woll. *Globodera pallida* is becoming the dominant species possibly due to two factors. The first is the lack of nematicides able to give effective control over this species, and the second is the lower temperature threshold of *G. pallida* enabling it to hatch earlier in the year. Both species of PCN originated from the Andean region of Peru and Bolivia (Evans & Stone, 1977). PCN were first recognised in the UK in 1917 but were not recorded in Jersey until 1938. PCN can cause crop losses both directly and indirectly. The direct damage caused by PCN is in the root, with the amount of damage caused being proportional to the numbers of invading juveniles. Tuber yield begins to decline when the population of PCN present at time of planting exceeds the tolerance threshold (Evans, 1993). The indirect cost of PCN infestation is measured in terms of the cost of chemical control applied.

The production of Jersey Royal potatoes is an important industry for the island. In 1998, 29% of the island was used for growing Royals, including steep south facing slopes called “côtils” that have to be ploughed by hand (Figure 1.1).

In 1998, 37,590 tonnes of Jersey Royal potatoes were exported from Jersey, with an average value per tonne of £824 giving a total value of £30.98 million. Fields are cropped with Jersey Royals annually, and occasionally biannually with Royals or with Royals followed by tomatoes or other potato cultivars, all of which are hosts of PCN (Mai & Lownsbery, 1948). Continuous cropping with a susceptible host can lead to multiplication of PCN. PCN infestation is regularly assessed by the Jersey Department of Agriculture and Fisheries.

Table 1.1 Number of PCN eggs per gram of soil in fields in Jersey in 1996 as categorised by the Department of Agriculture and Fisheries.

Category	I	II	III	IV
Eggs g soil⁻¹	PCN not found	1-6	7-60	>60
% of fields	20%	50%	25%	5%
area of land (ha)	619	1547.5	773.75	154.75

Use of granular nematicides is only recommended in category III and IV fields (Table 1.1). In 1996, it was estimated that 50% of potato fields were category II, 25% category III and 5% category IV. These figures have remained stable since 1991. In 1994 the Department of Agriculture and Fisheries in Jersey advised growers not to treat category II fields with nematicides, and this has not resulted in an increase in PCN.



Figure 1.1 Steep south facing slopes, called còtils in Jersey, covered in polythene to warm the soil and protect early crops from frost.

1.2 PCN LIFE CYCLE

Globodera pallida and *G. rostochiensis* are distinguishable by their colour when the females are present on the roots; the female of *G. pallida* is white and that of *G. rostochiensis* is golden. There are also morphological differences between the second stage juveniles and cysts of the two species. The life cycles of *G. pallida* and *G. rostochiensis* are similar, except that hatching occurs over a longer period for *G. pallida*. PCN have four juvenile stages before the adult stage (Figure 1.2). The first stage juvenile moults within the egg, to the second stage juvenile (J2), which will overwinter until spring when it hatches in response to hatching factors in the exudate from the potato roots (Clarke & Perry, 1977).

The hatched J2s enter the root via the root tip or at a lateral root and migrate away from the root tip, cutting through the cell walls and leaving a trail of ruptured cells. The J2 settles with its head towards the stele and begins feeding on pericycle, cortex or endodermis cells. At this point in its development the juvenile becomes sedentary, injecting saliva through its hollow mouth stylet and later withdrawing some of the cell's contents, forming a large syncytial transfer cell with dense, granular cytoplasm. Sex determination occurs at the third stage juvenile, and is controlled by nutrition (Trudgill *et al.*, 1987). The nematode remains in the transfer cell until it reaches maturity. The females enlarge to such an extent that they rupture the root cortex and are exposed on the outside of the root. The adult male remains vermiform and is only active for ten days, no longer feeds and dies after mating.

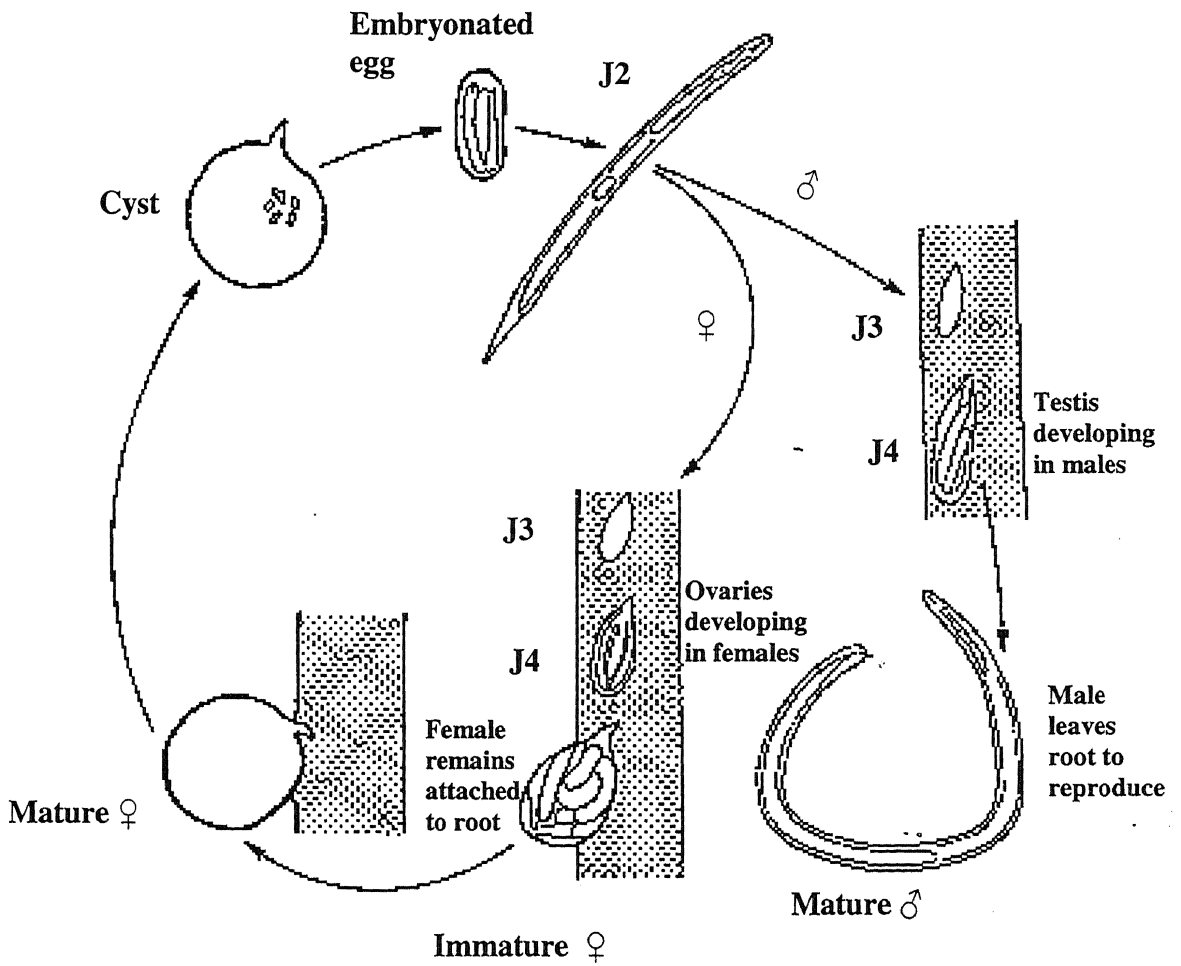


Figure 1.2 Life cycle of *Globodera* spp. (Taken from Evans & Stone, 1977).

The female dies when mature and the cuticle tans, becoming toughened to form a cyst that contains 200-400 embryonated eggs. Eventually the cyst will become detached from the root and fall into the soil. The eggs can remain viable within the cysts for up to twenty years and can withstand long periods of desiccation.

1.3 CURRENT CONTROL MEASURES

There are a number of control measures for PCN currently practised in the UK, but not all are suitable for application to potato cultivation on Jersey island.

1.3.1 CROP ROTATION

Rotation is an effective measure for control of PCN populations, as the number of nematodes declines in the absence of a host crop. The decline rate is dependent on a number of factors, such as soil type, but for comparison *G. rostochiensis* declined at 33% per annum compared to *G. pallida* which declined at 15% per annum in the same situation in the absence of a host crop (Evans, 1993). Adequate control can be achieved by growing three non-host crops in rotation with one resistant variety and one non-resistant variety potato crop treated with nematicides. This is not an option in Jersey, as only Jersey Royals can be exported, and therefore other varieties are not commonly grown. Due to the intensive cropping of Jersey Royals and the limited amount of land which is available, it is uneconomic and impractical to practise crop rotation.

1.3.2 RESISTANT CULTIVARS

Globodera rostochiensis can be controlled by the use of resistant potato cultivars containing the H1 gene which has been bred in to varieties such as Maris Piper, reducing the initial nematode population by 70-80% per annum (Trudgill *et al.*, 1987). Some varieties have partial resistance to *G. pallida*, for example Santé, Midas and Rocket, can reduce the multiplication of *G. pallida* by up to 80% compared to the initial population. Growing a cultivar resistant to *G. rostochiensis* together with cultivars with partial resistance to *G. pallida* will reduce the multiplication of both species. Jersey Royal potatoes are susceptible to both species of PCN.

1.3.3 NEMATICIDES

There are a number of nematicides available with various modes of action. Soil fumigants such as methyl bromide, 1,3 and 1,2-dichloropropane (DD) and 1,2 dibromo-3-chloropropane (DBCP) are broad spectrum pesticides which also adversely affect other organisms. Methyl bromide is not only damaging to organisms in the rhizosphere but is also ozone depleting, hence a world wide ban will be imposed by 2010. Fumigant nematicides control plant parasitic nematodes by the release of toxic gas which kills the juveniles within the cysts; some have to be applied prior to planting as they are toxic to plants, however DBCP is non phytotoxic. The total amount of DD used in Jersey in 1996 was 20,493 litres over 601 vergee (1 vergee = 0.17 hectare).

The other group of nematicides are non-volatile chemicals. Aldicarb (Temik 10G; 10% a.i. w/w aldicarb; RP Agric.) and oxamyl (Vydate 10G; 10% a.i. w/w; DuPont) are soil applied systemic carbamate/carbamoyloxime nematicides that prevent the nematode from finding the host by releasing nerve toxins. Granular nematicides have to be applied immediately before planting, as they start to break down to non-toxic products as soon as they are applied. As granular nematicides are non-phytotoxic, multiple cropping within a single season was introduced in the 1970's in the UK and USA, to increase output per hectare. However, the nematicides and their degradation products were subsequently found in drinking water in Long Island, New York (Kerry, 1993). Poor control of *G. pallida* by nematicides has been identified as the major contributor to the emerging dominance of this species over *G. rostochiensis* (Halford *et al.*, 1995). This is due to the spread of hatch over a longer period of time by *G. pallida* and greater reserves of lipids in their second stage juveniles, improving the chance of survival once the concentration of nematicide in the soil has declined. The nematicide oxamyl, for example, has a half-life of two or three weeks (Figure 1.3), whereas the peak hatch for *G. pallida* is at six weeks. In Jersey in 1996, the two granular nematicides Temik and Vydate, were applied over 218 hectares at a rate of 2.4 kg per hectare and 40 kg per hectare over 1071 hectares, respectively.

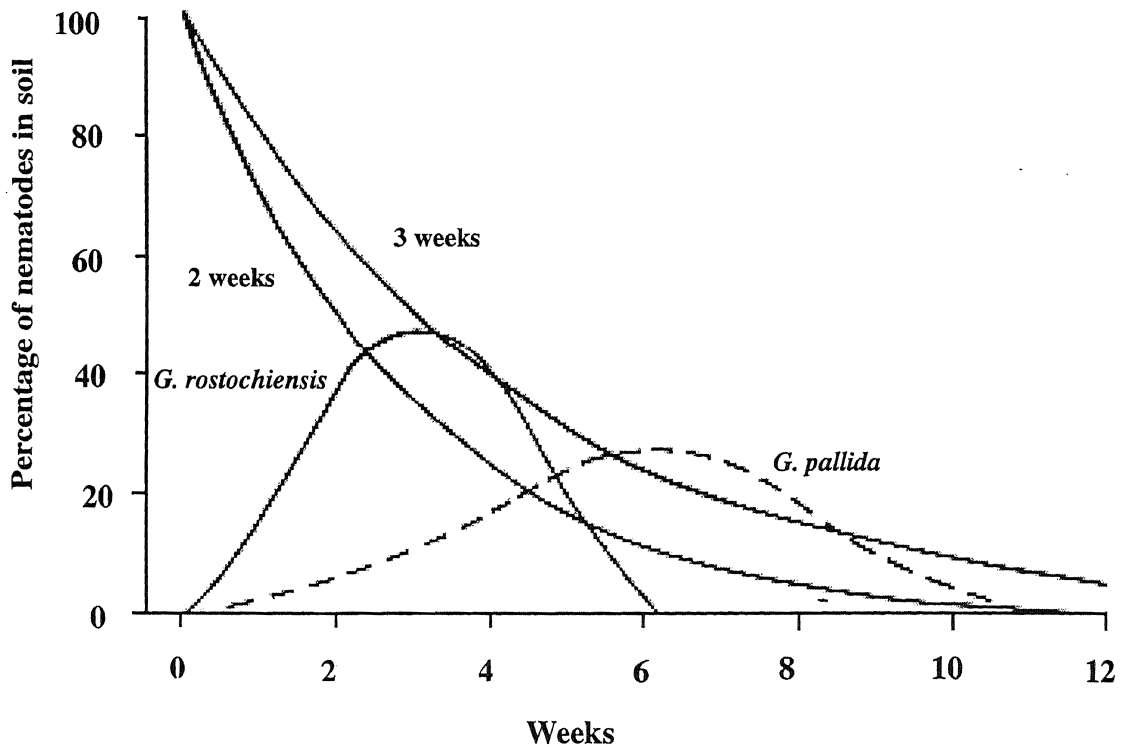


Figure 1.3 Hatching patterns for *G. rostochiensis* and *G. pallida* under a potato crop and decay curves for oxamyl preparations with two or three week half-lives. (Taken from Evans, 1993).

1.3.4 REMOVAL OF VOLUNTEERS

Volunteers are tubers which remain in the ground after harvest. Leaving the host in the ground bridges the interval between crops and allows the PCN to continue to reproduce and build up in the soil. Foliage and rotten and damaged potatoes which remain post-harvest can also add to the problem. The early harvest dates in

Jersey may serve to limit the rate of increase in population of PCN, as it is possible that not all the female PCN are reaching full maturity before harvest. This would make removal of volunteers particularly significant, as failure to remove them would negate the beneficial effect of the early harvest date. Removal of volunteer potatoes is actively encouraged in Jersey by the Department of Agriculture and Fisheries. Nematicide subsidies are withheld from farmers if this method of control has not been practised.

1.3.5 TRAP CROPPING

This method of control involves planting an early crop to stimulate nematode hatch. Once the nematodes have invaded the roots the plant is lifted. Trap cropping can potentially reduce PCN populations by 80%, but timing is crucial. If the crop is left in the soil for too long PCN numbers can multiply. Research by Halford *et al* (1999) found that trap cropping was time-consuming and labour intensive with few immediate benefits for the grower. Experiments so far have involved hand-lifting the potatoes and roots. It is not yet known if mechanical lifting of trap crops will remove enough root to prevent the nematodes developing further in the soil.

1.4 BIOLOGICAL CONTROL

“Biological control is the use of parasitoid, predator, pathogen, antagonist, or competitor populations to suppress a pest population, making it less abundant and

less damaging than it would otherwise be” (Van Driesche & Bellows, 1996). There are two types of biological control:- a) induced, resulting from application of the agent by man; and b) natural, resulting from a build up of natural enemies in the soil.

Due to intensive cropping practices of the single cultivar, the PCN populations would be expected to be higher than they are in Jersey, given that the expected rate of reproduction on a susceptible cultivar is approximately x 70 per growing season (Evans 1999, pers. com.). The use of nematicides has achieved some control, but since the cessation of the use of nematicides in Category II fields in 1994 the PCN populations have remained stable. This is possibly due to natural control by micro-organisms (fungi, bacteria, mites and other nematodes). Natural control has previously been shown to occur when susceptible crops are grown intensively in the presence of the pest, and the antagonist has had time to build up (Crump, 1989). Natural control has been well documented for cyst populations (Roessner, 1990; Heijbroek, 1983).

1.5 NEMATOPHAGOUS FUNGI

There are three types of nematophagous fungi which are categorised depending on their method of attack (Table 1.2). Firstly, predatory fungi trap nematodes by means of adhesive nets, hyphae, branches, knobs or nets, or non-adhesive constricting rings and non-constricting rings (Saxena *et al.*, 1991). Secondly, endoparasites of vermiform nematodes produce adhesive or non-adhesive spores

which remain dormant until either adherence to or ingestion by the nematode occurs. Once germination has occurred hyphae penetrate the cuticle and colonise the host. The hyphae of endoparasites are only present inside the nematode, with the exception of conidiophores which are produced externally, and do not parasitise the eggs. Both of these types of nematophagous fungi attack vermiform nematodes. The final group of nematophagous fungi, which are able to parasitise cyst nematodes and their eggs, includes the fungi *Plectosphaerella cucumerina* (Lindfors) W. Gams, *Paecilomyces lilacinus* (Thom.) Samson and *Verticillium chlamydosporium* Goddard, which are used in this investigation. The process of infection occurs by in-growth of vegetative hyphae. The host is exploited by the hyphae as a source of nutrition, thereby increasing fungal biomass and spore production which in turn increases the likelihood of survival of the fungus (Nordbring-Hertz, 1988).

Willcox & Tribe (1974) conducted a survey of plant parasitic nematodes infected with fungi in Great Britain. It was found that *G. rostochiensis* cysts were not extensively or even moderately infected with fungi. In a review, Dackman (1990), postulated that, due to the pest having been introduced, there were no indigenous antagonists. Fungi found on PCN in Great Britain are therefore more likely to be opportunistic saprophytes than highly adapted parasites. Saprophytic fungi are less effective as biological control agents than parasitic fungi. As saprophytes are capable of utilising other sources of nutrients they may not parasitise the pest if another food source is available. However, these fungi can be cultured artificially and survive in the absence of their host.

Strategy for infection	Mode of action	Example	Reference
Nematode trapping fungi	Adhesive networks	<i>Arthrobotrys oligospora</i>	Permark & Nordbring-Hertz (1997)
	Constricting rings	<i>Dactylaria</i> sp.	Nordbring-Hertz (1988)
	Adhesive knobs	<i>Dactylaria scaphoides</i>	Konig <i>et al.</i> (1996)
	Adhesive branches	<i>Monacrosporium cionapagium</i>	Saxena & Mittal (1995)
Endoparasites of vermiform nematodes	Adhesive spores	<i>Drechmeria coniospora</i>	Jansson (1993)
	Non-adhesive	<i>Harposporium anguillae</i>	Hodge <i>et al.</i> (1997)
Parasites of eggs and cysts	Egg parasite	<i>Verticillium chlamydosporium</i>	Crump & Irving (1992)
	Cyst parasite	<i>Paecilomyces lilacinus</i>	Morgan-Jones <i>et al.</i> (1993)
		<i>Acremonium</i> sp.	Nigh <i>et al.</i> (1980)
	Obligate parasite	<i>Plectosphaerella cucumerina</i>	Yu & Coosesman (1998)

Table 1.2 Examples of nematophagous fungi and their modes of action.

Obligate parasites are, by their nature, likely to give good levels of control and once the host population is infected the epizootic may be self-sustaining. However, it may be difficult to reintroduce the obligate parasite to the soil. A promising parasitic fungus, the obligate parasite *Nematophthora gynophila* Kerry and Crump, found in soil of the cereal cyst nematode (CCN), also gave a high level of control for PCN (Crump & Moore, 1990). Research and development of this fungus was hampered by the complex media needed for its culture.

1.5.1 *PLECTOSPHAERELLA CUCUMERINA*

Plectosphaerella cucumerina (Lindfors) W. Gams is very similar in morphology to *Acremonium* sp. (Palm *et al.*, 1995) and in fact the strain used in the project was initially identified as *Acremonium*. This hyphomycete has been found in arable soil and is frequently identified as a component of the rhizosphere. The only published finding that *P. cucumerina* infects plant parasitic nematodes was by Yu and Coosemans (1998), who found *P. cucumerina* was present on *Meloidogyne hapla* egg masses isolated from tomato fields in Belgium.

1.5.2 *PAECILOMYCES LILACINUS*

Paecilomyces lilacinus was originally classified in the genus *Penicillium* Link as *Penicillium lilacinus* Thom, but was reclassified *Paecilomyces* Bainier by Samson. This fungus is a facultative parasite of plant parasitic nematodes.

Paecilomyces lilacinus is a tropical or sub-tropical fungus that has not been isolated from nematodes on mainland UK. The strain used in these experiments was isolated from Jersey soils, where the warmer climate may favour the growth of this fungus.

Most reports of *P. lilacinus* infection on nematodes were for *Meloidogyne* spp. (Mertens & Stirling, 1993; Morgan-Jones *et al.*, 1984; Bonants *et al.*, 1995), but results from field trials have been variable probably due to the associated climate conditions and strain selection. Saifullah & Gul (1991) however, successfully controlled *G. rostochiensis* with *P. lilacinus* in Pakistan. This species has been produced as a biological control agent, 'Biocon', in the Philippines. The eggs of *Meloidogyne hapla* are infected by *P. lilacinus* by the production of lytic enzymes that degrade the vitellin which is located on the outside of the eggs and is the first barrier to the fungus (Bonants *et al.*, 1995). Djian (1991) detected acetic acid, which has nematicidal properties, in culture filtrates of *P. lilacinus*.

1.5.3 VERTICILLIUM CHLAMYDOSPORIUM

Verticillium chlamydosporium is a facultative parasite of *Meloidogyne* sp. (Bourne *et al.*, 1996) and is thought to be the cause of the natural decline of CCN in cereal monocultures (Crump & Irving, 1992). Strain selection is important when using *V. chlamydosporium* as a biological control agent since pathogenicity, optimum temperature for growth and production of chlamydo spores may vary between strains. The main proteinase produced by *V. chlamydosporium*, VCP1,

facilitates infection of the nematode egg by removing the outer layer of the shell. However, proteinases purified from *V. chlamydosporium* growing on *Meloidogyne* sp. do not attack *Globodera* sp., suggesting a host specific-determining factor (Segers *et al.*, 1996). *Verticillium chlamydosporium* produces chlamydo spores on aerial mycelia that are for dispersal and survival of the fungus. These spores are 20-25 µm in diameter and consist of walls, 6-9 cells deep. Conidia are also produced on phialides.

1.6 REGISTRATION

The three fungi used for this research were those most frequently isolated from PCN cysts in Jersey, and were all known to be parasites of plant parasitic nematodes. There were two reasons why isolates from Jersey were selected for these experiments; firstly, these fungi are the natural enemies of the Jersey PCN population and, therefore have co-evolved with the Jersey nematodes. Secondly, the fungi selected were indigenous to Jersey, which will facilitate registration.

For a biological control agent to be commercialised, a number of tests need to be conducted. The minimum amount of information needed to register a product is: define the active agent taxonomically; define the culture methods; demonstrate the commercial product is free from contamination by other dangerous microbes; and demonstrate that the product is not infectious to man or domestic animals. Depending on the individual country's requirements, more detailed information and further rigorous tests may be needed, for example: biological properties, *i.e.*

host spectrum; natural geographic occurrence; residues on the crop post-harvest and in the environment; infectivity and toxicity in mammals; or information on environmental hazards and effects on wildlife (bumble bees, parasitoids, earthworms and birds).

1.7 AIM AND OBJECTIVES

The overall aim of this thesis was to assess the efficacy of three nematophagous fungi, indigenous to Jersey and previously recorded as parasites of plant parasitic nematodes, for their suitability as potential biological control agents for controlling PCN in a commercial potato crop and in particular to determine if an agent based on one or more of these fungi could be incorporated in to the unique farming practices found on Jersey island. Specific objectives were:

- to determine whether there is variation in growth, development, and infectivity between monoconidial fungal isolates;
- to test the suitability of the nematophagous fungi for use in combination with fungicides and nematicides as part of an IPM strategy;
- to study colonial and hyphal interactions between the nematophagous fungi, plant pathogens and soil saprophytic fungi, to determine whether the nematophagous fungi will survive in the soil;
- to develop a formulation suitable for application of the nematophagous fungi in the field using existing machinery;

- to test the efficacy of the formulated nematophagous fungi in pot and field trials;
- to determine the composition of the PCN population in Jersey using RAPD-PCR and ELISA techniques to investigate whether one species of *Globodera* is more susceptible to nematophagous fungi than the other.

2 MATERIALS AND METHODS

2.1 ISOLATION OF NEMATOPHAGOUS FUNGI

The three fungal strains used in this study, *P. lilacinus*, *V. chlamydosporium* and *P. cucumerina*, were isolated from infected PCN females by plating the infected cyst on to WAA. Small sections of fungal mycelium, 3 mm², were cut out of the agar and transferred aseptically on to CMA containing antibiotics (section 2.2) to aid sporulation and identification. The fungi were then stored on PDA slopes under light white mineral oil (section 2.3.1). Sections, measuring 3 mm², of the agar containing the fungi were cut using a flame sterilised Borrodaile needle from the agar slope, transferred on to a PDA plate and incubated at 20°C for 3 weeks prior to experimentation. The identities of the fungi were confirmed by mycologists at CABI Bioscience and assigned an identification number: *P. cucumerina* (380408), *P. lilacinus* (380406) and *V. chlamydosporium* (380407).

2.2 ISOLATE AND GROWTH MEDIA

Potato dextrose agar (PDA, Oxoid, CM139) and Cornmeal agar (CMA, Oxoid, CM103) were made up according to the manufacturer's instructions. Water agar contained 8 g l⁻¹ agar, technical grade (Oxoid, LP013). Modified Pachlewski

medium (MPM) contained 9 g l^{-1} soluble starch, 1 g l^{-1} anhydrous glucose (AnalaR[®]), 1 g l^{-1} $\text{KH}_2\text{PO}_4 \cdot 7\text{H}_2\text{O}$ (AnalaR[®]), 0.65 g l^{-1} $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ (AnalaR[®]), 0.5 g l^{-1} $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g l^{-1} ammonium tartrate, 5 mg l^{-1} iron citrate and 0.5 mg l^{-1} thiamine hydrochloride. MPM was solidified where required by the addition of 1.2% (w/v) agar, technical grade. SNA (Synthetischer Nährboden Agar; Nirenberg, 1976) contained 1 g l^{-1} KH_2PO_4 , 1 g l^{-1} KNO_3 , 0.5 g l^{-1} $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g l^{-1} KCl , 0.2 g l^{-1} sucrose and 20 g l^{-1} agar, technical grade). Straw agar was prepared by boiling 40 g straw in 1 l of distilled water for 30 min and straining through a $53 \mu\text{m}$ sieve. Straw agar was solidified by the addition of 8 g l^{-1} agar, technical grade. Malt extract broth (MEB) contained 20 g l^{-1} malt extract (Oxoid, CM57). Media were sterilised by autoclaving at 121°C for 20 min. Water antibiotic agar (WAA) and modified Pachlewska antibiotic medium (MPAM) were prepared by the addition of each chloramphenicol, streptomycin sulphate and chlortetracycline (0.05 g l^{-1} respectively) to the agars when cooled to 45°C .

2.3 PRESERVATION OF CULTURES

2.3.1 UNDER MINERAL OIL

Fungal cultures were kept on PDA slopes under mineral oil. *Plectosphaerella cucumerina*, *V. chlamydosporium* and *P. lilacinus* were streaked across the surface of the agar and incubated at 20°C for 11 days. Light white mineral oil (Sigma) was sterilised by heating overnight at 80°C . The mineral oil was allowed

to cool and poured over the slope to a depth of 5 mm. Cultures were kept at room temperature in the dark.

2.3.2 LIQUID NITROGEN

Sections of agar (0.5 cm³) on which *P. lilacinus*, *P. cucumerina* or *V. chlamydosporium* were originally grown, were cut and placed into cryovials. The cryovial was filled with 10% glycerol and sealed. The samples were slowly cooled to -35°C by placing them in a polythene box with 400 g dry ice and 75 ml 90% ethanol. Samples were then plunged into liquid nitrogen for long term storage.

2.4 LARGE SCALE PRODUCTION OF FUNGAL BIOMASS

To produce large amounts of fungal biomass for the pot and field trials, a twenty litre fermenter was used (Figure 2.1 & 2.2).

2.4.1 PREPARATION AND INOCULUM

Approximately, 40 plugs (5 mm diameter) cut from colonies of either *P. cucumerina* or *P. lilacinus* growing on PDA were added to four universal bottles, 10 plugs per bottle, each containing 20 ml MEB. The bottles were whirlimixed for 45 s to dislodge the conidia from the agar. The resulting 80 ml of

conidial suspension, containing approximately 1×10^8 conidia ml^{-1} , was decanted from the plugs and used to inoculate 15 l of malt extract in a 20 l modified polypropylene carboy (Nalgene[®], UK) (Figures 2.1 and 2.2) which had been sterilised by autoclaving at 121°C for 40 min. This fermenter was maintained at 18-22°C and agitated and aerated by sparging with filter-sterilised air at 9 l min^{-1} . Approximately 0.02 ml antifoam 289 (Sigma, A8436) was added when the fermenter was inoculated to prevent the build up of foam.

2.4.2 VALIDATION OF METHODOLOGY

To determine the optimum growth period for *P. lilacinus* and *P. cucumerina* in liquid culture, the biomass dry weight and optical densities were determined.

2.4.2.1 Dry weight determination

Triplicate 10 ml samples of biomass were taken using the sample tube of the fermenter every 24 h for 7 d. The samples were passed through pre-dried 47 mm diameter glass microfibre filters (Whatman), that were held in a 3 piece filter funnel (47 mm diameter, Whatman). The biomass was washed three times with 10 ml distilled water, and dried for 24 h at 105°C. The filter papers were dried to constant weight in a desiccator (Gray & Markham, 1997) (Figure 2.3a & b).

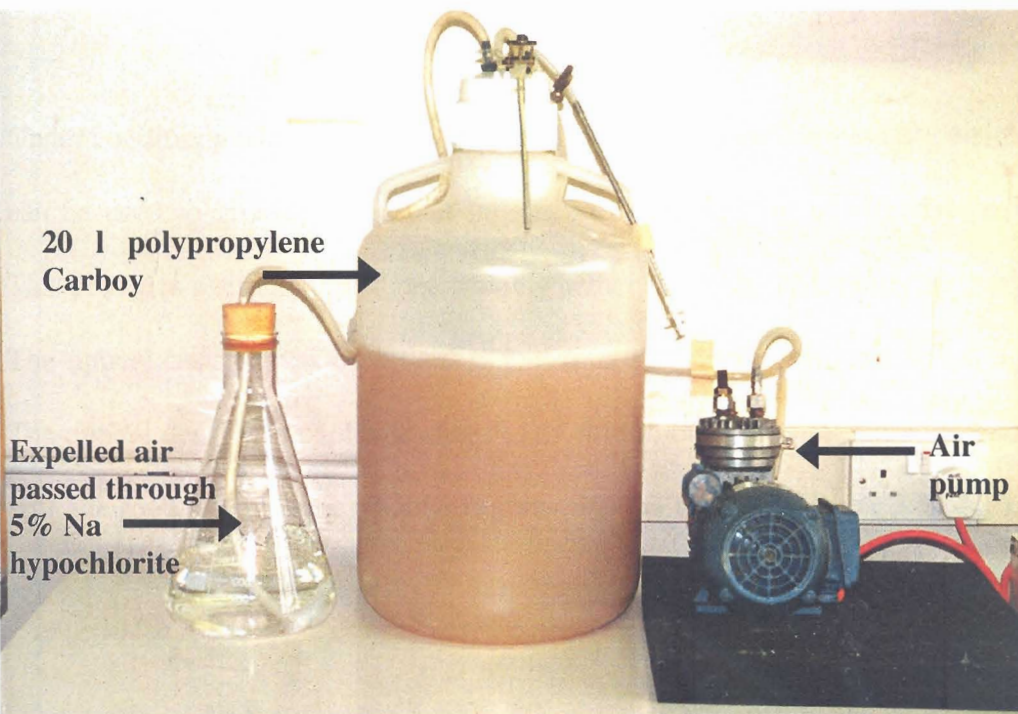


Figure 2.1 Twenty litre fermenter used for the production of fungal biomass.

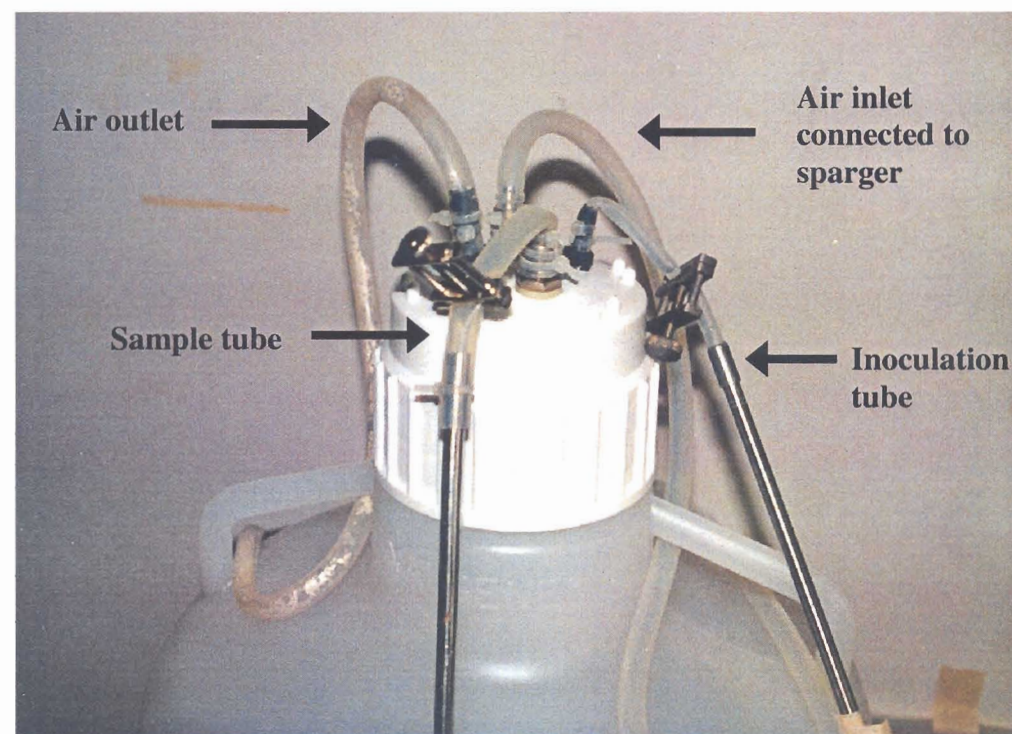
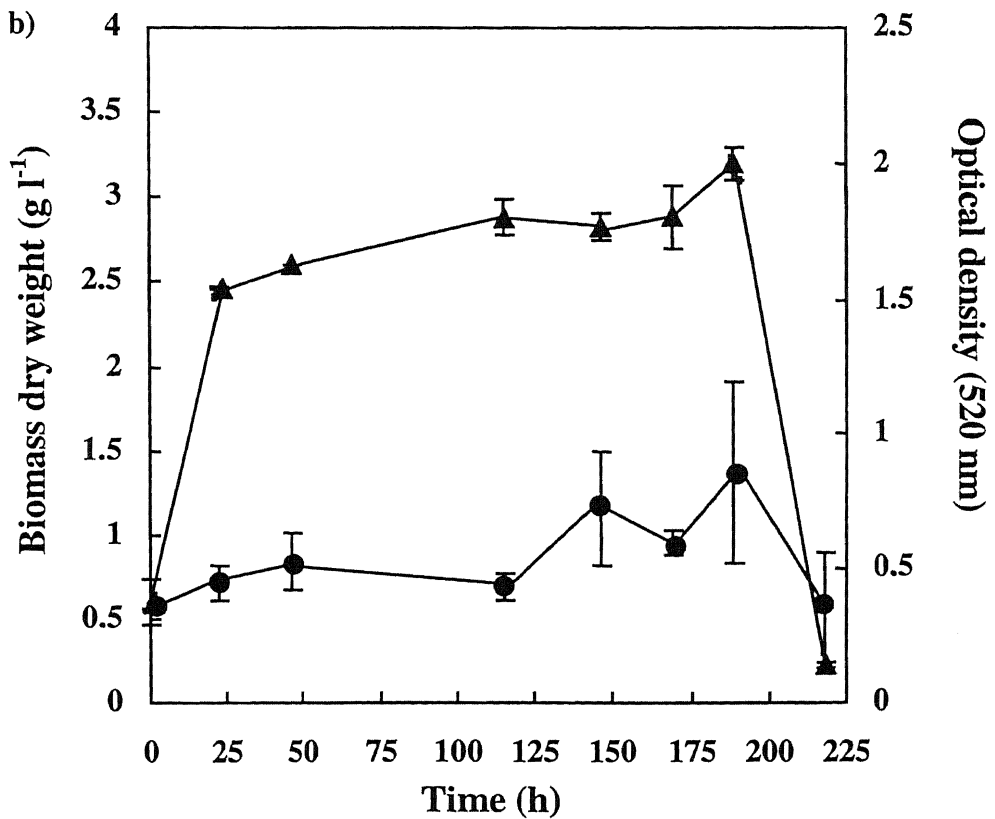
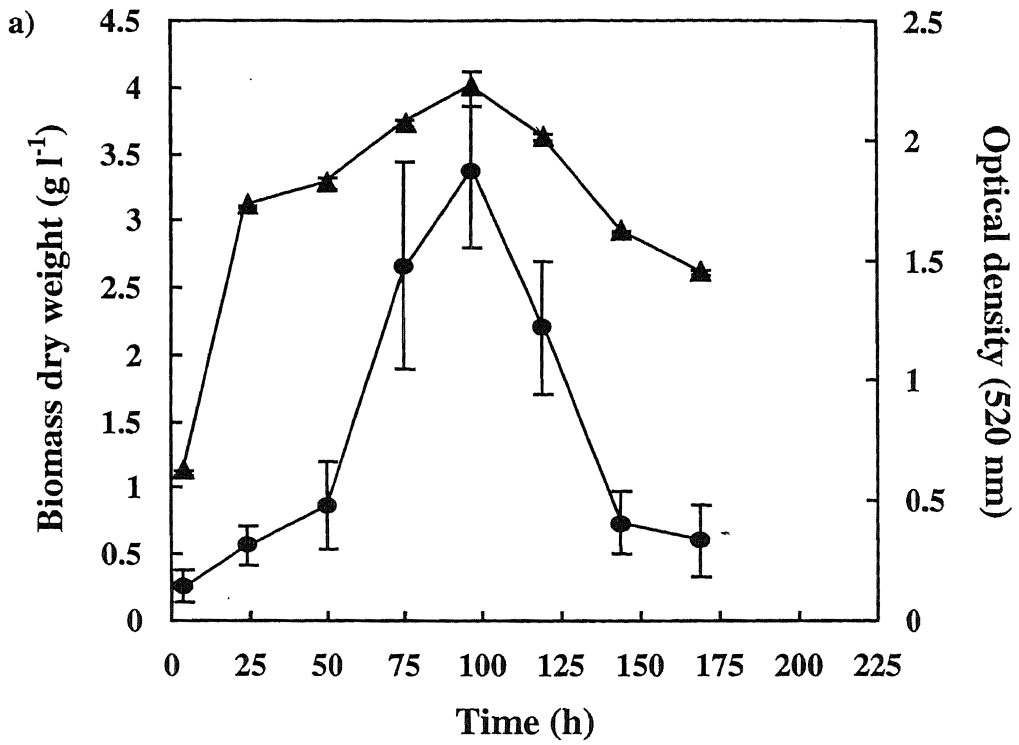


Figure 2.2 Lid of twenty litre fermenter showing inlet and outlet ports.

2.4.2.2 Optical densities of *Paecilomyces lilacinus* and *Plectosphaerella cucumerina* grown in batch culture

Under conditions where the optical density correlates to the biomass dry weight, it can be used to provide an instant indication of the amount of biomass present. This provides a simple test to determine whether the biomass is ready for harvest. The optical density was measured at the same time as the biomass dry weights. The optical density of triplicate samples of culture broth was measured at 520 nm with the spectrophotometer blanked against 2% malt extract (Figure 2.3a & b).

Figure 2.3 Biomass dry weight (●) and optical density (520 nm) (▲) for a) *Paecilomyces lilacinus* and b) *Plectosphaerella cucumerina*.



2.5 QUANTITATIVE ASSESSMENT OF PCN EGGS FROM SOIL SAMPLES

The soil samples were passed through a coarse sieve (4 mm in diameter pore size) to remove lumps and stones. The fraction of the soil that passed through the sieve was mixed and two 50 g samples were taken. One sample was put into a Trudgill fluidising column (Trudgill *et al.*, 1973) and floated for 45 s at a flow rate of 7 l min^{-1} , separating the cysts from the soil. The principle of operation of the Trudgill fluidising column is based upon the difference in density between the soil particles and the nematode cysts. A controlled upward flow of water contained in a column (7.5 cm x 42 cm) elutes the cysts from a soil sample. Cysts and other organic debris were collected on a sieve (250 μm pore size). The cysts were isolated from other debris by picking out with forceps under a dissecting microscope (x 120) and crushed using a glass rod to extract the eggs. The eggs were washed into a 100 ml measuring cylinder and mechanically disrupted using a vortex. The volume of water containing the eggs was noted and 1 ml of the egg solution was fed into a Fenwick multi-chamber counting slide (Doncaster *et al.*, 1966). Healthy eggs and juveniles were counted.

The second soil sample was used to determine the soil moisture content. The soil samples were air dried in cardboard trays and weighed once dry. The number of eggs per gram of dry soil was calculated as follows:

$$\frac{\text{No. of eggs} \times \text{volume}}{\text{known soil weight}} \times \frac{\text{known soil weight}}{\text{dry soil weight}} = \text{Eggs g soil}^{-1}$$

2.6 STATISTICAL ANALYSIS

Significant differences were assessed using Student's t-test (paired two tailed unless otherwise stated). One-way and two-way analysis of variance (ANOVA) were also used to assess significant differences using the statistical software package GENSTAT (Anon., 1987). Differences were classed as significant at the 95% level of confidence.

3 CULTURING AND SELECTION OF NEMATOPHAGOUS FUNGAL ISOLATES

3.1 INTRODUCTION

Considerable variation in pathogenicity between different strains of a single species of nematophagous fungi has been demonstrated for *V. chlamyosporium* (Irving & Kerry, 1986), *Fusarium sp.* and *Acremonium strictum* (Nigh *et al.*, 1980^a) and *P. lilacinus* (Villanueva & Davide, 1984; Tigano-Milani *et al.*, 1995). Kerry *et al.*, (1986) found strains of *V. chlamyosporium* varied in their pathogenicity towards *Heterodera avenae* eggs, as well as in optimum growth temperature and production of chlamyospores on agar. The fungal strains compared in these examples originated from different geographical regions or from different nematodes within a field.

To date, variation in pathogenicity between axenic cultures of nematophagous fungi isolated from a single nematode has not been studied. Monoconidial isolates of found the aphid-pathogenic fungus, *Erynia neoaphidis*, derived from a single aphid were to have different genotypes (Gray *et al.*, 1991). These isolates varied in their growth rates and in their ability to infect aphids. Therefore, a single conidium from an infected nematode could have a different genotype to another conidium from the same nematode. Careful strain and isolate selection is

important in the development of a fungal biological control agent, to ensure the efficacy and consistency of the agent is maintained.

The isolation of fungal isolates from their natural host on to a growth medium can affect the pathogenicity of the fungus through attenuation of its virulence due to a change in phenotype. During subculturing, genotypes with the ability for saprophytic growth on agar will be selected and dominate the population. If attenuation is due to a loss of some of the genotypes that make up an isolate, re-introducing the fungus to the natural host will not necessarily lead to that isolate recovering its original pathogenicity. If the loss in virulence is only due to a change in phenotypic expression, it is possible that the isolate can return to its original level of pathogenicity.

The pathogenicity of a nematophagous fungus should ideally be tested in pots or in the field. Pot tests allow for the effects of plant-nematode interactions and, to some degree, the influence of soil microorganisms to be investigated. Temperature and watering can easily be controlled in pot tests. Field tests demonstrate how the biological control agent will perform in a real situation, but there are many environmental factors which may influence the results. Pot and field tests can take a number of months before completion, so for logistical reasons, laboratory bioassays are often used.

3.2 SELECTION OF A SUITABLE MEDIUM FOR SPORULATION AND CONIDIATION OF NEMATOPHAGOUS FUNGI

3.2.1 INTRODUCTION

The nutritional requirements of the nematophagous fungi being studied are not fully characterised. Although they are saprophytic, the levels of different nutrients in the medium will affect the growth and development of the fungi. The numbers and infectivity of conidia produced were tested on five growth media. These media were in two categories: rich growth media (MPM and PDA); and low nutrient media traditionally used to encourage the production of conidia (CMA, straw agar and SNA).

3.2.2 NUMBER OF SPORES PRODUCED BY NEMATOPHAGOUS FUNGI ON DIFFERENT GROWTH MEDIA

3.2.2.1 Material and methods

Plectosphaerella cucumerina, *P. lilacinus* and *V. chlamydosporium* were grown on CMA for two weeks. Conidia of *P. cucumerina* and *P. lilacinus*, and both the chlamydospores and conidia of *V. chlamydosporium*, were then scraped from colonies using a Borrodaile needle and suspended in 5 ml sterile distilled water. The five different growth media (PDA, MPM, CMA, SNA and straw agar (2.2)) were each inoculated with 0.2 ml of the conidial suspension and grown at 20°C.

Triplicate plates for each fungus on each growth medium were incubated at 20°C for 176, 216 and 264 h for *P. cucumerina* and *P. lilacinus*, and 168, 214 and 296 h for *V. chlamydosporium*. Ten 3 mm diameter plugs were taken from each plate using a flame-sterilised core borer; five plugs from a transect of the plate and five plugs from a second perpendicular transect. The plugs were placed into 5 ml sterile distilled water and whirlmixed for 30 s (Campbell *et al.*, 1996). The concentration of conidia in suspension was measured using a haemocytometer. The numbers of conidia mm⁻² of colony was calculated as follow:

Surface area of a single plug (radius 2.5 mm) = 19.64 mm²

Therefore 10 plugs = 196.35 mm²

$$\text{Conidia mm}^{-2} = \frac{5 \text{ ml}}{196.35 \text{ mm}^2} \times \text{no. of conidia ml}^{-1}$$

3.2.2.2 Results

More conidia were present after 264 h when *P. cucumerina* was grown on PDA and MPM than when grown on straw agar, SNA or CMA. *Plectosphaerella cucumerina* produced significantly ($P < 0.05$, t-test) more conidia when grown on PDA than when grown on MPM (Figure 3.1). Fewer conidia were present on colonies of *P. cucumerina* on all media after 216 h growth than after 176 h growth. After 264 h, the number of conidia present on colonies of *P. cucumerina* increased compared to 216 h on PDA and CMA, but not on straw agar, SNA or MPM. The number of conidia mm⁻² of colony produced by *P. lilacinus* when

grown on different media increased with time (Figure 3.2). After 264 h the number of conidia produced when grown on PDA was significantly higher than any of the other media.

Verticillium chlamydosporium produced more spores mm^{-2} of colony after 269 h on PDA than on other media (Figure 3.3). The number of spores (conidia and chlamydo-spores) after 269 h was very similar for straw agar and MPM, 7.29×10^7 and 8.1×10^7 conidia mm^{-2} of colony respectively. The number of spores mm^{-2} of agar of *V. chlamydosporium* increased with time when grown on MPM, but the number of spores decreased when measured at 214 h for PDA, straw and CMA. The number of spore mm^{-2} agar for CMA, straw agar and PDA increased when measured at 269 h compared to 214 h.

Figure 3.1 The effect of the growth medium on the numbers of conidia produced by *Plectosphaerella cucumerina* grown on potato dextrose agar (PDA) (—▲—) low nutrient agar (SNA) (—■—), corn meal agar (CMA) (—◆—), straw agar (—X—) and modified Pachlewska medium (MPM) (—●—). N = 3.

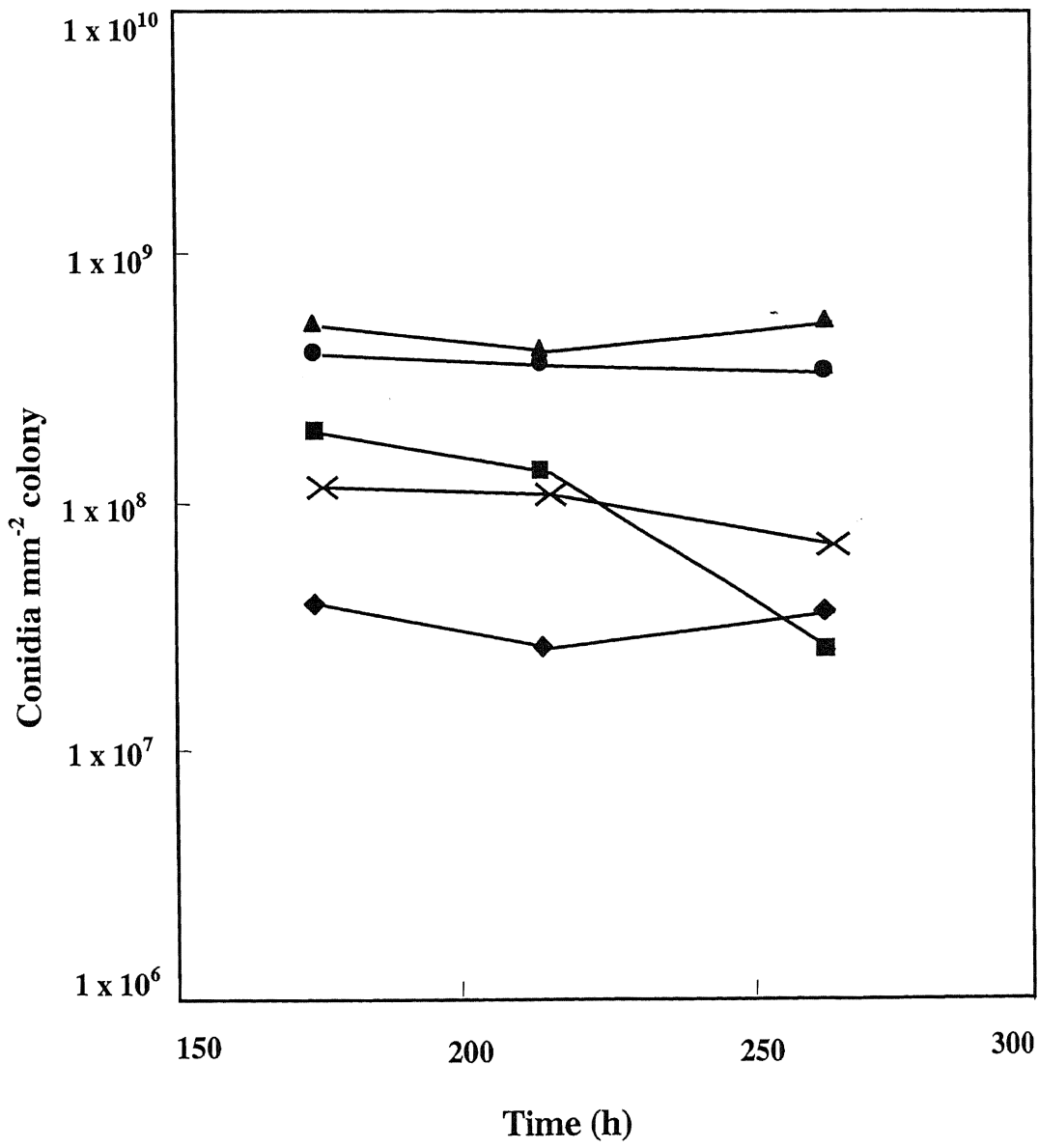


Figure 3.2 The effect of the growth medium on the numbers of conidia produced by *Paecilomyces lilacinus* grown on potato dextrose agar (PDA) (—▲—) low nutrient agar (SNA) (—■—), corn meal agar (CMA) (—◆—), straw agar (—X—) and modified Pachlewska medium (MPM) (—●—). N = 3.

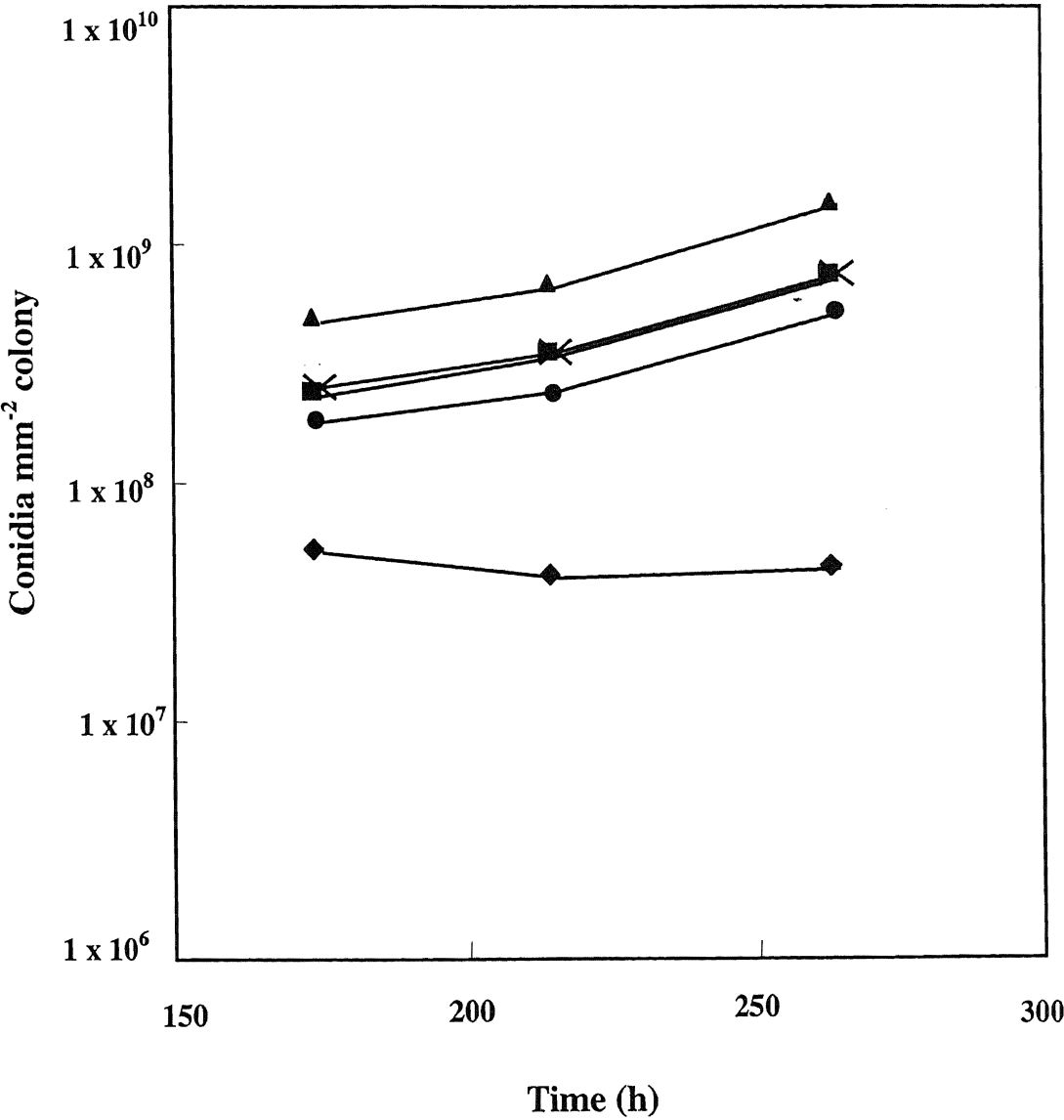
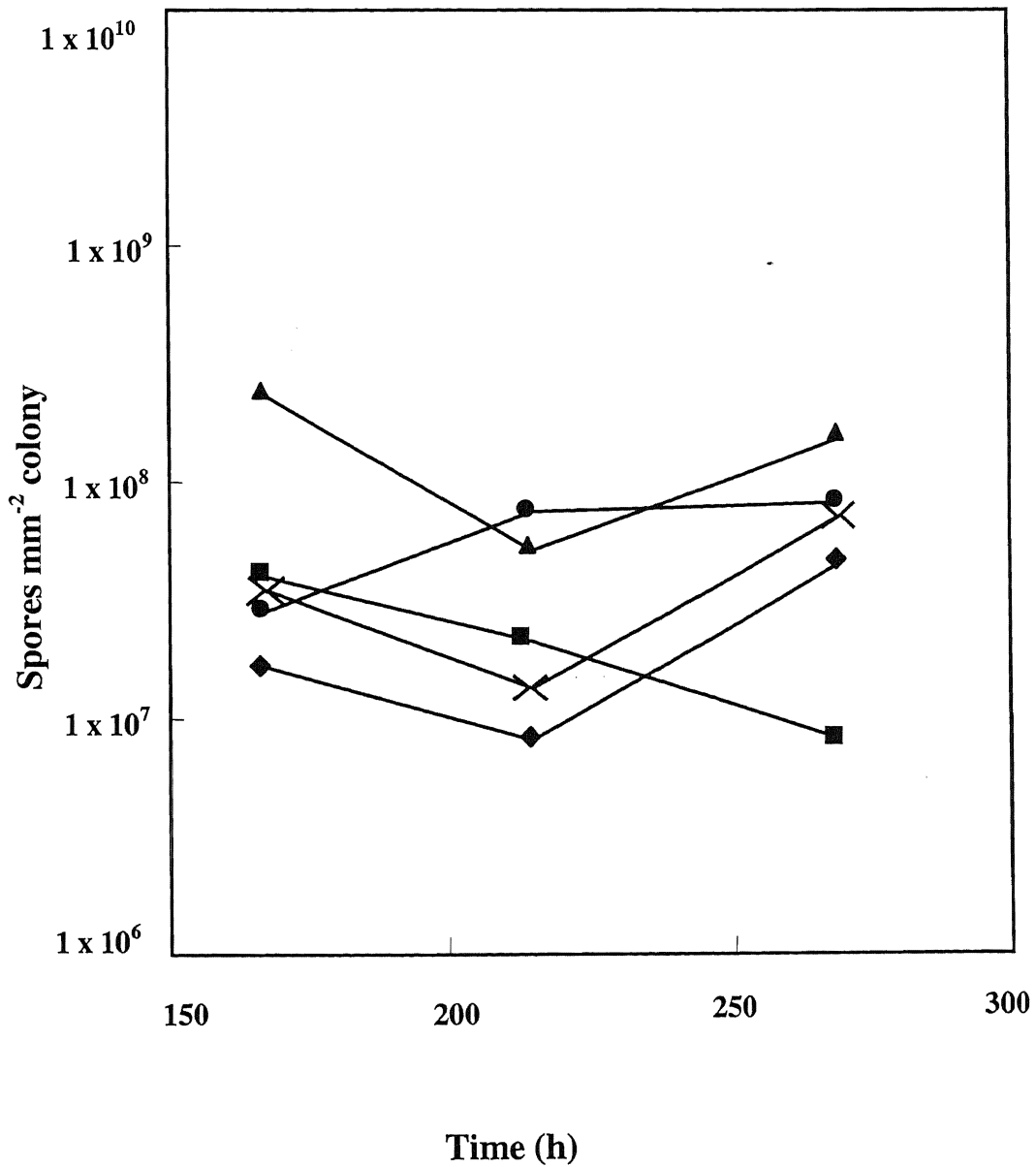


Figure 3.3 The effect of the growth medium on the numbers of conidia and chlamydo spores produced by *Verticillium chlamydosporium* grown on potato dextrose agar (PDA) (—▲—) low nutrient agar (SNA) (—■—), corn meal agar (CMA) (—◆—), straw agar (—X—) and modified Pachlewski medium (MPM) (—●—). N = 3.



3.2.3 EFFECTS OF DIFFERENT GROWTH MEDIA ON THE INFECTIVITY OF

P. CUCUMERINA

3.2.3.1 Introduction

One area of concern was the possible attenuation of the pathogenicity of the fungi when grown on artificial media. This is of importance when growing a fungus for field application to control a target pest. Even if the attenuation is reversible, a fungus may be less effective than the field isolate immediately post application, therefore allowing a pest to continue to cause damage to the crop. An artificial medium may not contain all the nutritional constituents required by a fungus to maintain its pathogenicity towards a particular organism. A change in the constituents can alter the growth and development and hence pathogenicity of a fungus (Latgé, 1975a; Latgé, 1975b). To measure the change in pathogenicity, a direct correlation with infectivity has been assumed.

3.2.3.2 Materials and methods

To produce large numbers of female PCN for the bioassay a two pot system was used. Four inch pots containing a layer of pea gravel and filled with Terra-Green® were placed on top of 5 inch pots filled with peat. Twenty dried cysts of *G. pallida* were placed 2 cm below the surface of the Terra-Green® (OIL.DRI UK Ltd., Cambridge, UK), together with a potato chit of cv. Desiree. Terra-Green, an inert montmorillonite mineral, was used as it absorbs water and allows maximum drainage. Osmacote slow release fertiliser (2 g l^{-1}) (15% nitrogen, 11% phosphoric

acid, 13% potassium oxide, 2% magnesium oxide plus trace elements (Scotts, UK)) was added to the surface of the upper pots, and the systems were watered daily. The female PCN were harvested after four to five weeks by washing the nematodes off the roots on to a 250 µm mesh sieve.

Female nematodes were surface sterilised in 1% sodium hypochlorite solution for 1 minute and then washed in distilled water. Three replicates of 10 females were plated on to WAA, for each isolate (unless otherwise stated). Nematodes were inoculated with 0.01 ml of fungal inoculum (1×10^5 conidia per ml sterile distilled water) or sterile distilled water only (as a control). Plates were incubated at in the dark 20°C for 7 days. The nematodes were then removed from the agar, surface sterilised (as above), plated onto moist filter paper to ensure the nematode was the only source of nutrients, and incubated as before. After 7 days the females were assessed for fungal infection. Abundant growth of hyphae from the cuticle of the nematode was taken as an indication of infection with the test fungus.

To test the variation in pathogenicity of *P. cucumerina* isolates that had been grown on different media, plugs (5 mm diameter) of *P. cucumerina* were cut from colonies (used in section 3.2.2), using a flame-sterilised core borer. For each growth medium, ten plugs were placed into separate vials for each media containing 5 ml of sterile distilled water. The samples were whirlimixed for 30 s to dislodge the conidia and the resulting suspension was diluted to 10^5 conidia ml^{-1} sterile distilled water. The female nematodes were each inoculated with

0.01 ml of the conidial suspension by dropping the suspension on to the nematode.

To allow for incidental contamination from sources such as soil and airborne conidia, the level of fungal infection in the control group was monitored. The results of the inoculated nematodes were adjusted for background infection using the following formula:

$$p(I) = p(B) + p(E) - p(B)p(E)$$

$$p(E) = \frac{p(I) - p(B)}{1 - p(B)}$$

$p(I)$ = probability of infection from any source.

$p(B)$ = probability of background contamination.

$p(E)$ = probability of experimental infection.

The number of nematodes infected in the bioassay can be due to background infection, experimental infection or both background and experimental infection.

An uninoculated control is assessed for background infection but when calculating the number of nematodes experimentally infected, some may be additionally contaminated by background infection. Therefore, to allow for females infected by both background and experiment, probabilities can be used to estimate total females experimentally infected. All results are adjusted for background infection unless otherwise stated.

3.2.3.3 Results

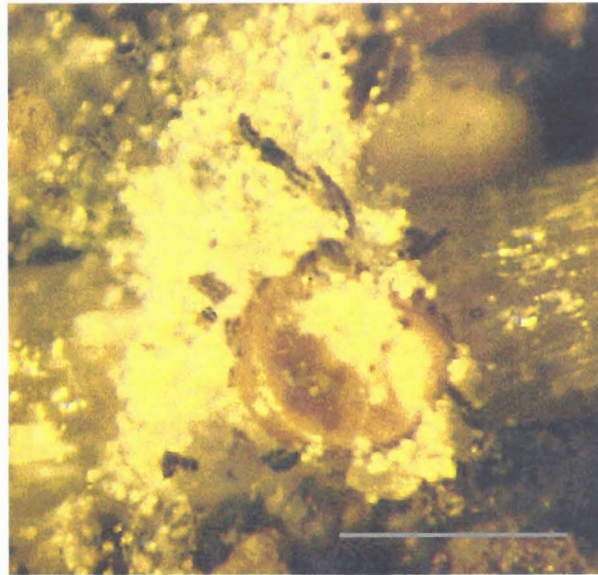
Figures 3.4 and 3.5 shows female PCN infected with *P. cucumerina*, *P. lilacinus* and *V. chlamyosporium*, females infected with other fungi were treated as contaminates. *Plectosphaerella cucumerina* conidia appeared to be most effective when grown on PDA and on MPM infecting 93.5% and 84.1% of female PCN in the bioassay respectively. These infections were not significantly different ($P < 0.05$, t-test) from those for the same fungus grown on any of the other media tested (Table 3.1).

Table 3.1 Infectivity of *Plectosphaerella cucumerina* grown on potato dextrose agar (PDA), synthetischer Nährboden agar (SNA), corn meal agar (CMA), straw agar and modified Pachlewski medium (MPM) against PCN (corrected for background infection).

Medium	No. of female PCN infected (out of 10)			% female PCN infected
	1	2	3	
Control	2	0	0	6.7 ^a
PDA	9	10	9	93.5
SNA	7	8	8	77.6
CMA	9	8	6	77.6
Straw	7	9	8	81.3
MPM	10	9	6	84.1

^a not corrected for effect of background infection.

a)



b)

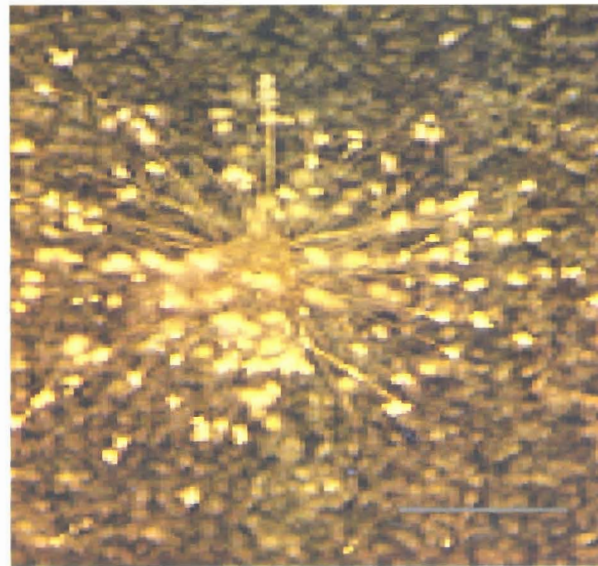


Figure 3.4 Female PCN infected with a) *V. chlamyosporium*. Bar = 700 μ m.
b) *P. lilacinus*. Photograph taken using light microscopy. Bar = 1 mm.

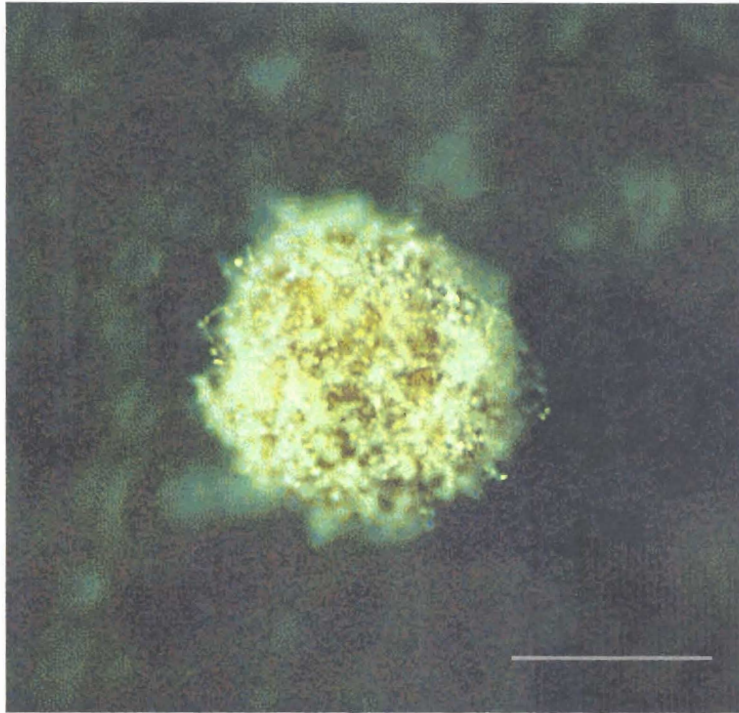


Figure 3.5 Female PCN infected with *P. cucumerina*. Photograph taken using light microscopy. Bar = 500 μm .

3.2.4 DISCUSSION

For the five media tested, all three fungi produced the greatest number of conidia on PDA (Figure 3.1, 3.2 and 3.3). This suggests that the nutritional requirements for spore production were best satisfied with this medium. *Paecilomyces lilacinus* increased its spore production over time when grown on all the media except CMA possibly indicating that a nutrient component essential for production of spores became exhausted after 214 h. This is a very different response to *P. cucumerina* and *V. chlamydosporium* (Figure 3.1 and 3.3), in which there was a decline in the number of spores present when grown on low nutrient agar for more than 178 h. Humphreys *et al.* (1990) found the entomogenous fungus *Paecilomyces farinosus* produced spores during the exponential and deceleration phase of growth, when grown in batch culture. This could explain why more spores were present at the first and third sample compared to the second time the fungi were sampled. Further work is needed to understand the relationship between the fungi and their nutrient requirements but the results indicated that the use of PDA was appropriate for the studies described in this thesis.

The pathogenicity of *P. cucumerina* conidia did not differ significantly on the five media tested ($P < 0.05$, t-test) (Table 3.1). Irving & Kerry (1986) also found the nutrient content of the agar did not affect the ability of *V. chlamydosporium* to infect *Heterodera avenae* eggs.

3.3 DOSE REQUIREMENT FOR INFECTING PCN FEMALES IN A BIOASSAY

3.3.1 INTRODUCTION

Producing large numbers of conidia can be time consuming and expensive (Cherry *et al.*, 1999). Therefore, field application of spores should be carried out at an optimum level where PCN infection is maximised but wastage of the biological control agent is minimised. As a preliminary investigation, a simple bioassay to compare the infectivity of the three nematophagous fungi was conducted.

3.3.2 MATERIALS AND METHODS

3.3.2.1 Growing monoconidial isolates

Monoconidial isolates were used for the majority of bioassays. Monoconidial isolates of *P. cucumerina* and *P. lilacinus* were obtained by scraping conidia from the original cultures using a sterile loop and whirlmixing for 30 seconds with 1 ml sterile distilled water, then spread plating 0.1 ml of this suspension on to MPM. When colonies appeared twenty colonies were plated individually on to fresh MPM plates and grown for 12 d at 20°C. Ten fungal plugs (6 mm in diameter) cut from each of these plates were put into 20 ml of sterile distilled water. The plugs in sterile distilled water were whirlmixed for 45 s, to dislodge the conidia from the agar, and the number of conidia was counted using a

haemocytometer. Conidial suspensions were centrifuged at 3000 rpm for 30 min to produce the high concentrations of conidia. The supernatant was discarded and 0.01 ml of the remaining conidia was used to inoculate individual female nematodes. The conidial suspension was adjusted to 1×10^5 conidia ml^{-1} sterile distilled water.

3.3.2.2 Separation of *Verticillium chlamydosporium* conidia from chlamydo spores

Ten plugs of agar (6 mm in diameter) were taken from the centre of a two week old *V. chlamydosporium* culture grown on PDA and whirlmixed in 20 ml of sterile distilled water. Conidia were separated from the chlamydo spores by centrifuging 10 ml of the spore suspension at 2000 rpm for 4 min to pellet the chlamydo spores. The supernatant which contained the conidia, was removed, and three 0.1 ml samples were examined in a haemocytometer to make sure no chlamydo spores were present. The suspensions were adjusted to 1×10^5 conidia ml^{-1} sterile distilled water.

3.3.2.3 Effect of inoculum dose

Three replicates of ten PCN females were inoculated with 0.01 ml of a suspension of conidia of *V. chlamydosporium*, *P. cucumerina* or *P. lilacinus* containing between 100 and 10^7 conidia ml^{-1} . This gave a dose of between 1 and 10^5 conidia per nematode. The bioassay was carried out as described in section 3.2.3.2.

3.3.3 RESULTS

The greatest percentage of infected female PCN occurred when *P. lilacinus* was applied as a dose rate of 10^3 conidia per nematode. *Plectosphaerella cucumerina* benefited from a higher dose of conidia (10^4) but the proportion of females infected only increased by 7%, which was not significantly different to the lower dose rates for that species ($P < 0.05$, t-test). For *P. lilacinus* and *V. chlamydosporium*, the proportion of females infected did not increase further when more than 10^3 conidia per nematode were applied. The batch of female PCN inoculated with *P. cucumerina* were the only group in which contamination was found, however the number of cysts infected in control treatment (due to background infection) was significantly lower than in any of the treated groups ($P < 0.05$, t-test).

Table 3.2 Dose requirement of *Plectosphaerella cucumerina*, *Paecilomyces lilacinus* and *Verticillium chlamydosporium* conidia for infection of female PCN. ND = not determined.

Dose (conidia per female PCN)	% Infected PCN		
	<i>P. cucumerina</i>	<i>P. lilacinus</i>	<i>V. chlamydosporium</i>
1	47.4	53.3	53.3
1 x 10 ¹	36.8	90	53.3
1 x 10 ²	46.7	90	60
1 x 10 ³	63.7	100	56.7
3.5 x 10 ³	ND	ND	54.8
1 x 10 ⁴	68.4	90	ND
1 x 10 ⁵	ND	90	ND
3.5 x 10 ⁵	ND	96.7	ND
Control	36.7 ^a	0 ^a	0 ^a

^a not corrected for background infection.

3.3.4 DISCUSSION

Paecilomyces lilacinus gave the highest level of infection. Ten conidia clearly infected PCN females better than a single conidium for this species; there was no further effect of increasing dose. *Plectosphaerella cucumerina* was most effective when 10^4 conidia were applied to a female. For *V. chlamydosporium* the dose rate did not correlate to the numbers of female PCN infected. This suggests that the conidia of *P. lilacinus* had a higher chance of infecting the nematodes successfully, when compared to the isolates of *P. cucumerina* and *V. chlamydosporium*. This could be due to a level of mortality and/or variation in pathogenicity between conidia. Further experiments are needed to determine the mechanisms determining infectivity of conidia against PCN. It was decided for future bioassays to use the maximum number of spores practically possible to ensure infection, therefore a dose rate of 10^5 conidia per female PCN for all fungi. As it was difficult to collect 10^5 conidia for *V. chlamydosporium* both conidia and chlamydo-spores were used to inoculate the female PCN.

3.4 VARIATION IN PATHOGENICITY BETWEEN MONOCONIDIAL ISOLATES AND PATHOGENICITY OF ISOLATES WHEN PASSAGED THROUGH PCN

3.4.1 INTRODUCTION

When selecting a fungal isolate for use in biological control it is important to determine whether there is variation between isolates or whether one isolate is as effective as another. This will reduce possible variation within a product and enable optimisation of its efficacy. In this section the term isolate refers to a fungal culture that was derived from a single nematode. The aim of this experiment was to remove single conidia from isolates of *P. cucumerina*, *P. lilacinus* and *V. chlamydosporium*, and grow them on agar, the progeny conidia were then used in a bioassay to determine whether there is variation within the isolate.

Pathogenicity of nematophagous fungi can be lost when grown continuously on artificial media. To prevent this from happening the fungi can be maintained on the natural host (as outlined in section 3.1). If attenuation does occur after growth on artificial medium, provided the change is purely phenotypic (not genotypic), pathogenicity can be restored by passage back through the host.

3.4.2 MATERIALS AND METHODS

PCN females were inoculated with twenty monoconidial isolates of *V. chlamydosporium*, *P. cucumerina* and *P. lilacinus* (section 3.2.3.2). Thirty females were inoculated per monoconidial isolate. Following assessment for numbers of infected females, any fungus growing out of the nematode's cuticle was plated on to MPAM using a sterile loop. These plates were incubated for a further 7 days at 20°C to allow the fungi to grow. The conidia produced by the resultant fungal growth were used to inoculate a further set of 30 PCN females per monoconidial isolate. This process was repeated once more leading to a final total of three passages of the three fungi through PCN females.

3.4.3 RESULTS

Variation in pathogenicity between monoconidial isolates was observed for each fungus (Figure 3.6 and 3.7), as was variation in infectivity of isolates after passage through female PCN (Table 3.3, mean values are shown in Figure 3.8). The pathogenicity of a particular monoconidial isolate relative to other isolates was not always consistent. This was observed for *P. lilacinus* where the range between the highest and lowest percentage infection for the first passage was 20%, but for the second passage was 61%. For example, *P. lilacinus* isolate 13 had the highest pathogenicity (80%) but when passaged to the second generation the pathogenicity decreased to 17%, one of the lowest recorded. For *P. cucumerina*,

Isolate 18 initially had the lowest pathogenicity (47%), but this increased to 73% when passaged through the nematodes.

The pathogenicity of fungal field isolates was reduced by passaging through the nematodes for each of the species tested (Figure 3.8). *Plectosphaerella cucumerina* was least affected, with an observed reduction of 14% (although this was not significantly different) in pathogenicity by the second passage, with no further reduction in pathogenicity following the third passage. Pathogenicity of *P. lilacinus* was most affected by passage, decreasing from 86% of nematodes infected after the first passage to just 7% following the third passage (Figure 3.8). Decline in pathogenicity after passaging the fungi through the female PCN was also evident in *V. chlamydosporium* with a statistically significant ($P < 0.05$, t-test) decrease of 98.5% in pathogenicity.

Figure 3.6 Variation in pathogenicity between 20 monoconidial isolates obtained from a single field isolate of each a) *Plectosphaerella cucumerina* and b) *Paecilomyces lilacinus*. Error bars show the standard errors of the means. N=30.

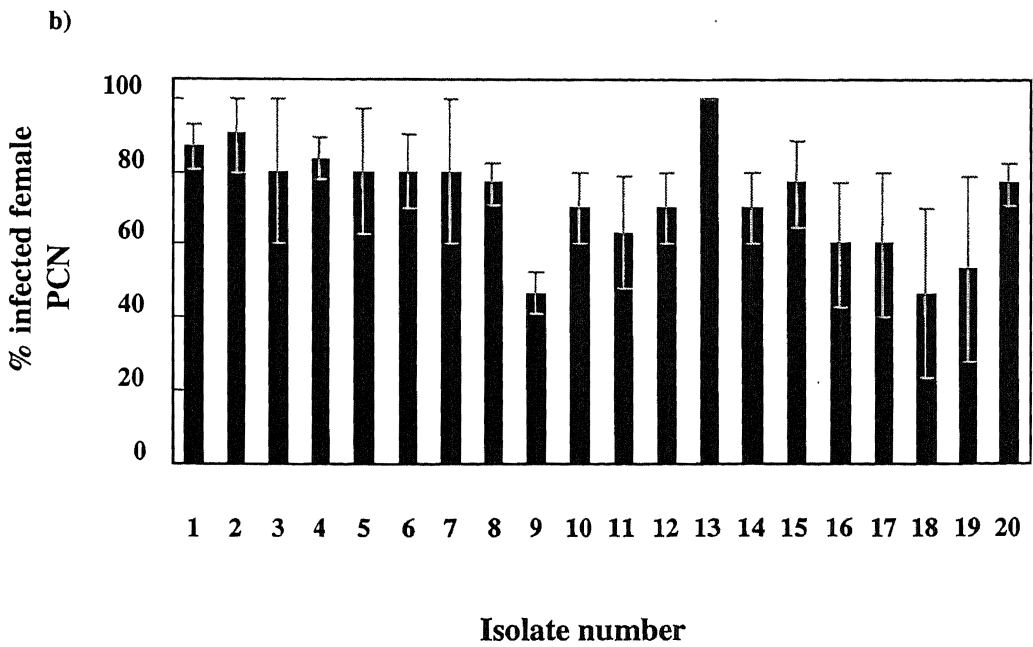
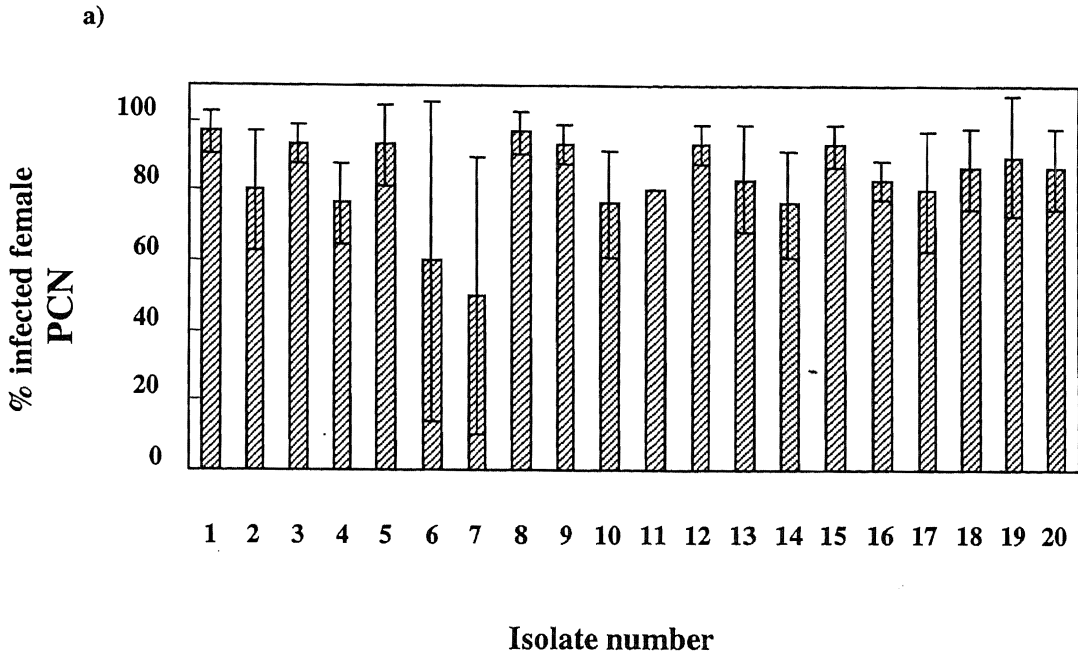


Figure 3.7 Variation in pathogenicity between 20 monoconidial isolates obtained from a single field isolate of *Verticillium chlamydosporium*. Error bars show the standard errors of the means. N=30.

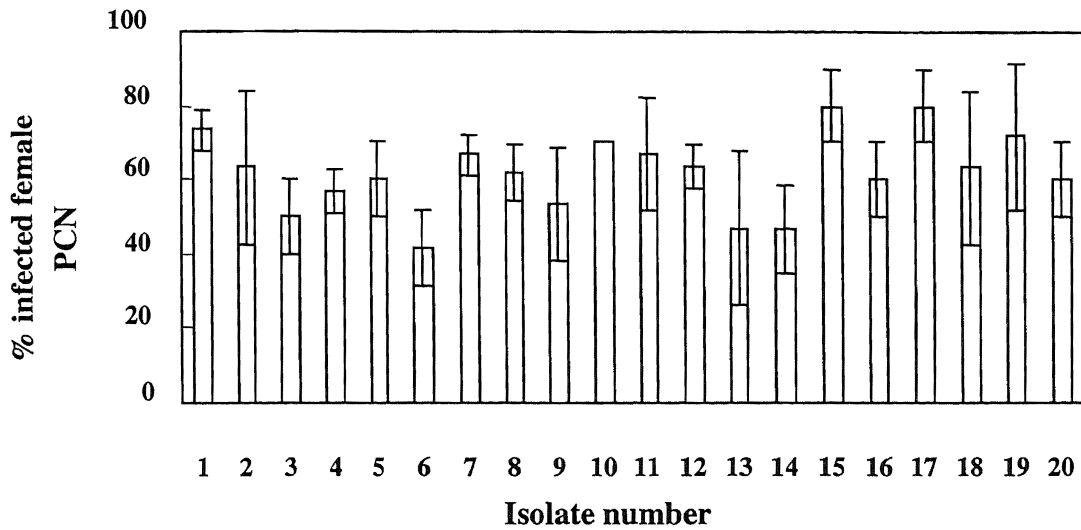
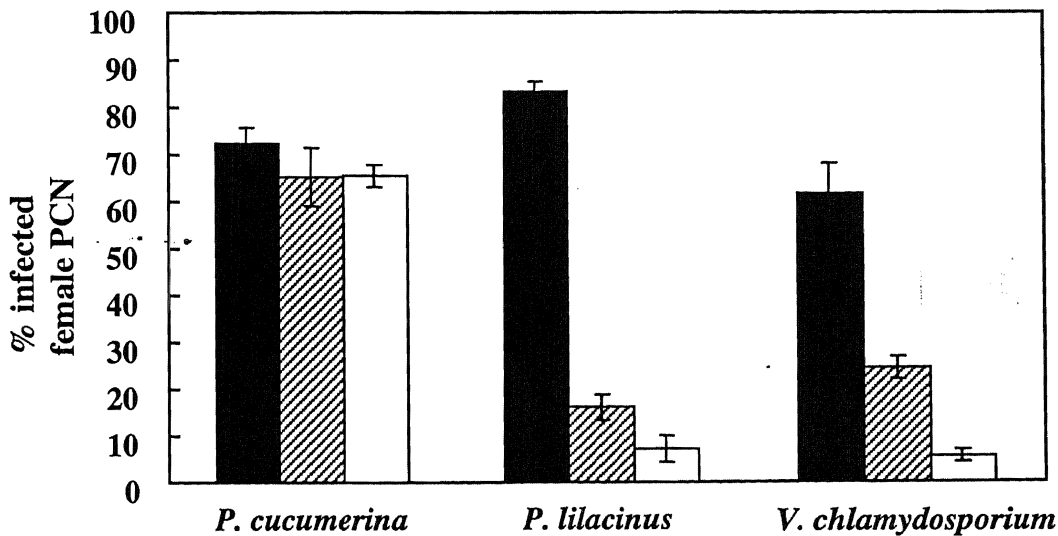


Figure 3.8 Infectivity of *Plectosphaerella cucumerina*, *Paecilomyces lilacinus* and *Verticillium chlamydosporium* against female PCN after passaging once (■), twice (▨), and three times (□) through female PCN. Error bars show the standard errors of the means. N = 20.



Isolate no.	<i>P. cucumerina</i>			<i>P. lilacinus</i>			<i>V. chlamydosporium</i>		
	1 st	2 nd	3 rd	1 st	2 nd	3 rd	1 st	2 nd	3 rd
1	86.7	70.0		96.7	6.7	0.0	73.3		
2	90.0	63.3		80.0	20.0	3.3	63.3	23.3	
3	80.0	50.0	93.3	93.3	20.0	10.0	50.0	40.0	
4	83.3	50.0		76.7	13.3	6.6	56.7		
5	80.0	61.7		93.3	23.3	13.3	60.0		
6	80.0	53.3		86.7	13.3	13.3	41.5		
7	80.0	62.6		76.7	26.7	14.1	66.7	16.7	
8	76.7	70.0		96.7	27.4	26.6	61.8	20.0	
9	46.7	69.2	73.3	93.3	23.3	0.0	53.3	20.0	
10	70.0	53.3	56.7	76.7	20.0	3.3	70.0	20.7	3.3
11	63.3	70.0	60.0	80.0	34.8	6.6	66.7	10.7	6.7
12	70.0	83.3	60.0	93.3	26.7	3.3	63.3	20.0	
13	100.0	63.3		83.3	46.7	3.3	46.7		
14	70.0	73.3		76.7	36.7	0.0	46.7	13.3	
15	76.7	63.3	70.0	92.9	20.0	16.6	80.0	17.0	
16	60.0	70.0	45.7	83.3	43.3	10.0	60.0	46.7	
17	60.0	73.3		80.0	56.7	0.0	80.0	23.3	6.7
18	46.7	73.3		86.7	43.7	3.3	63.3	23.3	
19	53.3	66.7		90.0	68.1	0.0	71.7	40.0	
20	76.7			86.7	62.2	13.3	60.0	33.3	

Table 3.3 Percentage of PCN females infected with either *P. cucumerina*, *P. lilacinus* or *V. chlamydosporium* after the fungus has been passaged through PCN females. No data indicates contamination has occurred.

3.4.4 DISCUSSION

The variation in pathogenicity observed for each species of nematophagous fungi could suggest that the monoconidial isolates were derived from a heterogeneous source, possibly due to the nematode being infected by more than one conidium to start with. An advantage of using a biological control agent that consists of a number of genotypes is that the pest is less likely to become resistant to the agent compared to a single isolate biological control agent. Genotypic variation within a population of a biological control agent can help to prevent resistance build up in the target organism, as long as all the individual isolates within the population are pathogenic. If not all the phenotypes are pathogenic, control may be patchy.

Some attenuation was probable as the fungi were not continuously passaged through nematodes but subcultured on to agar in between passages. However, the level of attenuation was much higher than expected and the addition of antibiotics in the agar was considered as an additional factor to explain the degree of attenuation observed in these experiments. This was investigated further (section 3.5).

3.5 EFFECT OF ANTIBIOTICS ON PATHOGENICITY

3.5.1 INTRODUCTION

Antibiotics are routinely used for isolation of fungi from field soils (Dackman, 1990; Mitchell *et al.*, 1987). Antibiotics prevent bacteria from growing by inhibiting a number of processes such as cell wall synthesis, protein synthesis, nucleic acid synthesis and intermediary metabolism. Many fungi produce antibiotics and will not necessarily have the sites targeted by different antibiotics, such as cell wall structures. However, the presence of sublethal concentrations of antibiotics can alter the physiology or exert selective pressure on fungi, which may lead to attenuation.

Attenuation of pathogenicity of the fungi against PCN was observed following passage of monoconidial isolates of nematophagous fungi were passaged through PCN females (Figure 3.8 and Table 3.3). It was hypothesized that this was due to the presence of antibiotics in the medium used when the fungi were cultured on agar. To test this hypothesis, *V. chlamydosporium* taken from a field isolate that had previously been plated on to agar, was grown on medium which contained antibiotics, and on medium deficient of antibiotics.

The attenuation may have taken place when the fungi were first isolated from the field and plated on to agar. To test this, *P. lilacinus* conidia that were isolated from a PCN cyst were compared to *P. lilacinus* conidia that had been grown on agar with and without the addition of antibiotics.

3.5.2 EFFECTS OF ANTIBIOTICS ON *V. CHLAMYDOSPORIUM*

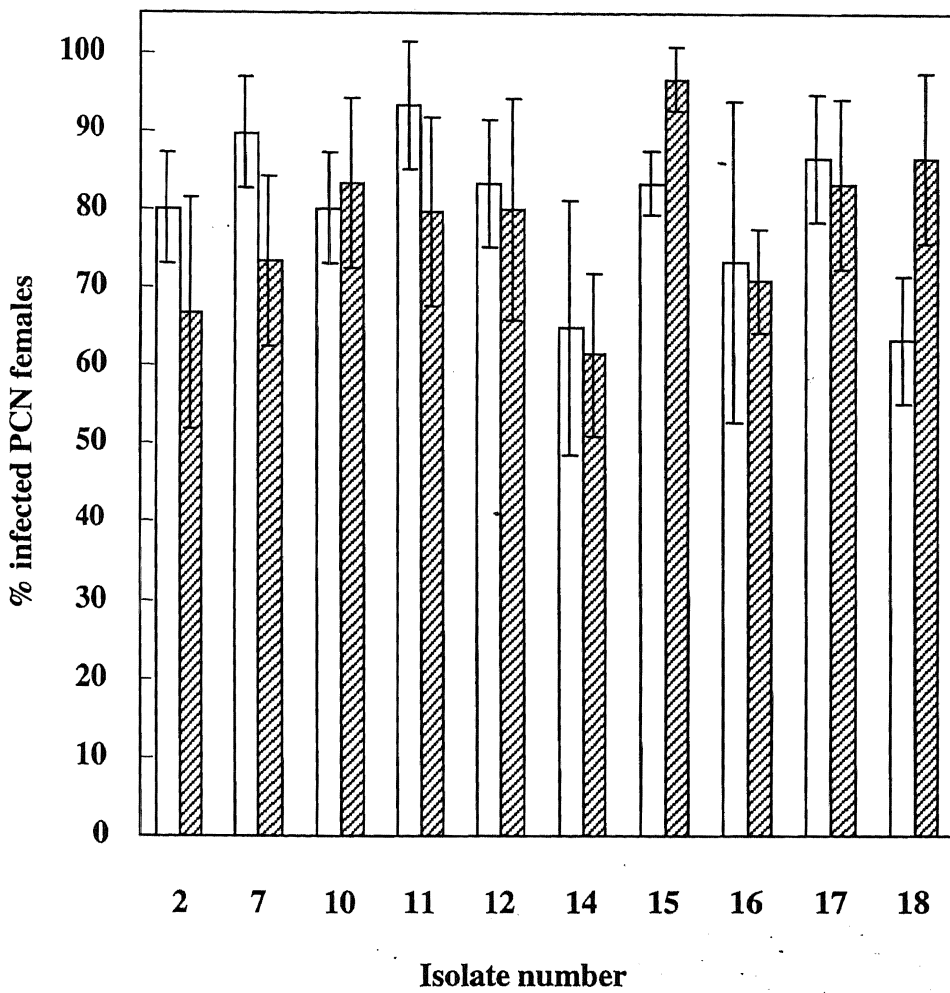
3.5.2.1 Materials and methods

Ten out of the twenty monoconidial isolates of *V. chlamydosporium* previously used in section 3.4.2 were grown on PDA. Plugs (6 mm diameter) were cut from plate cultures and inoculated on to MPM and MPAM. The fungus was grown for 12 d at 20°C. Ten plugs were cut from each agar plate and vortexed in 20 ml sterile distilled water. The conidia recovered from the suspension were used to inoculate nematodes as described in section 3.2.3.2.

3.5.2.2 Results

Six out of ten isolates of *V. chlamydosporium* had a lower pathogenicity when grown on MPAM than when grown on MPM. However, the differences were no greater than 16% (Figure 3.9). When isolates 10, 15 and 18 were grown on antibiotic agar, they were more infective than when grown on MPM, with differences of up to 23% for isolate 18. However, these differences were not statistically significant ($P < 0.05$, t-test).

Figure 3.9 Infectivity of *V. chlamydsporium* against PCN females grown on MPM (□) and MPAM (▨). Error bars show the standard errors of the means. N = 20.



3.5.3 EFFECTS OF ANTIBIOTICS ON *P. LILACINUS*

3.5.3.1 Materials and methods

Conidia of *P. lilacinus* were removed from the outside of an infected cyst collected from a Jersey soil using a sterile loop and streaked on to MPM and MPAM. The fungus was grown for 11 d at 20°C. Conidia were then recovered as in section 3.2.3.2., resulting in three suspensions of conidia derived from the three sources; MPM, MPAM and Jersey cyst, for the latter, a PCN cyst infected with *P. lilacinus* was put into 20 ml sterile distilled water and vortexed for 30 s. Each conidial suspension was used to inoculate twenty replicates of ten PCN females on agar.

3.5.3.2 Results

For *P. lilacinus* there was no statistical difference in infectivity between the isolate taken directly from a nematode in the field (58%) compared with the isolate grown on MPAM for 11 d (59%) (Table 3.4). Infectivity of conidia of *P. lilacinus* grown on MPM (69.7%) was higher than those grown on MPAM or derived directly from the field isolate ($P < 0.05$, ANOVA).

Table 3.4 Infectivity of an original field isolate of *P. lilacinus* compared to the same isolate grown on MPM and MPAM.

	Original isolate	Original isolate grown on	
	From cyst	MPM	MPAM
% infected female PCN	58	69.7	59
Standard deviation	3.9	4.9	4.1

3.5.4 DISCUSSION

Antibiotics do not appear to have an effect on the infectivity of nematophagous fungi when compared to the field isolate. However, when *P. lilacinus* was grown on MPM the percentage of infected females increased by 10% compared to the field isolate and isolate grown on MPAM. This could be due to a combination of factors such as the MPM supplying a more optimum source of nutrients for the fungus than that taken directly from a PCN female. Also, the female from which the field source isolate fungus was recovered may have been parasitised for some time and the available nutrients for the fungus depleted thereby affecting the ability of the conidia to infect. The difference between the fungus grown on the MPM and that grown on MPAM may have been due to a low level effect of the antibiotic used in the protocol.

3.6 GENERAL DISCUSSION

For each of the three fungi tested in this experiment, PDA was found to be the medium on which the fungi produced most spores, followed by MPM. Further experiments were therefore conducted on either MPM or PDA. After 220 h the number of conidia present on the surface of colonies of each of the fungi did not increase on any of the media tested. This was most likely due to a depletion in available nutrients. There was a small decrease in the number of conidia counted at the second sample time. This may have been due to the conidia being dislodged from the agar when the plates were moved, or to degeneration of conidia following nutrient exhaustion.

The minimum number of conidia needed to ensure optimum infection of female PCN was found to be 10^2 , 10^3 and 10^4 per female for *V. chlamydosporium*, *P. lilacinus* and *P. cucumerina*, respectively. This is unlikely to be achieved in an agronomic situation as it would be difficult to produce enough inoculum for these high dose rates. However, the purpose of the bioassays was to look for any variation between the monoconidial isolates and therefore the dose rate of 10^5 conidia per female was used as standard.

There was apparent variation between the monoconidial isolates in their ability to infect the female PCN (Figure 3.6 and 3.7). Differences of up to 54% in infectivity were seen from isolates obtained from the same culture. Tigano-Milani *et al.*, (1995) found genetic variation in isolates of *Paecilomyces fumosoroseus*

isolated from whitefly in Florida. Using molecular markers the populations of *P. fumosoroseus* were found to be genetically diverse. Tigano-Milani postulated that certain genotypes are more effective at different stages of epizootics and have a synergistic effect in combination. Many isolates would therefore need to be present for effective biological control, and a single isolate of one species of a nematophagous fungus may not be effective as a field inoculum.

As well as variation in pathogenicity between monoconidial isolates, there was high variability between replicates. This suggests the bioassay needs to be modified. Although the nematodes used were harvested after the same length of time, the growth rate and size of the PCN could have varied depending on the time of year due to variation in day length and temperature. Smaller (less mature) nematodes are more susceptible to infection by nematophagous fungi as the cuticle is more permeable than in larger nematodes. Cysts containing mature eggs tend not to become parasitised (Nigh *et al.*, 1980b). Where this was the case, comparisons within an experimental group may be valid but comparisons between experimental groups remain less reliable. A standard technique for the production of nematodes, that incorporates a controlled light and temperature regime, needs to be developed. A similar bioassay, to the one described in this thesis, was used by Chen *et al.*, (1996) to test the pathogenicity of a number of nematophagous fungi against the soybean cyst nematode *Heterodera glycines*. Chen used plugs of agar inoculated with nematophagous fungi to infect the female nematodes on agar plates, and counted the number of eggs infected with the test fungus, and the percentage reduction in hatch. This method gives a better indication of the extent

to which the fungus has reduced the nematode populations and the numbers of infected females.

The attenuation seen in all the fungi passaged through the three generations of nematodes was unexpected. Usually when a pathogen is reintroduced to its natural host having been cultured on a surrogate medium, a return of its pathogenicity is expected, as the fungus should either revert to its wild type or remain at its current level of attenuation. The fungi were grown on MPAM agar between nematode hosts to reduce the contamination by bacteria. It was suspected the addition of antibiotics in the agar may have caused the attenuation, however experiments 3.5.2.1 and 3.5.3.1 illustrated this was not the case. Mechanisms of attenuation are not clear and further work is needed in this area.

4 THE EFFECT OF AGROCHEMICALS AND OTHER SOIL FUNGI ON NEMATOPHAGOUS FUNGI

4.1 INTRODUCTION

Rhizoctonia solani Kühn is a major pathogen of potatoes. The fungus attacks the stems and stolons of growing potato plants (stem canker) and forms sclerotia on the tubers (black scurf), resulting in yield loss and also reduced market value due to blemishes on the tubers. Fungicides used to control *R. solani*, such as Rizolex (AgrEvo), Monceren (Bayer) and Gambit (Novartis), are applied to seed tubers prior to planting as a preventative measure and could have an adverse effect on fungi applied as control agents for PCN. Therefore, the effects of these fungicides on growth of the nematophagous fungi were investigated. The effects of foliar applied fungicides were not tested, as it is unlikely that these would affect the growth of the fungi in the rhizosphere (Larsen *et al.*, 1996).

Several species of fungi have been shown to be antagonistic towards *R. solani*. These include *Gliocladium roseum*, *G. virens*, *G. nigrovirens*, *Trichoderma hamatum*, *T. harzianum* and *Verticillium biguttatum* (Beagle-Ristanino & Papavizas, 1985). Turhan (1990) demonstrated that ten species of fungi, including *V. chlamydosporium*, were able to hyperparasitise *R. solani*, and *P. lilacinus* has

been reported to be antagonistic towards *R. solani* on Poinsettia (Cartwright & Benson, 1995).

For integrated pest management (IPM) to be effective, it is important to understand how a control agent for one pathogen interacts with non-target pathogens and other biological control agents. For example, individual applications of the nematicides oxamyl, ethoprofos and aldicarb in the field were found to increase the numbers of stems and stolons of potatoes infected with *R. solani* (Scholte, 1987), whereas Hide & Read (1991) found that the incidence of black scurf on tubers decreased after annual application of oxamyl for four years.

4.2 THE EFFECT OF AGROCHEMICALS ON NEMATOPHAGOUS FUNGI

4.2.1 INTRODUCTION

The active ingredients of fungicides currently used in Jersey are tolclofos-methyl (Rizolex; 10% a.i. w/w; AgrEvo), fenpiclonil (Gambit; 10% a.i. w/w; Novartis) and pencycuron (Monceren Flowable; 250 a.i. g/l; Bayer). These are applied separately to the tuber before planting. To determine whether application of the biological control agent to the tuber in the presence of the fungicides is feasible, the nematophagous fungi were grown on agar which contained these fungicides. The nematophagous fungi were also grown on agar containing the nematicide Vydate (active ingredient oxamyl 10G; 10% a.i. w/w; DuPont) to establish whether biological and chemical control agents could be used in combination.

4.2.2 MATERIALS AND METHODS

The three tuber-applied fungicides and the granular nematicide were tolclofos-methyl (Rizolex, AgrEvo); pencycuron (Monceren flowable, Bayer); fenpiclonil (Gambit, Novartis); and oxamyl (Vydate 10G, DuPont). PDA was prepared (section 2.2) and cooled to 45°C before addition of the agrochemicals at the following rates: 0.22 ml l⁻¹ Rizolex, 0.54 g l⁻¹ Monceren, 0.11 ml l⁻¹ Gambit and 3.6 mg l⁻¹ Vydate. The concentrations of agrochemicals added were equivalent to the rates of application recommended by the manufacturer, which are 0.25, 0.6 and 0.125 l tonne⁻¹ for Rizolex, Monceren and Gambit, applied as tuber dressings respectively, and 3.6 ppm for oxamyl applied to the soil. Sterilisation of the agrochemicals was unnecessary as they are inherently microbiocidal in concentrated form. Rizolex did not dissolve immediately in agar, so the flask was placed on a magnetic stirrer until the powder dissolved.

Plates of PDA to which each of the four agrochemicals had been added, and unamended PDA plates (controls), were inoculated centrally with 6 mm diameter plugs cut using a flame-sterilised cork borer from colonies of *V. chlamydosporium*, *P. cucumerina* or *P. lilacinus* that had been grown on PDA. The inoculated plates were then incubated at 20°C. Colony radius was measured as the mean of two perpendicular radii at approximately 48 h intervals until the colony reached 30 mm in diameter, with a minimum of 8 recordings. Colony radial growth rate (K_r ; Pirt, 1967) was calculated by linear regression of colony radius on time.

4.2.3 RESULTS

Monceren, Gambit and Rizolex all significantly ($P < 0.05$, t-test) reduced radial growth of *V. chlamydosporium* compared to the control, by 17.8%, 18.9% and 22.3% respectively (Figure 4.1). Radial growth of *P. lilacinus* was significantly reduced by the addition of Gambit (45.8%) and Rizolex (45.5%). Vydate, however, significantly increased the radial growth rate of *P. lilacinus* by 3% compared to the control. The addition of Gambit and Rizolex significantly reduced the radial growth rate of *P. cucumerina*, by 46.9% and 36.3% respectively.

For the first 650 h the growth rate of *V. chlamydosporium* on Vydate amended medium was the same as that on the control medium but then colony radial growth slowed (Figure 4.2). As the relationship between radius and time was not linear, it was not appropriate to calculate K_r . However, the increase in radius of colonies of *V. chlamydosporium* on Vydate amended medium between 650 h and 780 h was 16% less than on control plates.

The morphology of the nematophagous fungi was also affected by the presence of the fungicides in the agar (Figures 4.3 and 4.4). Sectors (formed by growth originating from mutated conidia) occurred on the majority of plates. To determine if the change in morphology was permanent, plugs were taken from a *P. cucumerina* isolate grown on PDA which had Rizolex incorporated into it (Figure 4.4b). The plugs were grown on new PDA plates with no fungicide

present. It was found that the fungi did not revert to the original form, indicating the morphology was permanently changed.

Figure 4.1 Colony radial growth rate (K_r) of nematophagous fungi *V. chlamydosporium*, *P. lilacinus* and *P. cucumerina* on PDA containing agrochemicals. Control (■), Monceren (0.54 g l⁻¹) (▨), Gambit (0.11 ml l⁻¹) (▩), Rizolex (0.22 ml l⁻¹) (▤) and Vydate (3.6 mg l⁻¹) (□). Error bars show the standard errors of the means. N=10. ND = not determined as the relationship between time and radius was not linear.

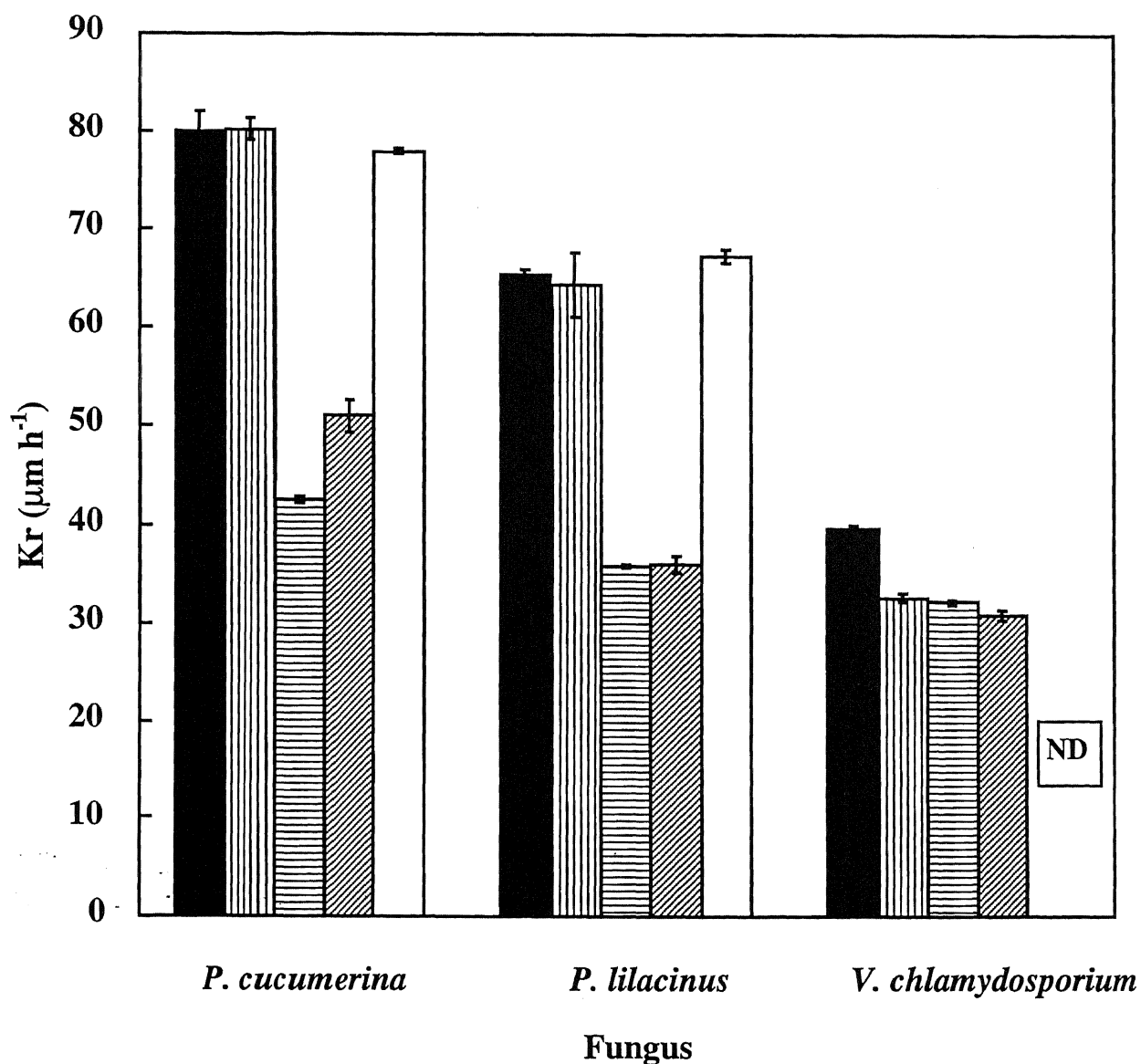
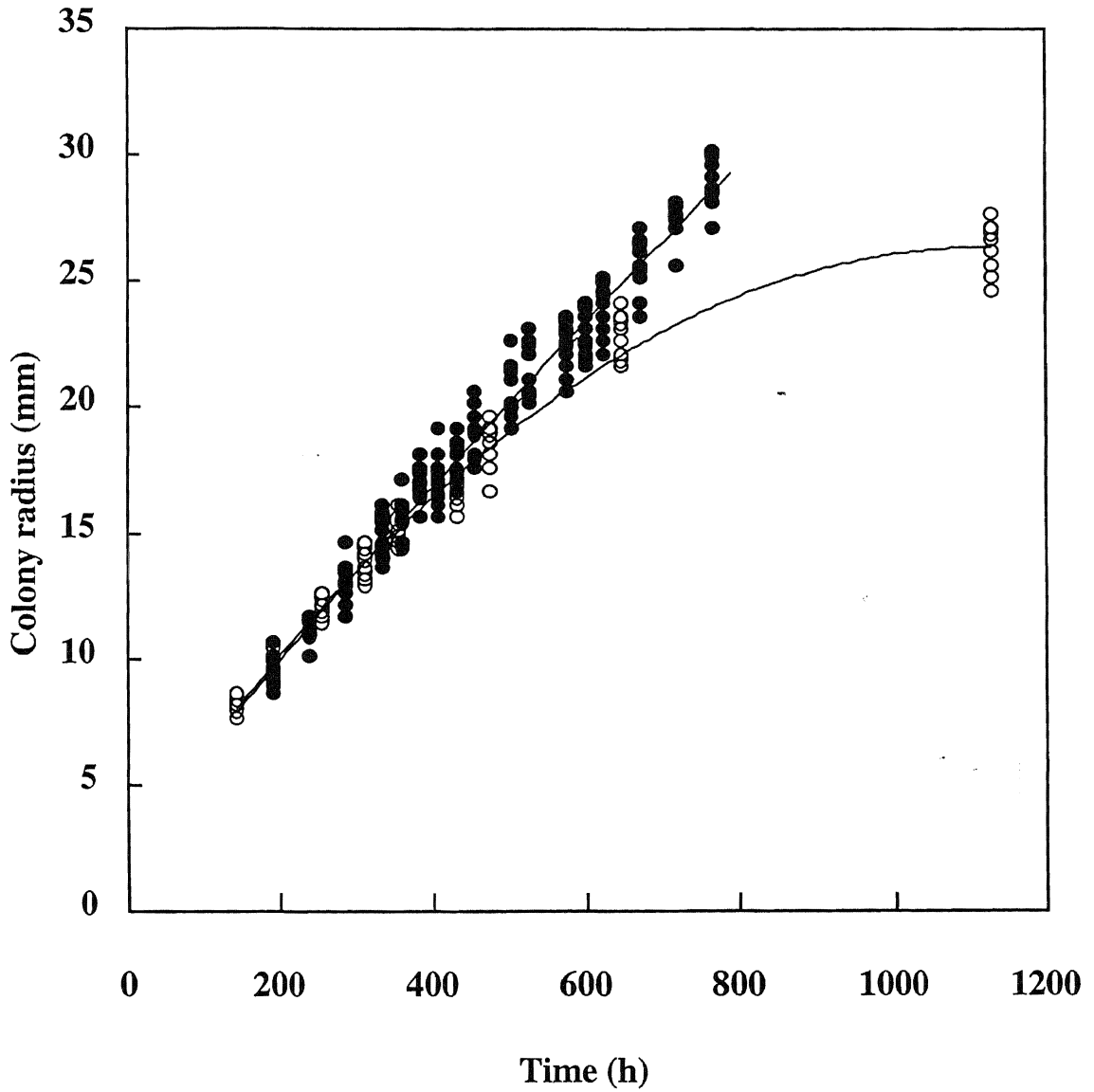
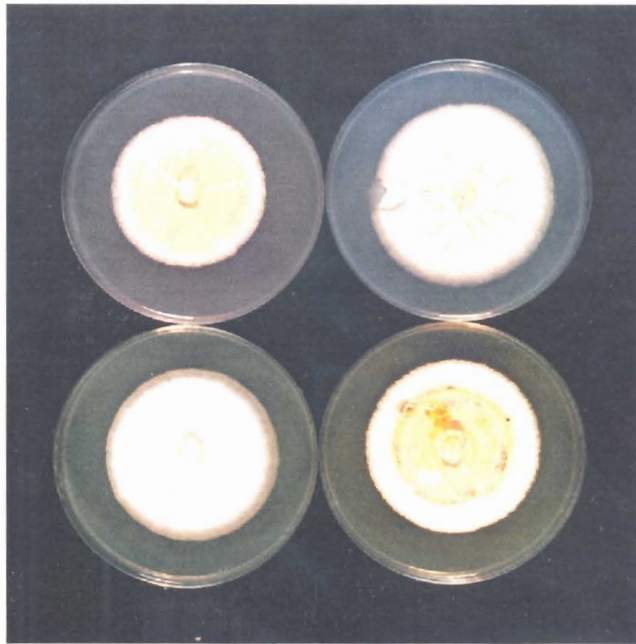


Figure 4.2 Colony radius of *V. chlamydosporium* (○) grown at 20°C on Vydade amended medium compared to that on unamended PDA (●).



a)



b)

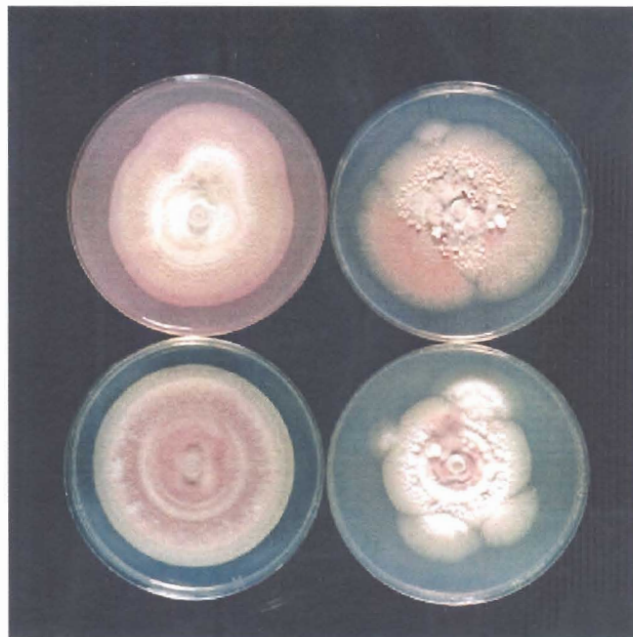
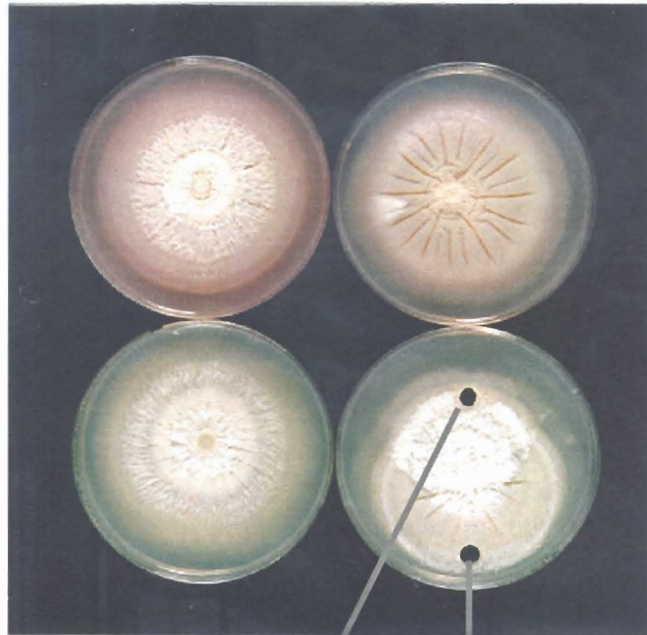


Figure 4.3 Effect of fungicides on the morphology of a) *Verticillium chlamydosporium* and b) *Paecilomyces lilacinus*. Fungicides were incorporated into the agar at the same rate at which they are applied to the soil. Treatments (from top left clockwise) were with Monceren, Gambit, Rizolex and control (no fungicide).

a)



b)



Figure 4.4 Effect of fungicides on the morphology of *Plectosphaerella cucumerina*. Fungicides were incorporated into the agar at the same rate at which they are applied to the soil. Treatments (from top left clockwise) were with Monceren, Gambit, Rizolex and control (no fungicide) (a). Sectors produced by *P. cucumerina* when grown on Rizolex, were subcultured on to new agar plates (b).

4.3 INTERACTIONS BETWEEN NEMATOPHAGOUS FUNGI, PLANT PATHOGENIC FUNGI AND SOIL SAPROPHYTIC FUNGI *IN VITRO*

4.3.1 INTRODUCTION

The interactions of the nematophagous fungi studied with other species of fungi are of relevance to their possible application as biological control agents for two reasons. Firstly, they may be applied in combination, and therefore need to be compatible with one another. Secondly, the effectiveness of a facultative parasite of PCN as a control agent, such as the fungi studied here, will depend partly upon its persistence in the soil microbial community, which in turn will depend on its ability to compete with other soil fungi.

The interaction between the nematophagous fungi and four soil saprophytic fungi was investigated. Common soil saprophytic fungi such as *Fusarium oxysporum*, *Penicillium bilaii*, *Chaetomium globosum* and *Trichoderma harzianum* could all potentially reduce the ability of the nematophagous fungi to infect the nematodes through competition and antagonism. *Penicillium bilaii* is commonly found in agricultural soils and has been produced commercially as a seed coating for wheat and other crops to increase phosphorus uptake (Goos *et al.*, 1994). *Chaetomium globosum*, also a soil saprophyte, is able to decompose straw (Halley *et al.*, 1996). *Trichoderma harzianum* is one of a number of *Trichoderma* species that have been found to be suitable for use as biological control agents of fungal pathogens. Isolates of *T. harzianum* have been used against *R. solani* (Thornton & Gilligan,

1999), and against *Phytophthora capsici* on pepper plants (Ahmed *et al.*, 1999). Strains of *Fusarium oxysporum* are classified as pathogenic or non-pathogenic to plants. *Fusarium oxysporum* has been isolated from a number of plant parasitic nematodes including *Heterodera schachtii*, *Meloidogyne hapla* and both *Globodera* spp. (Yu & Coosemans, 1998; Crump, 1989).

Reduced growth rates of the nematophagous fungi caused by the fungicides (section 4.2.2), led to the investigation into the interactions between the nematophagous fungi and *R. solani*. If the nematophagous fungi are able to reduce or inhibit the growth of *R. solani*, then fungicides could be applied at a reduced rate which would favour the survival of the nematophagous fungi in the field.

Studying interactions between fungi *in vitro* on agar plates is a simple method. Ahmed *et al.* (1999) used this method to evaluate *Trichoderma harzianum* for the control of *Phytophthora capsici* in pepper plants, and Siwek *et al.* (1997) used the same method to observe mycoparasitism of *Pythium ultimum* by binucleate *Rhizoctonia* isolates. Interactions within microfungal communities have also been observed using this protocol (Stahl & Christensen, 1992).

4.3.2 MATERIALS AND METHODS

4.3.2.1 A standard method for studying interactions between fungi *in vivo*

A modification of the method of Fokkema (1973), described by Robinson *et al.* (1993a), was used for studying the interactions between fungi (Figure 4.5). Plugs (8 mm diameter) taken from the edge of the colony of the opposing species, were cut using a flame-sterilised core borer from plate cultures growing on PDA and placed 20 mm apart (measured from the edges of the plugs) on a fresh PDA plate. Eight plates were inoculated for each pairing; four replicates were incubated at 10°C and four at 20°C. Two measurements of colony radius were made from the centre of each colony, one directly away from the challenging colony (R_1), and the other towards the challenging colony (R_2) (Figure 4.5). A minimum of 8 measurements of R_1 and R_2 were made at regular intervals until the two colonies met, usually after about 10 d at 10°C and 3 d at 20°C. The degree of inhibition of colony radial growth was calculated using the equation:

$$\% \text{ inhibition} = 100 \times (R_1 - R_2)/R_1$$

Positive results indicate that radial growth of the colony is inhibited in the presence of the challenging colony; negative results indicate that radial growth is increased (Table 4.1).

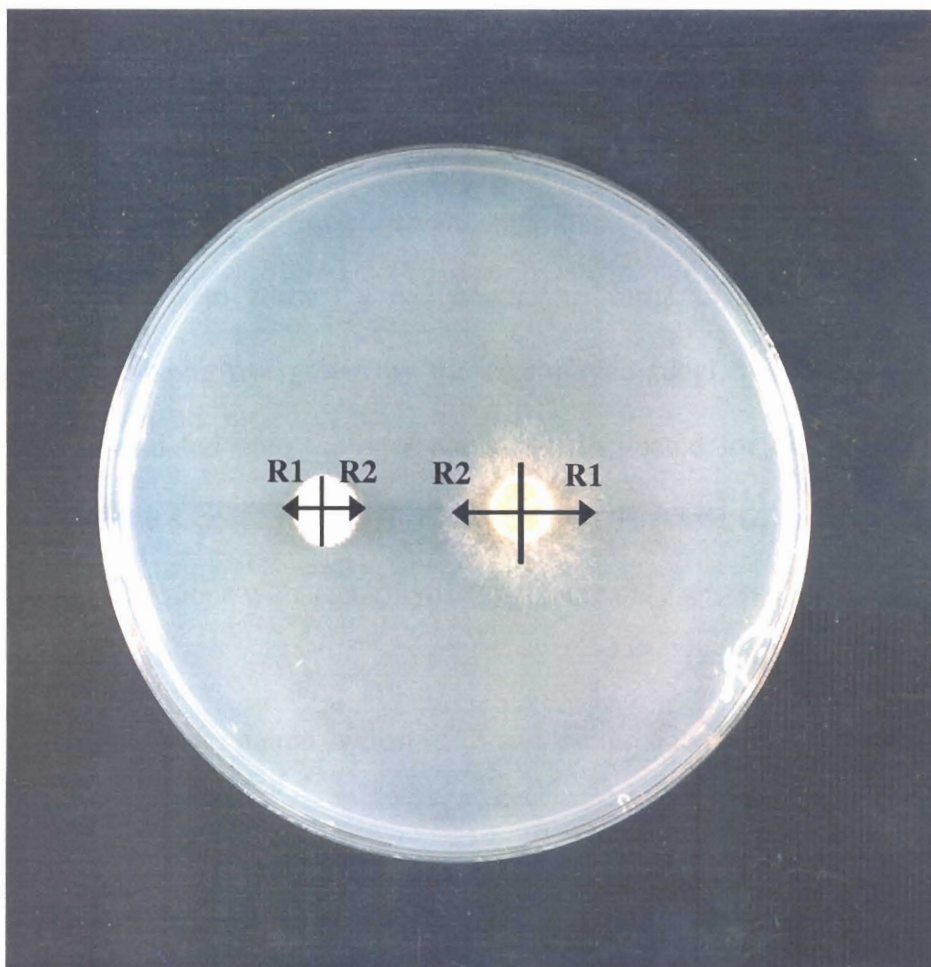


Figure 4.5 Standard method for measuring interactions between two opposing fungi. Two measurements of colony radius were made from the centre of each colony: directly away from the challenging colony (R_1), and towards the challenging colony (R_2). Percentage inhibition was calculated as $(R_1 - R_2) / R_1 * 100$. (Fokkema, 1973).

The three nematophagous fungi, *P. cucumerina*, *P. lilacinus* and *V. chlamydosporium*, were paired against themselves and against each other, interactions were recorded as above (section 4.2.3.1).

In a separate experiment, the nematophagous fungi were grown paired against four saprophytic fungi. From visual observations, it was evident that the saprophytic fungi had a faster growth rate than the nematophagous fungi. Therefore, in order to allow the nematophagous fungi to grow a measurable distance before being overgrown by the saprophytic fungi, the nematophagous fungi were inoculated onto the agar plates and incubated for one week before inoculation of the saprophytic fungi, *F. oxysporum*, *P. bilaii*, *C. globosum* and *T. harzianum*. Interactions with saprophytic fungi were only observed at 20°C.

Rhizoctonia solani was paired against itself and the three nematophagous fungi.

4.3.2.2 Hyphal interactions between nematophagous fungi and *Rhizoctonia solani*

Sterilised microscope slides were coated thinly with water agar. Fungal plugs (4 mm discs) were cut from cultures of *R. solani* and placed at one end of the slide opposite either *V. chlamydosporium*, *P. cucumerina* or *P. lilacinus*. Slides were placed in 90 mm diameter plastic Petri dishes sealed with Parafilm to prevent drying out and incubated at 20°C until the two colonies met. Hyphal interactions

were observed using light microscopy and Nomarski light interference microscopy at x 400 magnification under a Olympus BH2 microscope.

Visual observations of pairwise interactions of nematophagous fungi were made at 7 d and against *R. solani* at 13 d, to obtain more information about the nature of the interactions which took place once the two opposing colonies had met.

4.3.3 RESULTS

4.3.3.1 Interaction between nematophagous fungi

Colony radial growth of *P. lilacinus* was significantly inhibited by both *P. cucumerina* (by 15.5%) and *V. chlamydosporium* (by 31.4%) at 20°C, but was not inhibited by either species at 10°C. Indeed, colony radial growth of *P. lilacinus* was increased by 18% in the presence of *V. chlamydosporium*, although this increase was not statistically significant ($P < 0.05$, t-test) (Table 4.1). Colony radial growth of *P. cucumerina* was reduced in the presence of both *P. lilacinus* and *V. chlamydosporium* at both 10°C and 20°C, by between 16 and 45%; these effects were all statistically significant ($P < 0.05$, t-test), except for that of *P. lilacinus* at 10°C (Table 4.1). In addition to spreading more slowly, colonies of *P. cucumerina* produced copious aerial hyphae when paired against *V. chlamydosporium*. Radial growth of *V. chlamydosporium* at 20°C was inhibited in the presence of *P. lilacinus*, by 28.8% (although this difference was not significant), but was unaffected by *P. cucumerina*. Radial growth of *V. chlamydosporium* at 10°C was negligible.

‘Responders’								
‘Challengers’	<i>R. solani</i>		<i>P. cucumerina</i>		<i>V. chlamydosporium</i>		<i>P. lilacinus</i>	
	10°C	20°C	10°C	20°C	10°C	20°C	10°C	20°C
<i>R. solani</i>	0±11.1	19.8±5.5*	12.5±12.5	-14.6±16.8	NG	0±0	41.7±4.8*	-1.7±13.4
<i>P. cucumerina</i>	20.8±12.6	2.1±8.6	-10.4±4.3*	4.6±4	NG	-5±6.7	-8.8±24	15.5±1.3*
<i>V. chlamydosporium</i>	20.8±12.6	51.1±1.8*	20.7±8.5*	44.9±4.7*	NG	8.3±2.3*	-18.3±8.2	31.4±1.4*
<i>P. lilacinus</i>	39.4±2.2*	60.9±1.6*	25.2±12.1	16.2±1.9*	NG	28.8±9.7	-2.77±6.5	10.6±3.2*

Table 4.1 Inhibition of colony radial growth of *R. solani* and three species of nematophagous fungi when paired against themselves and each other. Percentage inhibition was calculated as $(R_1-R_2)/R_1*100$. Results are given \pm standard errors of the means. N=4. * indicates percentage inhibition significantly different from zero ($P<0.05$ t-test). N = negligible growth.

Colony radial growth of all the species studied except *P. cucumerina* was significantly inhibited ($P < 0.05$, t-test) when paired against themselves at 20°C. The only statistically significant effect on radial growth when the four species were paired against themselves at 10°C was a 10% increase for *P. cucumerina*.

It was evident from visual observation of a change in colony morphology compared to the control (Figure 4.6a) that, after only 24 h incubation, radial growth of *R. solani* was inhibited in the presence of *P. lilacinus* and *V. chlamydosporium* (Figure 4.6d & c). At this time the edges of the colonies of *P. lilacinus* and *V. chlamydosporium* were still 13 mm and 11 mm away from the *R. solani* colony respectively (Figure 4.6). Inhibition between the nematophagous fungi was evident after 120 h for *P. cucumerina* paired against *V. chlamydosporium*, and *P. lilacinus* paired against *V. chlamydosporium*. When the colonies were paired against themselves, inhibition occurred after 200 h.

Radial growth of *R. solani* at 20°C was significantly inhibited in the presence of *P. lilacinus* and *V. chlamydosporium*, by 61% and 51% respectively, but was not inhibited by *P. cucumerina* (Figure 4.6). At 10°C, only *P. lilacinus* significantly reduced colony radial growth of *R. solani*, by 39%; however, at this temperature *R. solani* significantly inhibited radial growth of *P. lilacinus* in return, by 42%. The presence of *R. solani* did not significantly affect the K_r of *P. cucumerina* at 10°C, nor that of any of the three species of nematophagous fungi at 20°C (Table 4.1). However, *R. solani* did eventually overgrow and replace challenging colonies of *P. cucumerina* at 20°C.

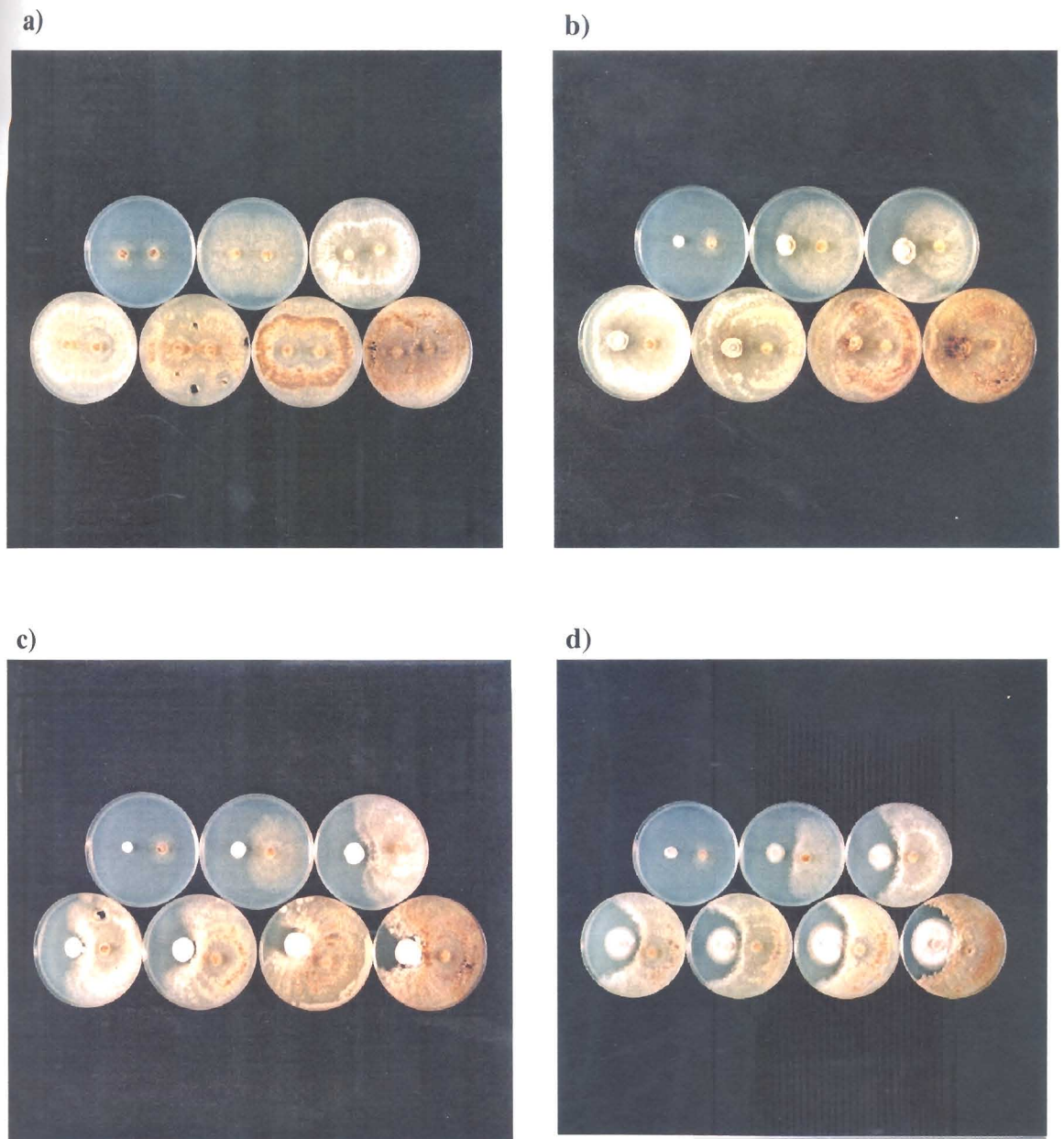


Figure 4.6 Antagonism between nematophagous fungi and *R. solani* on nutrient medium at 20°C over a 13 day period. a) *R. solani* against *R. solani*. b) *P. cucumerina*. (left) against *R. solani* (right), c) *V. chlamydosporium* (left) against *R. solani* (right). d) *P. lilacinus* (left) against *R. solani* (right). The first plate (top row left) shows fungal growth after 24 h, subsequent plates show fungal growth every 48 h from left to right.

Colony radial growth of *P. cucumerina*, *P. lilacinus* and *V. chlamydosporium* was significantly inhibited in the presence of each of the species of saprophytic fungi tested, except for the pairings of *P. lilacinus* against *T. harzianum* and *V. chlamydosporium* against *P. bilaii* (Table 4.2). Growth of *F. oxysporum*, *C. globosum* and *P. bilaii* was significantly inhibited in the presence of each of the three nematophagous fungi, but growth of *T. harzianum* was only significantly inhibited by *V. chlamydosporium*. *Verticillium chlamydosporium* was most effective in inhibiting growth of the saprophytic fungi, and was also least susceptible to being inhibited by them.

4.3.3.2 Hyphal interactions

When *R. solani* was grown in the presence of *P. lilacinus* on an agar coated microscope slide, the mycelium of *R. solani* appeared to be damaged. Abnormal swelling was observed in the penultimate compartment (Figure 4.7). This was only observed in the minority of hyphae.

Coiling of hyphae of *V. chlamydosporium* around *R. solani* hyphae was observed when these fungi were grown together on agar coated slides (Figure 4.8). Approximately, a quarter of *R. solani* hyphae were hyperparasitised by *V. chlamydosporium* in the form of coiling.

No change to the morphology of hyphae of *P. cucumerina* or *R. solani* was observed when these species were paired against one another.

‘Responders’							
‘Challengers’	<i>P. cucumerina</i>	<i>V. chlamydosporium</i>	<i>P. lilacinus</i>	<i>F. oxysporum</i>	<i>C. globosum</i>	<i>P. bilaii</i>	<i>T. harzianum</i>
<i>F. oxysporum</i>	13.5±1.8*	5.9±1.9*	10.8±2.7*	ND	ND	ND	ND
<i>C. globosum</i>	16.6±2.1*	9.6±1.7*	19.6±2.7*	ND	ND	ND	ND
<i>P. bilaii</i>	20.9±1.2*	4.7±3	18.3±2*	ND	ND	ND	ND
<i>T. harzianum</i>	9.4±1.3*	11.5±2.9*	2.9±7.1	ND	ND	ND	ND
<i>P. cucumerina</i>	ND	ND	ND	24.9±2*	24.4±2.1*	29±4.8*	-0.1±7.8
<i>V. chlamydosporium</i>	ND	ND	ND	39.9±1.6*	31.9±2.7*	57±2.4*	27.1±3.1*
<i>P. lilacinus</i>	ND	ND	ND	21.7±3*	24.5±2*	34.5±0.7*	8.8±10.4

Table 4.2 Inhibition of colony radial growth of the saprophytic fungi *F. oxysporum*, *P. bilaii*, *C. globosum* and *T. harzianum* and three species of nematophagous fungi when paired against each other. Percentage inhibition was calculated as $(R_1 - R_2)/R_1 * 100$. Results are given \pm standard errors of the means. N=4. * indicates percentage inhibition significantly different from zero ($P < 0.05$ t-test). ND = not determined.

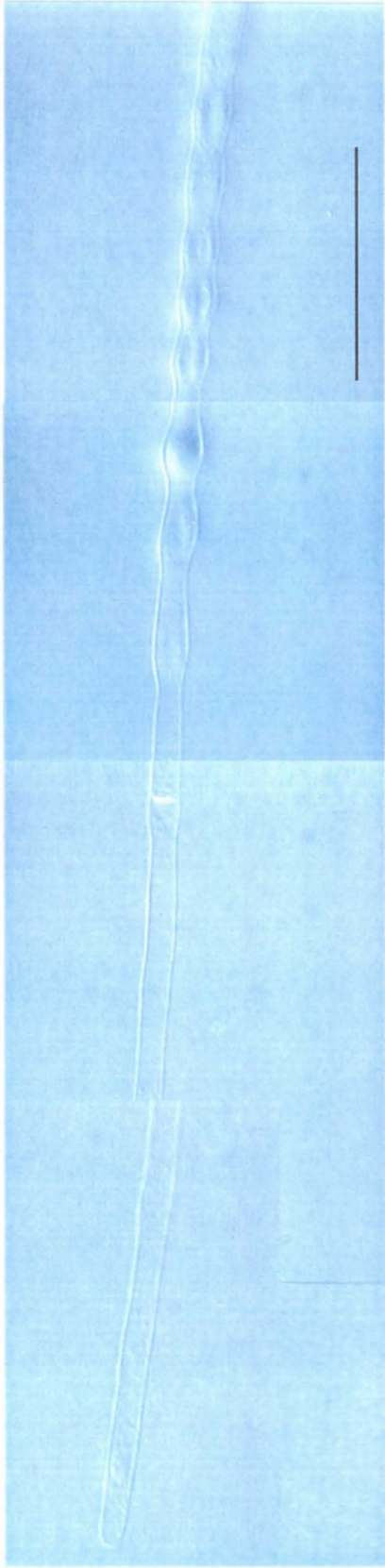


Figure 4.7 *Rhizoctonia solani* mycelium showing swelling when grown in the presence of *Paecilomyces lilacinus* on agar.
Photograph taken using Nomarski differential interference microscopy. Bar = 50 μ m.

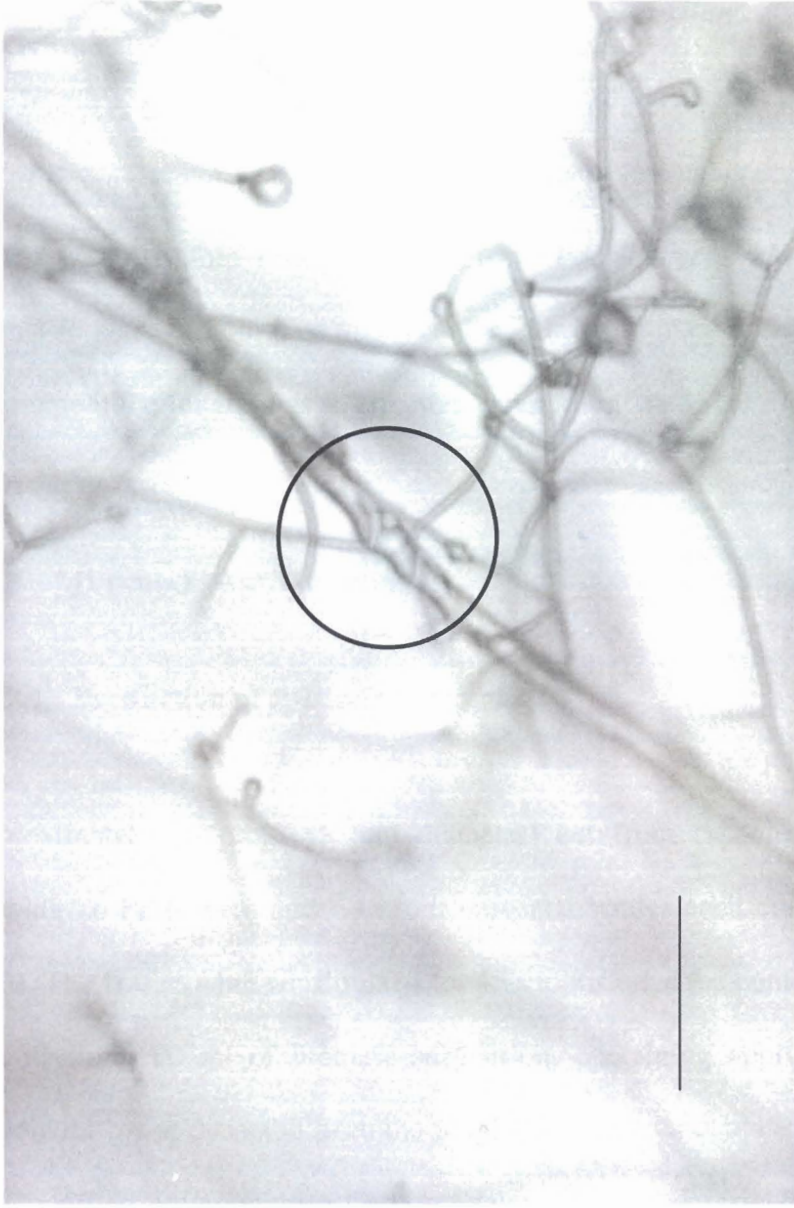


Figure 4.8 *Verticillium chlamydosporium* hyphae observed coiling around a *Rhizoctonia solani* hypha. Photograph taken using light microscopy. Bar = 100 μm .

4.4 BIOASSAY OF THE EFFECTIVENESS OF *P. LILACINUS* FOR THE CONTROL OF *RHIZOCTONIA* DISEASE

4.4.1 INTRODUCTION

Paecilomyces lilacinus showed the greatest potential as an antagonist towards *R. solani* *in vitro* compared to *P. cucumerina* and *V. chlamydosporium*. Its efficacy *in vivo* was therefore tested in a pot trial. *Paecilomyces lilacinus* was tested against *R. solani* as biomass and formulated in to alginate pellets (Chapter 5), to determine if the fungus was as effective when formulated.

4.4.2 MATERIALS AND METHODS

4.4.2.1 Production of *P. lilacinus* biomass

Approximately 40 plugs (5 mm diameter) cut from colonies of *P. lilacinus* growing on PDA were added to four universal bottles each containing 20 ml of MEB. The bottles were whirlmixed for 45 s to dislodge the conidia from the agar. The resulting 80 ml of biomass suspension, containing approximately 1×10^8 conidia ml^{-1} , was decanted from the plugs and used to inoculate 15 l of MEB in a 20 l modified polypropylene Carboy (Nalgene[®], UK) described in section 2.4.1. This fermenter was maintained at ambient temperature (18-22°C) and agitated and aerated by sparging with filter-sterilised air at 9 l min^{-1} . The culture was harvested after 7 d, when the culture was in late exponential phase (Figure 2.3).

4.4.2.2 Production of alginate pellets

Excess spent growth medium was removed from *P. lilacinus* liquid culture, section 4.4.2.1., by straining on a sterile 1 mm mesh sieve. This reduced the culture volume from 15 l to 10 l. Alginate pellets were produced (as in 5.2.2.3) except that 160 g l⁻¹ sodium alginate and 400 g l⁻¹ milled barley were used. The pellets were stored for one month at room temperature until needed.

4.4.2.3 Production of *Rhizoctonia solani* inoculum

Rhizoctonia solani inoculum was produced in the same way as *P. lilacinus* biomass in a 20 l fermenter. Biomass was harvested after 12 d, when visually the biomass was at its densest, and strained as above. The fungus was mixed with compost (similar to John Innes No. 3, as used at IACR-Rothamsted) (1 part fungus:10 parts compost) and stored at 10°C until needed.

4.4.2.4 Bioassay

Potato tubers (cv. Desiree disease free minitubers) were planted 5 cm from the bottom of 13 cm diameter pots (1 litre) filled with compost. Five treatments were used: no inoculum (disease free control); inoculation with *R. solani* only (diseased control); addition of 30 g uninoculated alginate pellets to uninoculated compost; addition of 30 g alginate pellets containing *P. lilacinus* together with *R. solani* inoculated compost; and the addition of 15 g *P. lilacinus* biomass together with

R. solani inoculated compost. Pots were soaked by standing in 10 cm depth of water for 2 h before being placed in the dark under polythene at 10°C. Once the potato shoots had appeared the polythene was removed, and a 30 cm long section of polyethylene pipe, 10 cm in diameter, was placed on top of the compost and filled with compost to the top to simulate earthing up of the potato plants. Plants were grown for 3 months and then assessed for disease.

4.4.2.5 Disease assessment

Stem canker disease was assessed using the method described by Lootsma & Scholte (1997). Their disease index (DI=0 to 100) was calculated to determine the severity of *Rhizoctonia* disease in each treatment group.

4.4.3 RESULTS

The incidence of stem canker in potato plants grown in soil inoculated with *R. solani* was reduced from a disease index of 48.5 to 28.8 when *P. lilacinus* biomass was added to the soil (Table 4.3). Treatment with *P. lilacinus* incorporated into alginate pellets significantly ($P < 0.05$, t-test) reduced the incidence of stem canker, to a disease index of 16.8, compared with the *R. solani* disease control. The control treatment, with no inoculum of either *R. solani* or *P. lilacinus*, resulted in a disease index of 28.2 (Table 4.3) and the plants developed reddish-brown to brown lesions characteristic of stem canker, indicating that the compost had been

contaminated with *R. solani*. Adding uninoculated alginate pellets to the control soil reduced the stem canker disease index from 28.2 to 12.

Table 4.3 Efficacy of various formulations of *P. lilacinus* for the control of potato stem canker caused by *R. solani*. * = significantly different to *R. solani* inoculated compost (P<0.05, t-test), N = 10.

Treatment	Disease index (0-100)
Disease free control (no inoculum)	28.2
Control alginate pellets in uninoculated compost	12
<i>R. solani</i> inoculated compost (disease control)	48.5
<i>R. solani</i> inoculated compost with <i>P. lilacinus</i> biomass	28.8
<i>R. solani</i> inoculated compost with <i>P. lilacinus</i> alginate pellets	16.8*

4.5 DISCUSSION

Sensitivity to commonly used chemical pesticides is likely to be undesirable in a biological control agent intended for use in IPM. On the basis of K_r , *P. lilacinus* and *P. cucumerina* did not appear to be sensitive to the fungicide Monceren, a protective fungicide, or the nematicide Vydate, but were sensitive to the fungicides Rizolex, which inhibits phospholipid biosynthesis, and Gambit, a long-lasting fungicide. Although K_r is not a direct measure of specific growth rate as it is also inversely proportional to branching frequency, it is unlikely that the K_r of a fungus sensitive to a pesticide would be unaffected by the incorporation of that pesticide in its growth medium. Thus, Monceren would be the fungicide of choice for use in IPM incorporating either of these fungi, which could be applied in combination with a chemical nematicide. The colony radial growth rate of *P. lilacinus* was increased by 3% when grown on Vydate amended media, possibly due to the Vydate acting as a paramorphogen, changing the spatial distribution of the organism's biomass but not its rate of production (Trinci, 1984). The nematicide, aldicarb, has also been found to increase the colony radii of *V. chlamydosporium* and another nematophagous fungus, *Cylindrocarpon destructans* (Crump & Kerry, 1986). Crump & Kerry (1986), detected that oxamyl had a fungicidal effect on *C. destructans* at all tested concentrations of the nematicide (1, 10 and 100 ppm) and for *V. chlamydosporium* at 100 ppm. Radial growth of *V. chlamydosporium* was inhibited by all the pesticides tested here (Figure 4.1). It could be argued that this makes *V. chlamydosporium* unsuitable for use as a biological control agent for PCN in the field. However, Wilding

(1982), found that fungicides known to be toxic to entomophthoraceous fungi in laboratory studies had little effect on fungal infection of aphid populations in the field, and none of the pesticides tested entirely prevented growth of *V. chlamydosporium*, so use of this fungus in an IPM programme may still be feasible.

Radial growth of *R. solani* was inhibited *in vitro* by *P. lilacinus* at both 10°C and 20°C. Twenty-four hours after inoculation of agar plates with *P. lilacinus* and *R. solani* plugs, it was evident from the speed with which an effect could be observed, and the large gap still remaining between the colonies, that some substance had diffused through the agar from the *P. lilacinus* colony to inhibit the growth of *R. solani*. *Paecilomyces lilacinus* produces an antibiotic, P-186, that has a wide anti-microbial activity (Siddiqui & Mahmood, 1994). This antibiotic, or a related substance, may have inhibited growth of *R. solani*. Although, as stated above, inhibition of colony radial growth may be due to increased branching frequency rather than reduced biomass generation, this could still result in control in the field situation. For example, Wakae and Matsuura (1975), found rice sheath blight could be controlled by the fungicide Validamycin A. This antifungal agent prevents the pathogen spreading to the upper parts of the plant, where it reduces crop yield. However, at 10°C *R. solani* in turn inhibited the growth of *P. lilacinus*. Chand & Logan (1981) found the incidence of disease on potato sprouts caused by *R. solani* was highest when the plants were grown at 13°C. *Paecilomyces lilacinus* grows more slowly at this temperature than at 20°C, therefore at the higher temperature *P. lilacinus* is either able to colonise the plate

faster than at 10°C or the metabolites used to inhibit *R. solani* are produced at a faster rate. In contrast, the inhibition of radial growth that occurred when species were paired against themselves was only observed when the colonies were in close proximity (<2 mm). This was consistent with competition for nutrients (Pirt, 1967).

Although it did not grow at 10°C, *V. chlamydosporium* apparently released a compound that inhibited the growth of both *R. solani* and *P. cucumerina* despite the fact that radial growth of *V. chlamydosporium* ceased at this temperature. *Verticillium chlamydosporium* produces several antifungal compounds one of which is monorden (syn. radicol). Monorden has been found to cause morphological abnormalities when fungi are grown in its presence (Leinhos & Buchenauer, 1992).

The four soil saprophytic fungi tested all suppressed the growth of the nematophagous fungi to varying degrees, except for *T. harzianum* against *P. lilacinus* and *P. bilaii* against *V. chlamydosporium*. This may be a survival strategy, in which the slow-growing fungi inhibit the growth of faster growing fungi in order to compete for nutrients. *Trichoderma harzianum*, *C. globosum* and *F. oxysporum* all had faster growth rates than the nematophagous fungi, therefore the nematophagous fungi were unable to colonise the agar. To determine whether the nematophagous fungi are able to inhibit the growth of the saprophytic fungi, the nematophagous fungi were allowed to grow on the agar before the saprophytes were added. This allowed the nematophagous fungi to release antifungal

compounds into the agar before the saprophytic fungi had colonised the plate (Table 4.2). *Penicillium bilaii* had a different competitive strategy to the other saprophytic fungi. *Penicillium bilaii* grew more slowly than the other saprophytic fungi but produced compounds which inhibited the growth of all the nematophagous fungi. In the rhizosphere the nematophagous fungi have adapted to parasitise nematodes, therefore they may not be in complete competition with saprophytic fungi. However, isolates of *F. oxysporum* have been found to colonise PCN (Yu & Coosemans, 1998; Crump, 1989).

Paecilomyces lilacinus grew at both 10°C and 20°C, and its radial growth was inhibited in half of the pairings made, listed in Table 4.1. It inhibited the radial growth of all fungi tested, possibly through production of an antibiotic substance, and it can therefore be considered an efficient competitor and likely to persist in soil (Pierson & Pierson, 1996). Of the fungi tested, *V. chlamydosporium* was least susceptible to inhibition by other species and showed some evidence of production of antimicrobial substances which would facilitate competition with other soil fungi. However, it did not grow at 10°C, which would be a disadvantage for a control agent for early season use in mainland UK but might imply a role as a late season control agent. For example, in the Channel Island of Jersey, potatoes are grown early on in the year while soil temperatures are around 10°C, but harvesting of the main crop continues until July when the average soil temperature is 15°C and can rise to 21°C during the day (Figure 6.9). At harvest the PCN females are still developing on the roots which are left in the ground. This would be an opportune time to apply *V. chlamydosporium*.

Plectosphaerella cucumerina was the poorest competitor of the nematophagous fungi tested, being inhibited by both *V. chlamydosporium* and *P. lilacinus* at both 10°C and 20°C. As there was a degree of inhibition between the three nematophagous fungi, application of a combination of fungal species for control of PCN may be less successful than either application of a single species, or successive applications of individual species – for example, *P. lilacinus* for early season control followed by *V. chlamydosporium* for late season control.

In vitro tests on agar are a useful tool to demonstrate interactions between fungi. Whipps (1987) tested a range of media to show the effects on growth and interactions between pathogens and antagonistic fungi. It was found that the choice of medium had a significant effect on the growth rates, production of and the response to volatile and non volatile antibiotic compounds. Fungi grown on a low nutrient agar, for example tap water agar or soil extract agar, may demonstrate more realistic interactions than when grown on a high nutrient agar, where the high concentrations of available nutrients greatly exceed those present in most soils. A single *in vitro* screening may not conclusively establish the nature of all interactions likely to take place in the soil, but will give some indication of any mycotoxins produced. Different fungal strains may also influence the interactions, for example Chand & Logan (1981) found that variations in pathogenicity of *R. solani* were linked to cultural morphological characteristics.

Hyphal interactions between the nematophagous fungi and *R. solani* demonstrated that *P. lilacinus* produces a compound that causes hyphae of *R. solani* to lyse and

form swellings along the compartment before the septa (Figure 4.7). *Verticillium chlamydosporium* also showed antagonistic behaviour to *R. solani* by coiling around the host (Figure 4.8). Coiling can precede penetration of the antagonist penetrating directly into the host causing slow death of the host hyphae in some fungi. Turhan (1990) also observed parasitic development of *V. chlamydosporium* on the host *R. solani*.

The experiments outlined in this chapter have shown that *P. lilacinus* and *V. chlamydosporium* are suitable for use as a biological control agent due to their ability to compete with other fungi and grow in the presence of agrochemicals. *Plectosphaerella cucumerina*, however, may not be as suitable due to its poor saprophytic abilities.

5 FORMULATIONS OF NEMATOPHAGOUS FUNGI

5.1 INTRODUCTION

Formulation of microbial biological control agents is needed to maintain viability and infectivity during storage of the organisms concerned, to aid distribution of the agent and therefore to maximise the contact with the target pest. Also, the formulation should protect the agent from adverse physical conditions and extend the period of time for which it is effective. Formulations and delivery systems for biological control agents have always been problematic. Lack of commercially acceptable application techniques has limited the introduction of fungal biological control agents into commercial agriculture (Schuster & Sikora, 1992a). For a biological control agent to be suitable for commercialisation it must be able to be produced on a large scale, remain viable for long periods of time, be easily stored and transportable; and must be able to survive and infect the target organism in its augmented form. Therefore, the success of a fungal biological control agent is dependent on the formulation.

There are a number of different formulations and application methods for nematophagous fungi, including adding the fungi to liquid as a soil drench (Perveen & Ghaffar, 1998) and dipping the potato tubers in a liquid application. Surfactants used for tuber dressings may reduce spore attachment and spore

viability, and liquid formulations can cause conidia to germinate prematurely (Van Driesche & Bellows, 1996).

Nematophagous fungi have also been incorporated into organic matter such as leaf extracts (Siddiqui & Mahmood, 1994), animal manures (Abu-Laban & Saleh, 1992), water lilies, grains of wheat, rice and oats. For more commercial purposes, where large quantities of the agents are required, the fungi have been formulated in vermiculite and clay such as Pyrax[®] (pyrophyllite, hydrous aluminum silicate) (R. T. Vanderbilt Co., Norwalk, CT, USA) (Fravel, 1985).

Alginate has been widely used as a carrier for microbes used in biological control. Production methods for alginate pellets have been investigated including aspects such as nutrient modifications of the pellet content (Schuster & Sikora, 1992b), the effect of sterilisation and pH (Daigle & Cotty, 1997) and the effect pellet size has on distribution of the fungi (Shah *et al.*, 1999). Alginate pellets can be uniformly distributed throughout the soil, increasing the chances of contact between nematode and fungus. Nutrient sources such as milled barley can be added to alginate granules to increase the sporulation and period of time the pellet can support the fungus. Connick *et al.* (1990) found that alginate pellets could support fungal growth and sporulation for up to 6 weeks after application to the soil.

The aim of the work outlined in this thesis was to develop a product that could ultimately be used in the field and, therefore, it was necessary to find a

formulation for the nematophagous fungi that would satisfy field application requirements. Much of the previous work in this area has concentrated on laboratory preparations to enable research to be conducted on the efficacy and relationships between the target organism and the biological control agent (Lewis & Papavizas, 1985; Daigle & Cotty, 1997; Fravel *et al.*, 1985)).

5.2 EXPERIMENT COMPARING DIFFERENT FORMULATIONS OF NEMATOPHAGOUS FUNGI

5.2.1 INTRODUCTION

Three formulation methods were tested to determine which was the most effective for application of the nematophagous fungi *P. cucumerina* and *P. lilacinus* for control of PCN. These were: incorporating the fungi into alginate pellets; adding Terra-Green[®] which had been inoculated with fungus underneath the tubers; and coating the tubers with a spore suspension. Alginate pellets and Terra-Green[®] were selected as they have been shown to be effective formulations in which to apply fungi (Shah *et al.*, 2000; Crump 1998, pers. com.). In Jersey, the tubers are handled individually and dipping them is a practical alternative to field application, which may have an additional benefit by preventing other pathogens from attacking the tubers.

Verticillium chlamyosporium was not put into a formulation as chlamyospores can be applied in water as a soil drench (Kerry *et al.*, 1993).

5.2.2 MATERIALS AND METHODS

5.2.2.1 Production of fungal conidia for application around the tuber

To enable pure samples of fungal conidia free from any growth medium, fungal colonies were grown on cellophane. Circles of cellophane (80 mm in diameter) were deplasticised by boiling in water, then sandwiched between sheets of filter paper in a glass Petri dish so they could easily be separated. The sheets of cellophane were autoclaved at 121°C for 20 min and, once cooled, placed individually on to the surface of MPM agar using sterile forceps. Plugs (5 mm in diameter) of either *P. lilacinus* or *P. cucumerina*, taken from the edge of a growing colony, were inoculated on to four cellophane-covered plates per species, and incubated at 20°C. After 15 d growth, the cellophane was removed from the agar and the biomass was scraped off the cellophane using a Borrodaile needle into 1 l of water. The concentration of conidia, measured in a haemocytometer, was $4.5 \times 10^9 \text{ l}^{-1}$ for *P. cucumerina* and $2.5 \times 10^{10} \text{ l}^{-1}$ for *P. lilacinus*. The tubers were dipped into the spore suspension for two minutes before being planted.

5.2.2.2 Production of inoculated Terra-Green®

A mixture of 800 g of Terra-Green® and 500 ml of 2% malt extract solution (section 2.2) was autoclaved in a bag at 121°C for 20 min and cooled. Seven plugs (5 mm in diameter) of either *P. lilacinus* or *P. cucumerina* taken from the edge of a colony were added to the Terra-Green® and incubated at 20°C for 13 d. During

the incubation period, the bags containing the Terra-Green[®] were shaken daily. The control did not have any fungus added. Post incubation of the inoculum, 10 g of the inoculated Terra-Green[®] was applied under each tuber at planting.

5.2.2.3 Production of alginate pellets

Conidia from *P. lilacinus* and *P. cucumerina*, grown on MPM, were scraped using a Borrodaile needle into separate vials of 1 ml MPM liquid culture and whirlimixed for 45 s. The spore suspension was added to two conical flasks each containing 250 ml MPM liquid media and shaken at 100 rpm, 20 mm throw for 7 d at ambient temperature. The liquid culture was homogenised in a sterilised blender before it was added to the alginate pellets.

Sodium alginate (20 g) was dissolved in 500 ml water at 40°C on a hot plate. Milled barley (50 g, passed through a 4 mm aperture sieve) was mixed with 250 ml of water, homogenised in a sterilised blender, autoclaved at 121°C for 20 min and cooled. The liquid culture, milled barley and alginate were homogenised in a Waring blender (model 38BL45, Waring, New Hartford, Connecticut, USA) at 20,000 rpm until smooth. The alginate mixture was then processed through a device to produce the pellets (Figure 5.1). A hopper containing the mixture was connected by silicone tubing to a polyurethane cylindrical chamber (4 cm diameter x 3 cm depth). A peristaltic pump was used to force the mixture into the chamber. Four, 1 ml polyurethane pipette tips were mounted in a cork sealing the base of the chamber. The mixture was forced

through the pipette tips, producing drops which were deposited into a beaker containing 0.25M calcium chloride (Fravel *et al.*, 1985). The drops formed gel like pellets on contact with the calcium chloride solution (Rodriguez-Kábana *et al.*, 1994) (Figure 5.2). The pellets were left in the solution for 20 min then recovered on a sieve, washed in water and allowed to dry on tissue paper in the laminar flow cabinet for 24 h. The control alginate pellets contained no fungus. Pellets were added to the soil at 1% (w/w).

5.2.2.4 Glasshouse experiment to test the efficacy of the different formulation methods

Hemispherical potato chits cv. Desiree were planted in Kettering loam in 10 cm pots (500 ml) following either addition of alginate pellets or Terra-Green[®], or coating of the tuber with conidia of *P. lilacinus* or *P. cucumerina* prepared as above. Controls consisted of: alginate pellets with no fungus; Terra-Green[®] with no fungus; and untreated tubers respectively. Fifty PCN cysts (*G. rostochiensis*) were added around the chit, and Osmacote fertiliser (2 g l⁻¹ soil) was sprinkled on the surface of the soil. Treatments were allocated into randomised blocks in the glasshouse and each treatment was replicated seven times.

5.2.2.5 Egg counts

After growth, maturation and natural senescence of the potato plants had occurred, (approximately 3 months) the PCN cysts were harvested and the concentration of PCN eggs calculated, using the method described in section 2.5.

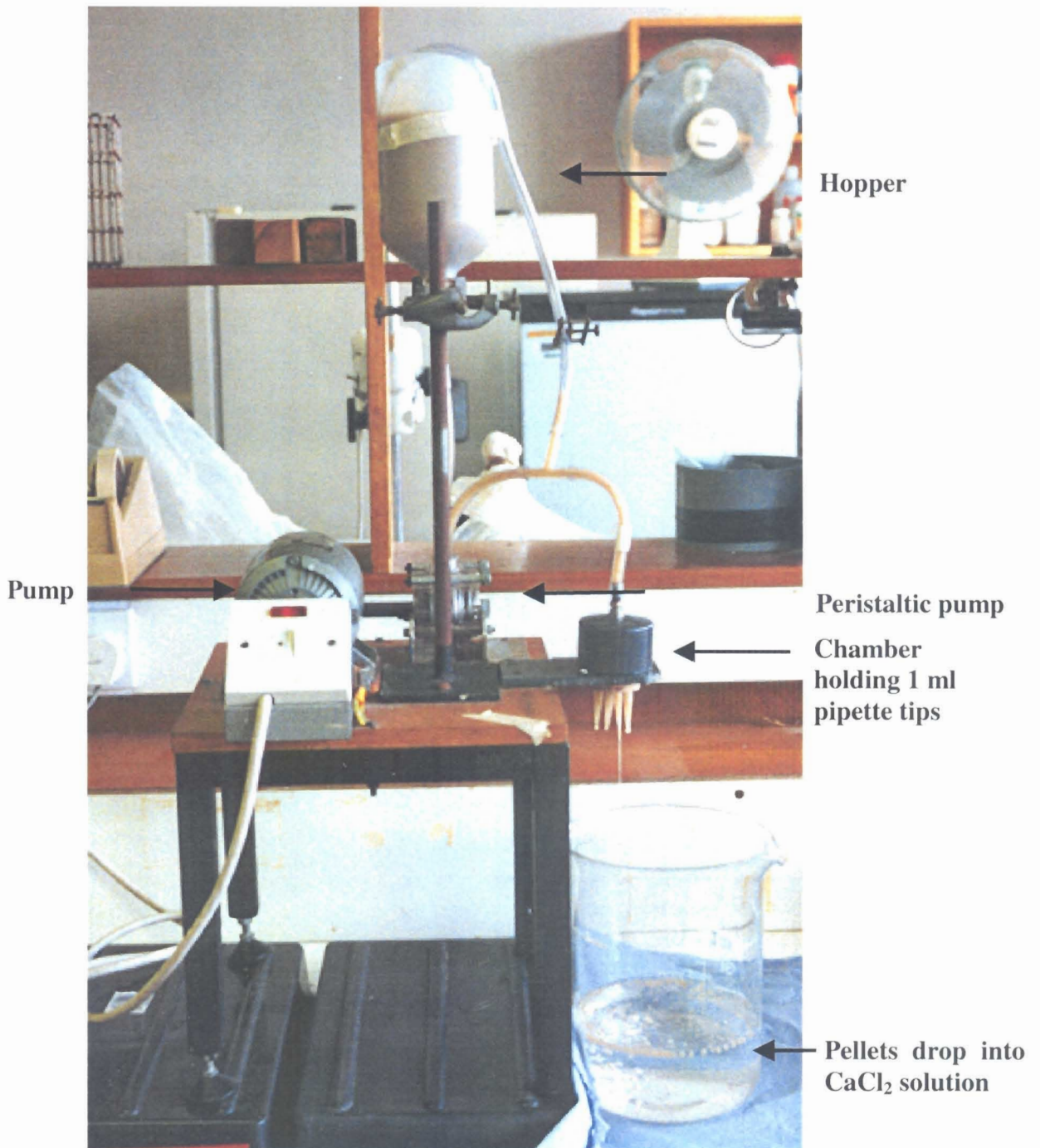


Figure 5.1 Equipment used for the production of alginate pellets.

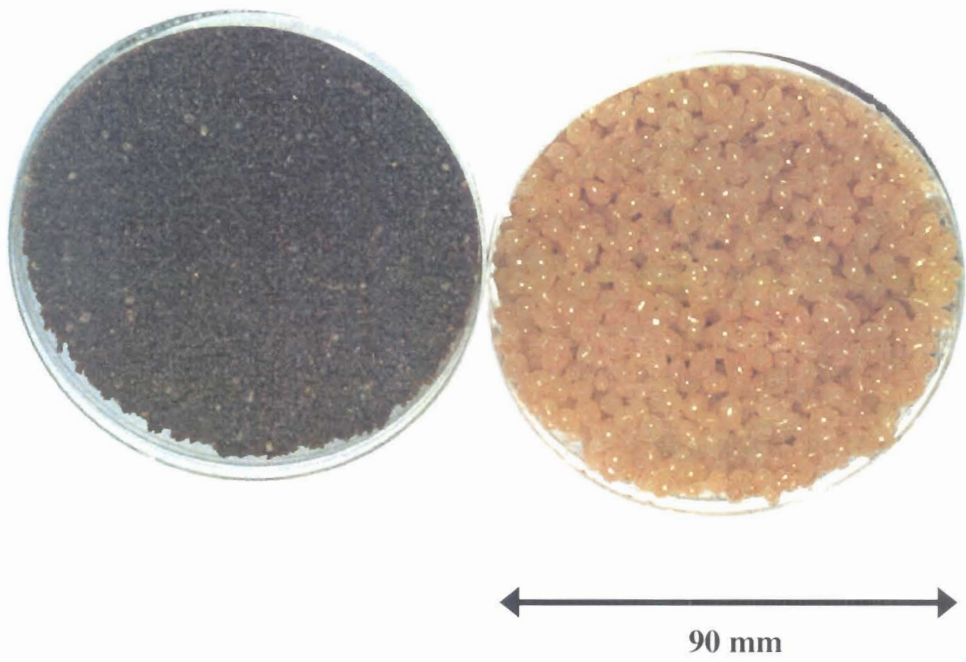
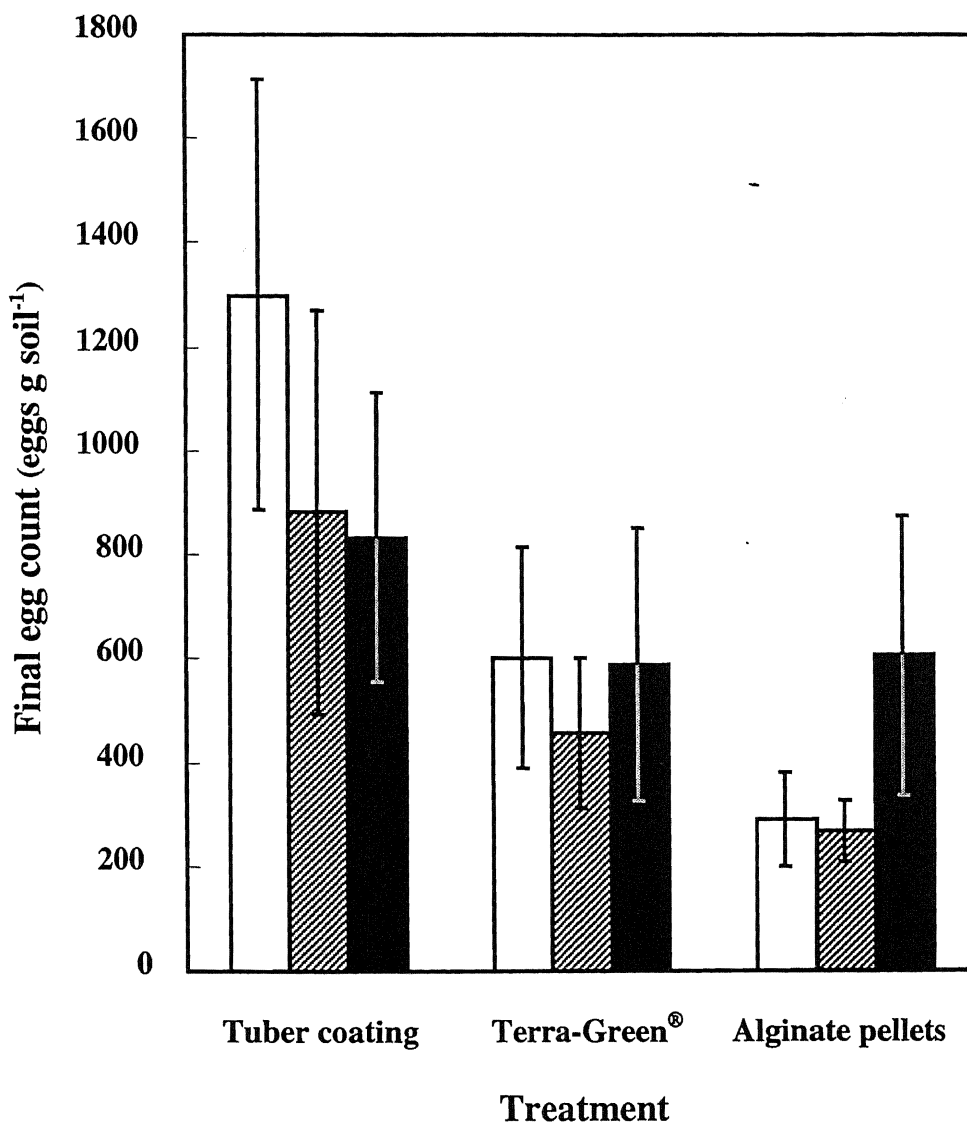


Figure 5.2 Dried alginate pellets, approximately 3 mm in diameter (left) and freshly produced alginate pellets (right).

5.2.3 RESULTS

When no fungus or carrier was added to the soil (tuber coated control), the concentration of viable PCN eggs after harvesting was 1300 eggs g soil⁻¹ (Figure 5.3). Tuber applications of *P. lilacinus* or *P. cucumerina* conidia resulted in final concentrations of PCN eggs of 880 and 884 eggs g soil⁻¹, respectively. The control Terra-Green[®] treatment and control alginate pellets gave final concentrations of 601 and 290 eggs g soil⁻¹, respectively. Treatment with Terra-Green[®] inoculated with *P. lilacinus* and *P. cucumerina* resulted in final PCN egg concentrations of 457 and 587 eggs g soil⁻¹ respectively. Alginate pellets inoculated with *P. lilacinus* reduced the final PCN egg counts to 266 eggs g soil⁻¹. This was the most effective of the treatments tested, significantly reducing the final PCN egg numbers ($P < 0.05$, ANOVA) compared to the tuber coated control by 79.5%. *Plectosphaerella cucumerina* incorporated into alginate pellets gave less nematode egg control (604 eggs g soil⁻¹) than alginate pellets alone (290 eggs g soil⁻¹).

Figure 5.3 Comparison of biological control formulations, incorporating the nematophagous fungi *P. cucumerina* (■) and *P. lilacinus* (▨), for the control of PCN eggs. The formulations were: fungus incorporated into alginate pellets mixed into the soil; Terra-Green[®] inoculated with nematophagous fungi placed under the tuber; and a tuber dip containing biomass suspended. The controls (□) for each formulation were as the treated groups with the fungi omitted. Error bars show the standard errors of the means.



5.3 FURTHER DEVELOPMENT OF ALGINATE PELLETS

5.3.1 INTRODUCTION

As alginate pellets proved to be the most effective of the methods tested for reducing PCN egg counts, it was decided to use this method for further experiments. The alginate pellets had a number of advantages over the other treatments; they were easy to apply, did not have to be used immediately and were not as bulky as the Terra-Green[®] formulation. Further tests were carried out to improve the formulation of the alginate pellets, determine the effect the physical form of the pellets has on plant growth and to test the effects of storage on the viability of the fungi within the pellets. *Paecilomyces lilacinus* was used for the majority of these tests, as it was found to be the most effective fungus for control of PCN in the previous experiment (5.2). The effect on plant growth was investigated as Schuster & Sikora (1992b) reported reduced root weight of potatoes when grown in the presence of alginate.

5.3.2 MATERIAL AND METHODS

5.3.2.1 Different formulations

Different proportions of alginate and milled barley were added to the pellets to investigate whether this made them more effective. Table 5.1 shows the different formulations tried. Formulation 1 was the original formulation used in the previous experiment (section 5.2.2.3). The alginate and milled barley need to be

mixed with water to form a paste before they can be homogenised. The aim was to increase the amount of fungal biomass within the pellets and to reduce the amount of liquid incorporated into the alginate and milled barley. Water was replaced with growth medium, as the latter contains nutrients to support the survival and growth of the fungus. The amount of milled barley in the pellets was also increased for the same reason. The formulations were tested for retention of viability and appropriateness of consistency for pellet production.

Table 5.1 Different formulation mixtures for alginate pellets. The table shows the quantities of alginate and milled barley per litre fungi grown in liquid culture. Formulation 1 is the standard method used in experiment 5.2.2.3.

Formulation	Alginate	Volume added	Milled barley	Volume added
	(g l ⁻¹)	(ml)	(g l ⁻¹)	(ml)
1	40	1000	200	500
2	80	333	200	333
3	160	125	400	125
4	160	100	400	100
5	40	500	800	125

5.3.2.2 Survival of nematophagous fungi in alginate pellets

Air-dried pellet preparations of *P. cucumerina* and *P. lilacinus* were prepared as above (section 5.3.2.1) and stored in an air tight container at room temperature. At

intervals, ten pellets of each preparation type were placed onto PDA and incubated at 20°C. After 5 d the pellets were visually assessed for growth of the test fungi. This procedure was carried out daily for the first month, weekly for the second month and monthly for a further sixteen months.

5.3.2.3 Effect of alginate pellet pre-treatment on plant and nematode development

Alginate pellets containing *P. lilacinus* were produced as described in section 5.2.2.3. The alginate pellets were divided into three treatment groups; standard pellets, crushed pellets (ground in a coffee grinder) and pellets soaked in 50 ml of distilled water for 1 h. Approximately, 15 ml of the water was absorbed by the soaked pellets. Pots, 12 cm in diameter, were filled with Kettering loam. Potato tubers, cv. Desiree, were placed 5 cm from the bottom with 50 cysts of *G. rostochiensis*. The pellets (15 g dw) were placed on top of the tuber and the pots then topped up with loam. Nine replicates were used for each of the three treatments and were placed in randomised blocks in the glasshouse. Plants were watered daily.

The emergence of the first shoot for every plant was recorded. Once natural senescence had occurred the *G. rostochiensis* cysts were collected. The soil was dried for 7 days, and cysts were extracted for egg counts (section 2.5).

5.3.3 RESULTS

5.3.3.1 Performance of different formulation mixtures

It was decided that formulation 4 (Table 5.1) was the most suitable for large scale pellet production as the amount of liquid was reduced to the minimum and the pellets were therefore easier to dry. Another consideration was the consistency of the formulation for flow through the machine and formation of pellets in the CaCl_2 . Formulation 5 did not flow through the machine easily as the consistency was too dense.

5.3.3.2 Survival of nematophagous fungi in alginate pellets

The fungi formulated within the alginate pellets were found to survive encapsulation for at least 18 months for both species of fungi.

5.3.3.3 Effect of alginate pellet pre-treatment on plant and nematode development

Shoots of potato plants that had been treated with crushed alginate pellets started to emerge nine days after planting and all the plants had emerged after fifteen days. Shoot emergence also occurred nine days after planting for the plants treated with soaked alginate pellets and, by day sixteen, all the plants had emerged. Plants

treated with standard alginate pellets did not start to emerge until day eleven with all plants emerging by day eighteen (Figure 5.4).

Final egg counts in pots treated with crushed alginate pellets were lower than in pots treated with either soaked or standard pellets (Figure 5.5). This difference however was not significant ($P < 0.05$, t-test).

Figure 5.4 Effect of the pre-treatment of alginate pellets on plant emergence.

Alginate pellets were either untreated (\square), crushed (▨) or soaked (\blacksquare). N = 9.

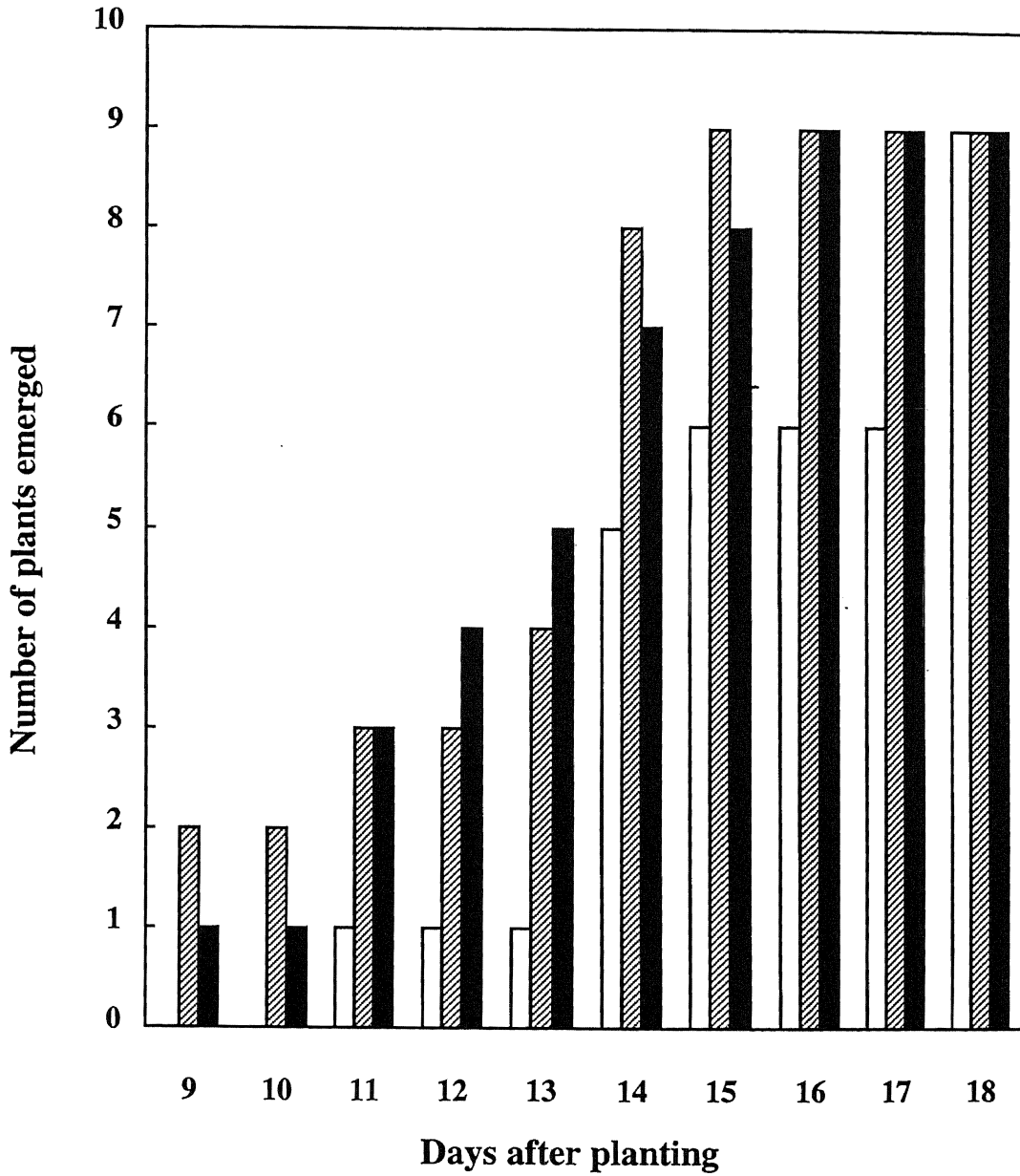
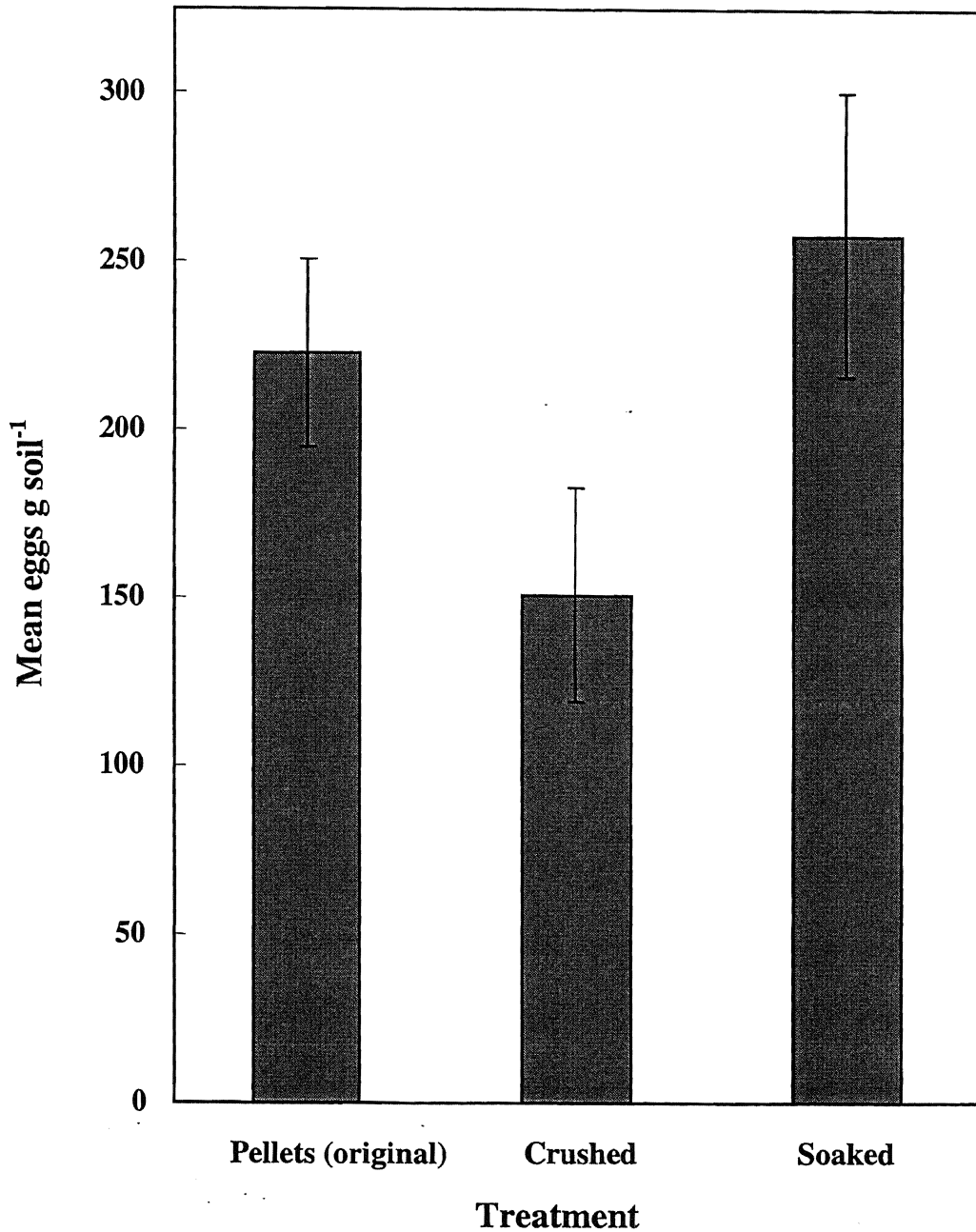


Figure 5.5 The effect of pre-treatment of alginate pellets on final numbers of *G. rostochiensis* eggs. Error bars show the standard errors of the means. N = 9. (Raw data shown in Appendix 2.1).



5.4 DISCUSSION

Fungal biological control agents may be added to a base or carrier to help bulk up the agent, increase longevity during storage and supply the fungus with a nutrient source. The application of either *P. lilacinus* or *P. cucumerina* without a carrier gave control of PCN. However, the carriers Terra-Green® and sodium alginate alone also reduced the PCN egg numbers (Figure 5.3). *Plectosphaerella cucumerina* reduced PCN egg numbers when applied as a tuber coating, but when formulated with Terra-Green® or alginate did not appear to increase the degree of control of PCN achieved by the uninoculated formulations. In contrast, incorporating the *P. lilacinus* into either, alginate pellets or Terra-Green®, reduced the mean nematode egg numbers when compared to the level of control of the carrier substance alone and the *P. lilacinus* tuber coating. The lack of significant differences between many of the treatments may have been a consequence of high within-sample variation.

Although, the alginate pellets alone gave comparable control to pellets incorporating the fungi, it is likely that this effect will be short-lived and that the inoculated pellets will break down leaving just the fungi as a soil inoculum. Further studies (Chapter 6) have shown this to be the case. Having a short term control will reduce PCN nematode numbers but, as mentioned in Chapter 1, the early, single cohort type hatch pattern of *G. rostochiensis* means that over time the use of alginate could lead to a predominance of *G. pallida* over *G. rostochiensis*.

The fact that alginate pellets alone reduced PCN egg numbers may have been due to either the alginate or the milled barley component of the pellets. Alginate is derived from the seaweed *Laminaria hyperborea*. Seaweed extract used as a soil drench has been shown to reduce the number of *Meloidogyne javanica* and *M. incognita* eggs (Whapham *et al.*, 1994; Wu *et al.*, 1997). Therefore, the alginate pellets may share a common component with seaweed which is either nematocidal or a plant growth stimulant. However, Schuster & Sikora (1992b) hypothesised that alginate pellets reduced root growth due to extensive fungal and bacterial growth around the alginate, which altered the CO₂/O₂ ratio in the rhizosphere. This led to suppression of root emergence and extension, reducing invasion of the roots by PCN. Another theory to explain the apparent control of PCN eggs when treated with uninoculated alginate pellets, is that when the dried pellets rehydrated, the moisture content was reduced in the soil, preventing the rehydration of the PCN cysts. Tests showed plants grown in the presence of dried alginate pellets took longer to produce their first shoot, compared to soaked or crushed pellets, but the final number of PCN eggs recovered between the different treatment groups was not significantly different. Therefore, it was decided the standard alginate pellets would be used in further experiments as preparation time was also less.

The addition of a nutrient resource within a biological control formulation can give an increase in crop yield which could be attributed to apparent nematode control, although PCN numbers may have increased. Rodriguez-Kabana & Morgan-Jones (1988) found the addition of an uninoculated substrate, such as oats

or rice, gave the same yield responses as the inoculated group. The plants are, to some extent, able to locate enough nutrients to increase yield as well as support a level of parasitisation by PCN. If there were no or reduced numbers of nematodes in comparison to a positive control, the yield response would be even greater. The apparent control of PCN by uninoculated Terra-Green[®] may be due to the fact that the substrate is coated with malt extract broth, which is providing the plant with nutrients.

Comparing the different methods of applying fungi showed that surface application by dipping of the tuber was not as effective as the application of formulated fungi under the tuber in reducing the number of PCN eggs. This may have been due to tuber dipping not leading to such a high concentration of conidia in the soil where the roots would develop in comparison to the other formulations, thereby decreasing exposure of the nematodes to the fungi. It is also possible that irrigation may have washed any conidia or biomass off the tuber and below the growing roots and away from the PCN. In future, suspending the fungi in a paste may be more successful in achieving adherence of conidia to the tuber.

There are a number of advantages to using alginate pellets rather than the other formulation methods tested: ease of production; better control of PCN egg numbers; retention of viability during storage over at least one and a half years at room temperature; and the fact that alginate pellets can be used in existing machinery designed for the distribution of granular nematicides. Nematophagous fungi applied in the form of alginate pellets will be distributed within the

rhizosphere and will not come into contact with tuber applied fungicides. Fungi applied using this method are therefore, less likely to be adversely affected by tuber applied fungicides than a tuber dressing.

There is continuing research into formulations for fungal control agents and with many different sources of nutrients and stabilising agents, species specific formulations are possible (Lewis & Larkin, 1998; Quimby *et al.*, 1999). The results clearly show that of the combination of formulations and agents considered here, *P. lilacinus* amended alginate pellets gave the best control of PCN in pot tests.

Hebbar *et al.* (1998) listed four criteria for a biological control agent of weeds to be effective, these are: low losses in viability during the formulation process; satisfactory shelf life at room temperature; abundant secondary (chlamydo)spore production; and rhizosphere colonisation. This list can also be applied to biological control agents of plant parasitic nematodes. *Paecilomyces lilacinus* and *P. cucumerina* fulfil all of these criteria when incorporated into alginate pellets.

6 THE EFFICACY OF FORMULATED NEMATOPHAGOUS FUNGI TESTED IN A PLUNGE AND FIELD TRIAL

6.1 INTRODUCTION

To determine the efficacy of a biological control agent it needs to be tested in the environment in which it will finally be used. Therefore, the nematophagous fungi being studied should be tested in the field or in conditions as close to the field situation as possible. Environmental factors such as soil type, pH, indigenous microflora, water content and temperature will all have an effect on the ability of the biological control agents performance (Gray, 1985).

The ideal situation for the biological control of plant parasitic nematodes is to establish a soil that is permanently suppressive for the target species. However this may not be commercially acceptable. Soils suppressive to cereal cyst nematodes, due to the presence of nematophagous fungi, were developed incidentally through the monocropping of some cereals during the second world war (Kerry *et al.*, 1982). Other soils have also been identified as suppressive to plant parasitic nematodes and this has been attributed to the presence of relatively high numbers of nematophagous fungi and other antagonists (Kerry & Crump, 1998). For example, Jaffee and Zehr (1982) attributed the decline of *Criconebella xenoplax* on peach trees to natural control by the fungus *Hirsutella rhossiliensis*.

It was only possible to conduct one field trial in Jersey during this research. As a supplement to this a plunge trial was designed in which pots containing soil from a potato field in Jersey were used to test the efficacy of formulated nematophagous fungi at IACR-Rothamsted. Jersey soil was used because, as well as containing indigenous PCN populations, it also contains other microflora which may affect the efficacy of the nematophagous fungi. Although a plunge trial can not fully simulate, for example, the climatic conditions in the field, it has some advantages over a field trial in Jersey, in that plant growth can be monitored daily, watering can be regulated and the amended soils can be used for a second season. For example, Van den Boogert *et al.* (1994) used microcosms to manipulate the soil environment by adding layers of sterilised soil which contained either nematodes or nematophagous fungi.

6.2 PLUNGE TRIAL TO DETERMINE THE EFFECTIVENESS OF COMBINED USE OF *P. CUCUMERINA* AND *P. LILACINUS* AS CONTROL AGENTS OF PCN OVER TWO SEASONS

6.2.1 INTRODUCTION

An experiment was designed to determine both the effectiveness of *P. cucumerina* and *P. lilacinus* as control agents for PCN over a single season, and the cumulative effect on PCN populations and potato yield of the addition of fungus to the soil over a number of seasons. Uninoculated alginate pellets were added to see whether the addition of the alginate alone had an effect on plant growth. A combination of the two fungi was also tested to determine whether there was any synergistic effect.

6.2.2 EXPERIMENTAL DESIGN

Two litres of peat was put into the base of each of forty, 25 cm diameter, plastic pots (5 litre). These were then filled with soil naturally infected with PCN obtained from a category III field in Jersey, and mixed with Terra-Green[®] (3:1). The pots were submerged in an outdoor sand plunge (Figure 6.1). Chitted Jersey Royal seed tubers were planted 20-25 cm deep, just above the peat layer, and 28 g of alginate pellets (produced as described in 5.2.2.3) were placed around the tuber. Five treatment regimes were used: control (no additions); addition of uninoculated alginate pellets; addition of 28 g (dw) *P. cucumerina* formulated within alginate



Figure 6.1 Plunge trial. Pots containing Jersey field soil were sunk into sand, 40 cm deep, at IACR-Rothamsted. Jersey Royal seed tubers were planted

pellets; addition of 28 g (dw) *P. lilacinus* formulated within alginate pellets; and addition of 14 g (dw) of pellets for each formulation. Treatments were allocated in random blocks using random numbers, with eight replicates per group. The trial was set up in May 1998, plants were grown for 80 days and watered when necessary. Prodigy tubers were weighed at harvest in August, after which the experiment was repeated using the same soils.

6.2.3 NEMATODE COUNTS

Five soil cores, 15 cm deep and 3 cm in diameter, were taken per pot in the pattern of one central core and four peripheral cores spaced evenly around the pot, before planting and at harvest. The nematodes were extracted from 50 g of soil using the Trudgill fluidising column described in section 2.5.

6.2.4 ASSESSMENT OF PCN MULTIPLICATION

Changes in nematode population density can be described using the ratio P_f/P_i ; where P_i is the initial population density and P_f is the final population density. Therefore a P_f/P_i that is greater than unity indicates an increase in the population density.

6.2.5 RESULTS

6.2.5.1 The effects of alginate formulation on PCN multiplication

For the first season alginate pellets were tested in the plunge, it was found that the control treatment with no additional treatment had a Pf/Pi of 4.3, this was slightly lower than the uninoculated alginate pellets treatment which gave a Pf/Pi of 4.8. *Plectosphaerella cucumerina* gave the best control of PCN with a Pf/Pi of 2.2, which was significantly different ($P < 0.05$, t-test) to both the control group and the control alginate pellet group. Treatment with *P. lilacinus* resulted in a Pf/Pi of 3.0, which was significantly different to the control alginate pellet group. The effect of a combined treatment of *P. cucumerina* and *P. lilacinus* (Pf/Pi = 3.4) was not significantly different to any other treatment (Figure 6.2).

The plants set up in August 1998 did not grow due to old seed tubers being used and lack of fertiliser. Therefore the results have not been analysed.

6.2.6 THE EFFECTS OF ALGINATE FORMULATION ON POTATO YIELDS

The control treatment resulted in the highest mean tuber weight per plant (193 g) compared to the other groups (Figure 6.3). This was significantly different ($P < 0.05$, t-test) to plants treated with *P. cucumerina* (129 g) and significantly different to plant treated with *P. lilacinus* (152 g). Plants treated with uninoculated alginate pellets gave the second highest mean yield of potatoes (170 g) which was

significantly different to plants treated with *P. cucumerina*. The combined *P. cucumerina* and *P. lilacinus* treatment resulted in a mean tuber yield per plant of 163 g, which was not significantly different to any of the other treatments.

Figure 6.2 Ratios between final and initial egg counts (Pf/Pi) for PCN after one season's growth of potatoes in untreated Jersey soil, and in Jersey soil treated with alginate only, alginated *P. cucumerina*, alginated *P. lilacinus*, or both *P. cucumerina* and *P. lilacinus*. Error bars show the standard error of the mean. N = 8.

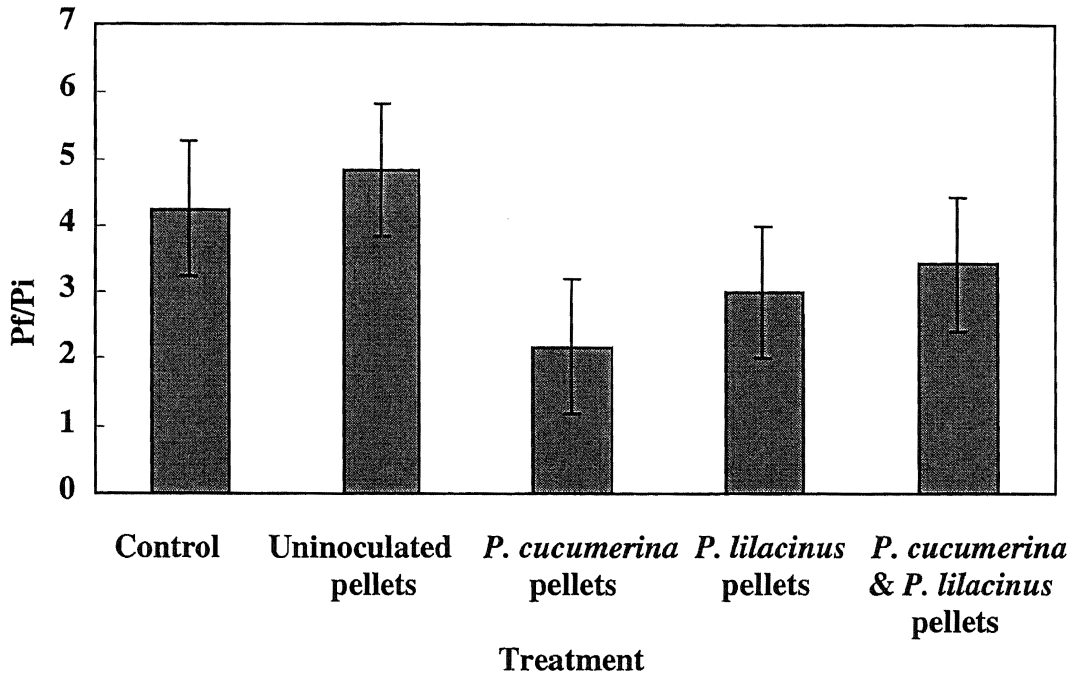
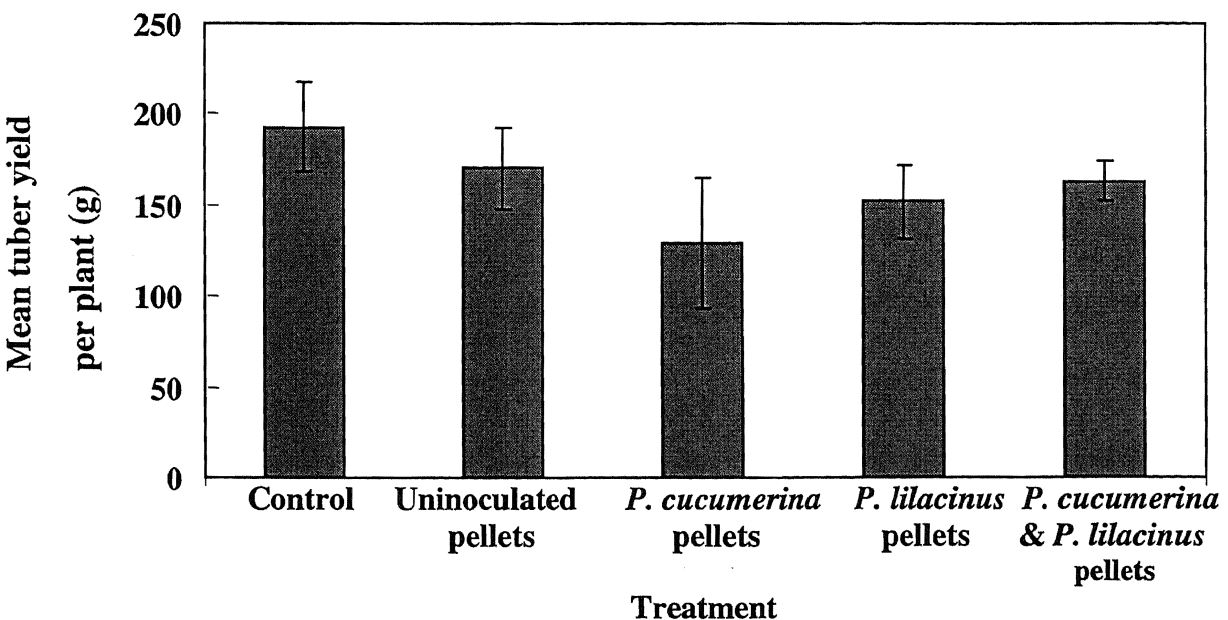


Figure 6.3 Potato yields from plants grown in untreated Jersey soil, and Jersey soil treated with uninoculated alginate pellets, alginated *P. cucumerina*, alginated *P. lilacinus*, or both *P. cucumerina* and *P. lilacinus*. Error bars show the standard error of the mean. N= 8.



6.3 PLUNGE TRIAL INVESTIGATING THE LONGEVITY OF NEMATOPHAGOUS FUNGI IN SOIL

6.3.1 INTRODUCTION

This experiment was set up to determine whether the nematophagous fungi could survive from the previous year to give nematode control for a second year, or whether the soil needed a second application of fungus.

6.3.2 MATERIALS AND METHODS

Soil from the previous year's plunge trial (6.2.2), treated with either *P. cucumerina* or *P. lilacinus* for two growing cycles or left untreated (control), was put into pots of 20 cm in diameter. Five pots previously treated with *P. cucumerina* were given a second dose of 30 g *P. cucumerina* alginate pellets (5.2.2.3), and five pots of the same soil were left untreated. Soil to which *P. lilacinus* had previously been applied was treated in the same way. Five further pots were filled with soil from the previous year's control treatment and were left untreated. Chitted Jersey Royal seed tubers were planted 5 cm from the bottom of each of the pots. Alginate pellets (30 g) inoculated with either *P. cucumerina* or *P. lilacinus* were added. Pots were sunk into the plunge and watered when necessary. Tuber yields were recorded after 80 days growth. Nematode counts were made as described in section 6.2.1.3. Roots were harvested, washed and weighed for use in Chapter 7. This trial was planted in June 1999.

6.3.2.1 Results

The results of this trial did not follow any obvious pattern (Figures 6.4 and 6.5). The Pf/Pi value for the control treatment decreased from 4.3 to 1.3 after potatoes were grown for a second season. A second addition of *P. lilacinus* inoculated alginate pellets decreased the Pf/Pi value from 3.0 in the first season to 0.9 in the second season, however, the Pf/Pi value for soil treated with *P. lilacinus* in the first season but left untreated in the second season decreased to 0.8. With the addition of a second application of *P. cucumerina* inoculated alginate pellets the Pf/Pi value increased from 2.2 for the first season to 3.9 in the second season. Soil treated with *P. cucumerina* in the first season but not the second season also had an increased Pf/Pi value (Pf/Pi = 3.7).

Figure 6.4 Pf/Pi values for PCN grown for a second season in untreated Jersey field soil, or in Jersey soil treated previously with *P. cucumerina* (Pc) or *P. lilacinus* (Pl) alginate pellets, with (+ dose) and without (- dose) a second application of *P. cucumerina* and *P. lilacinus* alginate pellets. Error bars show the standard errors of the means. N = 5.

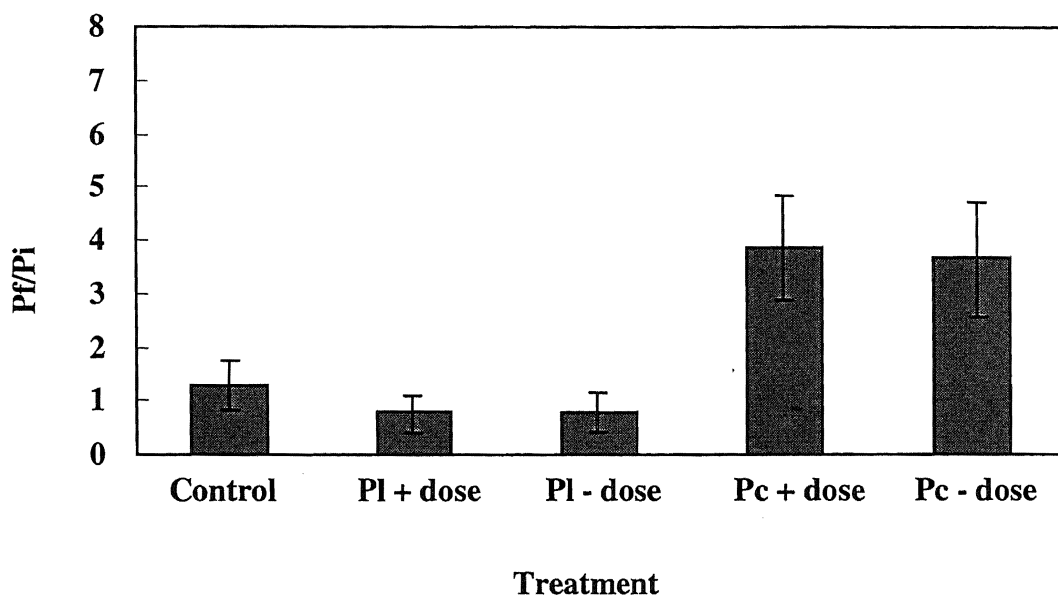
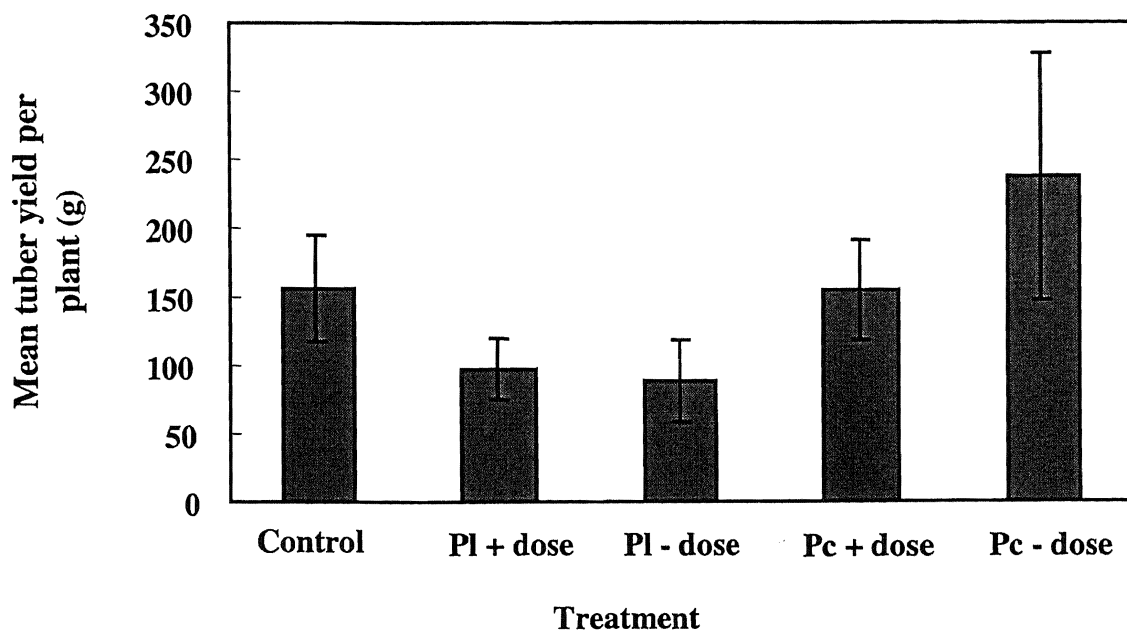


Figure 6.5 Yields of potatoes grown for a second season in untreated Jersey field soil, or in Jersey soil treated previously with *P. cucumerina* (Pc) or *P. lilacinus* (Pl) alginate pellets, with (+ dose) and without (- dose) a second application of *P. cucumerina* and *P. lilacinus* alginate pellets. Error bars show the standard errors of the means. N = 5.



6.4 PLUNGE TRIAL TO DETERMINE THE EFFECTS OF THE RATE OF APPLICATION OF ALGINATE PELLETS FOR CONTROL OF PCN

6.4.1 INTRODUCTION

When applying the formulated fungus it is important to add the correct amount of the biological control agent, so it is economically viable but is also effective. The cost of producing and applying the agent must be below that of the expected value of yield increase. High dose rates (Van Driesche & Bellows, 1996), may be impractical as well as uneconomic. However, uneconomic dose rates do not necessarily mean high dose rates, as this is directly dependent on cost of production.

6.4.2 MATERIALS AND METHODS

Pots were set up as in 6.2.2, but with fresh soil taken from the field that the field trial was conducted in (6.5). Alginate pellets containing either *P. cucumerina* or *P. lilacinus* were applied under the chitted Jersey Royal seed tubers at the following rates; 15 g, 30 g or 100 g per tuber. Potatoes grown in the absence of pellets were treated as the control. At harvest the pots were harvested and sampled in the same way as in section 6.3.2.

6.4.3 RESULTS

Both species of fungi formulated in alginate pellets reduced multiplication of PCN compared to the control, at all application rates tested the effects of the treatments were statistically significant ($P < 0.05$, t-test) except for that of *P. cucumerina* applied at a rate of 30 g tuber⁻¹ (Figure 6.6). The most effective treatments were the addition of *P. cucumerina* and *P. lilacinus* applied at a rate of 100 g tuber⁻¹, resulting in Pf/Pi's of 0.5 and 0.9 respectively. Larger doses of alginate pellets gave better nematode control, and there was evidence of a dose response effect.

There was no obvious relationship between dose of the biological control treatment and tuber yield. Plants treated with 100 g of *P. cucumerina* gave the lowest tuber yields (126.5 g), compared to the other dose rates (Figure 6.7). The highest yields for both groups were observed when 30 g of pellets containing either of the fungi were added. There were no significant differences ($P < 0.05$, t-test) between treatment groups.

Figure 6.6 Effect of the addition of different amounts of alginate pellets containing *P. lilacinus* (Pl) or *P. cucumerina* (Pc) on the multiplication of PCN in a plunge trial. Error bars show the standard error of the mean. N = 5.

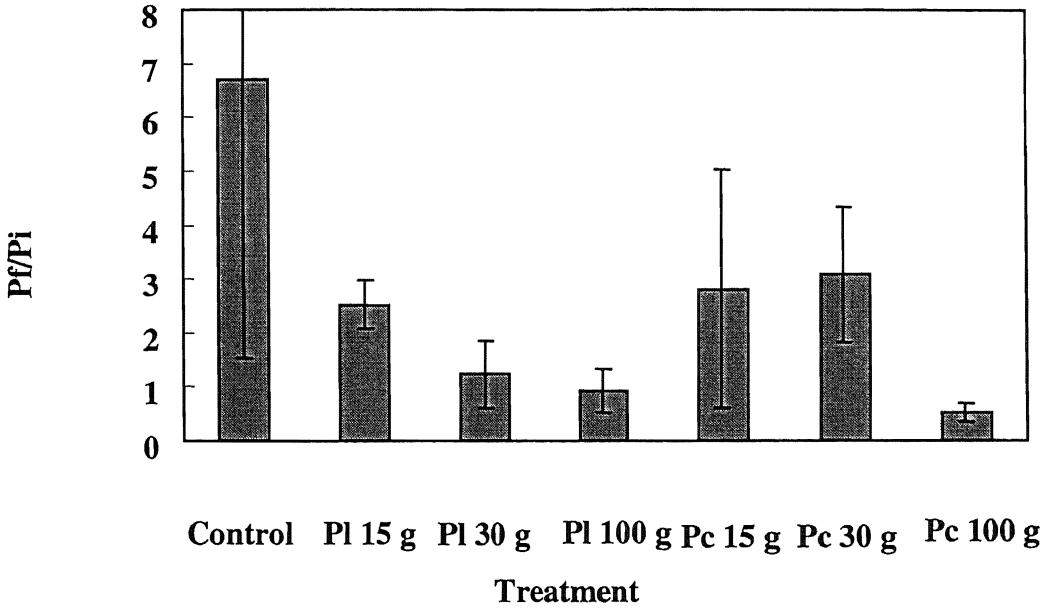
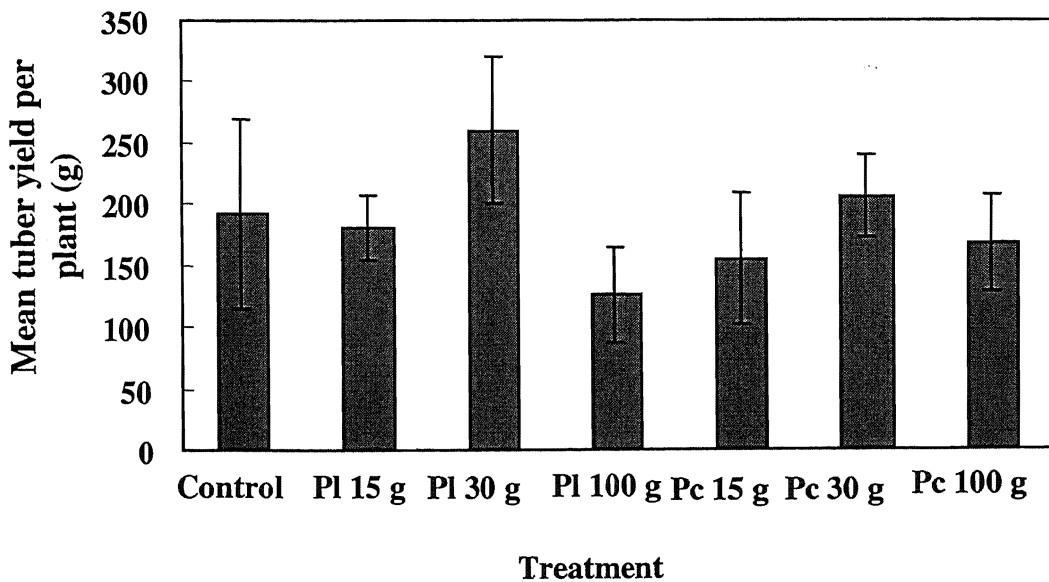


Figure 6.7 Effect of the addition of different amounts of alginate pellets containing *P. lilacinus* (Pl) or *P. cucumerina* (Pc) on the yields of Jersey Royal potatoes in a plunge trial. Error bars show the standard error of the mean. N = 5



6.5 FIELD TRIAL

6.5.1 INTRODUCTION

The field trial was carried out in April 1999 in field no. J 146, Le Canibut, St John, Jersey. The field was selected as it was a Category III field and the number of nematodes was expected to increase in the control plots as the initial samples showed PCN to be present at concentrations of up to 44 eggs g⁻¹ soil (Lane & Trudgill, 1999).

6.5.2 METHODS

6.5.2.1 Preparation of alginate pellet inoculum

Alginate pellets were prepared using the method described above (5.3.3.1). Pellets were formulated with either *P. cucumerina* or *P. lilacinus* grown in the bubbler (2.4). The control was not treated with alginate pellets.

6.5.2.2 Field trial

Although the field was treated with Vydate by the farmer, a central strip was left untreated, and within this a single ridge was left unplanted for our trial. The ridge was divided into plots, each of which consisted of three chitted seed tubers planted in the ridge. The plots were 90 cm apart along the row to ensure that the

treatments did not interfere with one another. Soil samples, 10 cores taken down the centre of the ridge were taken per plot, 15 cm deep and 2 cm in diameter, were taken to allow determination of the initial egg counts. Potatoes were planted at the bottom of the ridge. The biological control agent, 30 g per tuber of either *P. cucumerina* or *P. lilacinus* incorporated into alginate pellets, was added and forked gently into the soil prior to placing the tuber on top. Each treatment was replicated thirteen times. Soil around the tuber was built up to re-form the ridge, approximately 20 cm high. Location of treatments along the ridge was randomised. A Tiny Talk™ temperature probe was placed at the bottom of the ridge (20 cm deep) to monitor the soil temperature throughout the growing season.

After 3 months growth the plants were harvested (Figure 6.8). Tubers were weighed and soil samples were taken (as for the initial egg counts in this trial). Egg counts of PCN were determined using the method described in section 2.5.

6.5.3 RESULTS

6.5.3.1 Soil temperature

The mean soil temperature over the growing season was 12.5°C. The lowest temperature recorded was 5°C in April and the highest temperature was 21.6°C at the end of May. The mean temperatures for each month were 9.5°C, 10.6°C, 14.9°C and 14.5°C for March to June respectively (Figure 6.9).



Figure 6.8 Harvesting tubers at the end of the field trial in Jersey. The ridge was separated with a guard row either side from the rest of the field that had been treated with nematicides. Plots consisted of three potato plants.

6.5.3.2 Effect of the addition of alginate pellets containing *P. lilacinus* or *P. cucumerina* on the multiplication of PCN in soil in the field

The initial egg counts varied considerably along the ridge, but did not follow any clear pattern (Figure 6.10). Counts ranged from 0.6 to 43.6 eggs g soil⁻¹. The mean initial egg counts were 10.8, 20.4 and 8.7 eggs g soil⁻¹, respectively, for plots treated with *P. lilacinus*, *P. cucumerina* and the control.

Treatment with *P. lilacinus* or *P. cucumerina* limited the multiplication of PCN in soil around the potato tuber (Pf/Pi = 8.8 and 7.4, respectively) compared to that in control plots (Pf/Pi = 21.7) (Figure 6.11). The effect of the fungal treatments was significant ($P < 0.05$, t-test; data normalised by log₁₀ transformation).

The tuber yield for the untreated plots was significantly higher ($P < 0.05$, t-test) (1.93 kg) than for the *P. lilacinus* treated plots (1.53 kg) and the *P. cucumerina* treated plots (1.59 kg) (Figure 6.12).

Figure 6.9 Soil temperature during the growing season in Jersey. Recordings were taken every 2 h from the base of a ridge in the centre of the field.

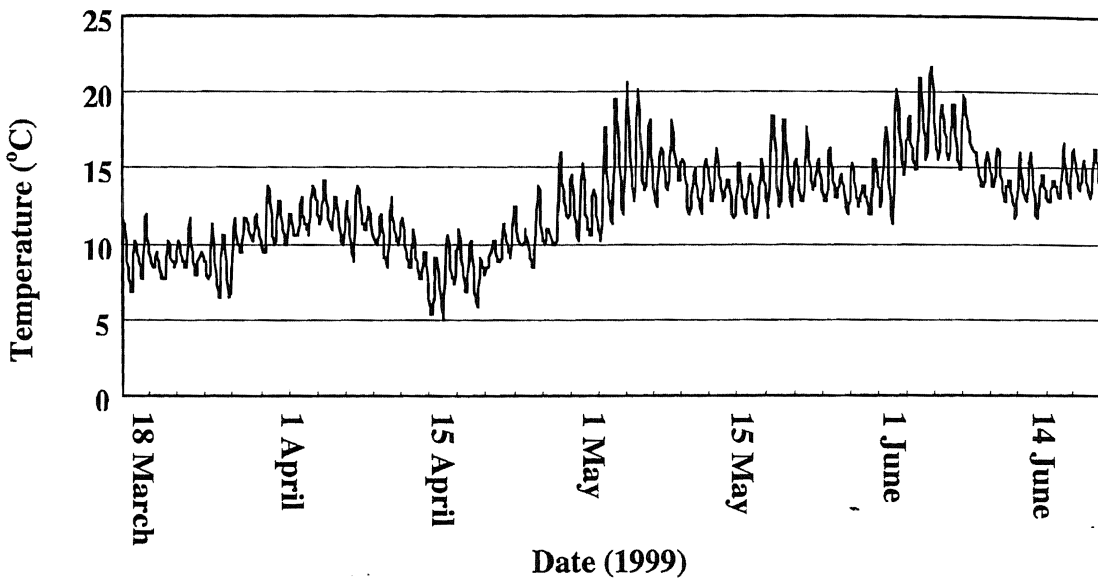


Figure 6.10 Initial PCN egg counts along the ridge used for the field trial. Plots were subsequently treated with *Paecilomyces lilacinus* (▨), control (□), and *P. cucumerina* (■).

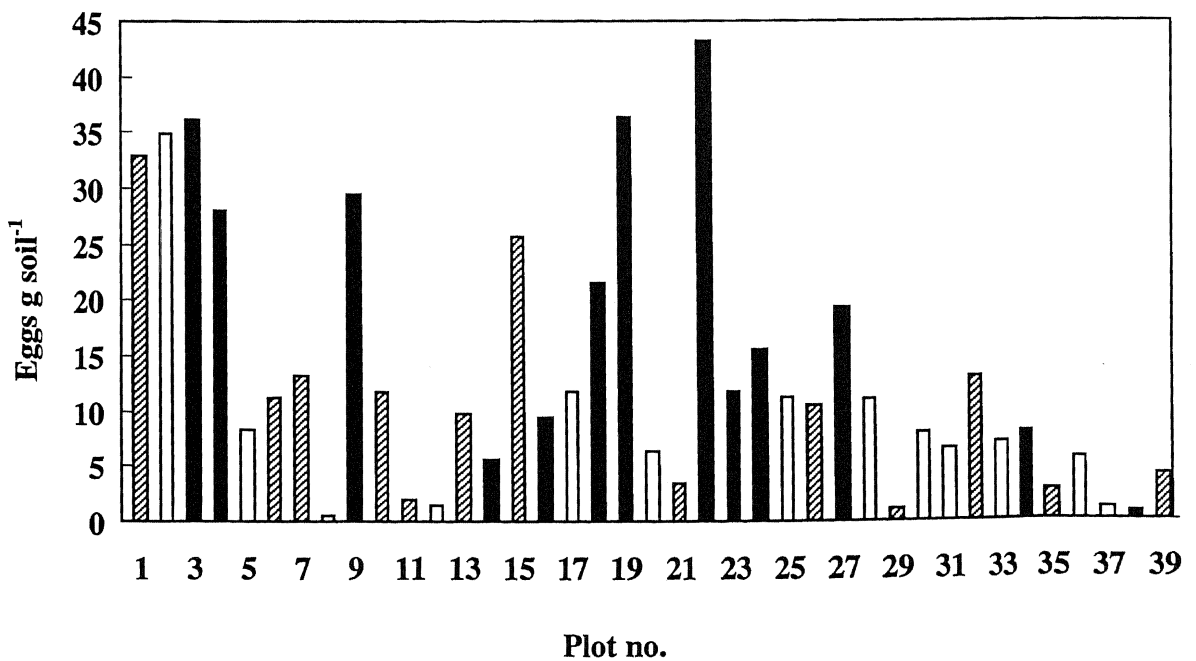


Figure 6.11 Effect of the addition of alginate pellets containing *P. lilacinus* or *P. cucumerina* on the multiplication of PCN in soil in the field. Error bars show the standard error of the mean. N = 13.

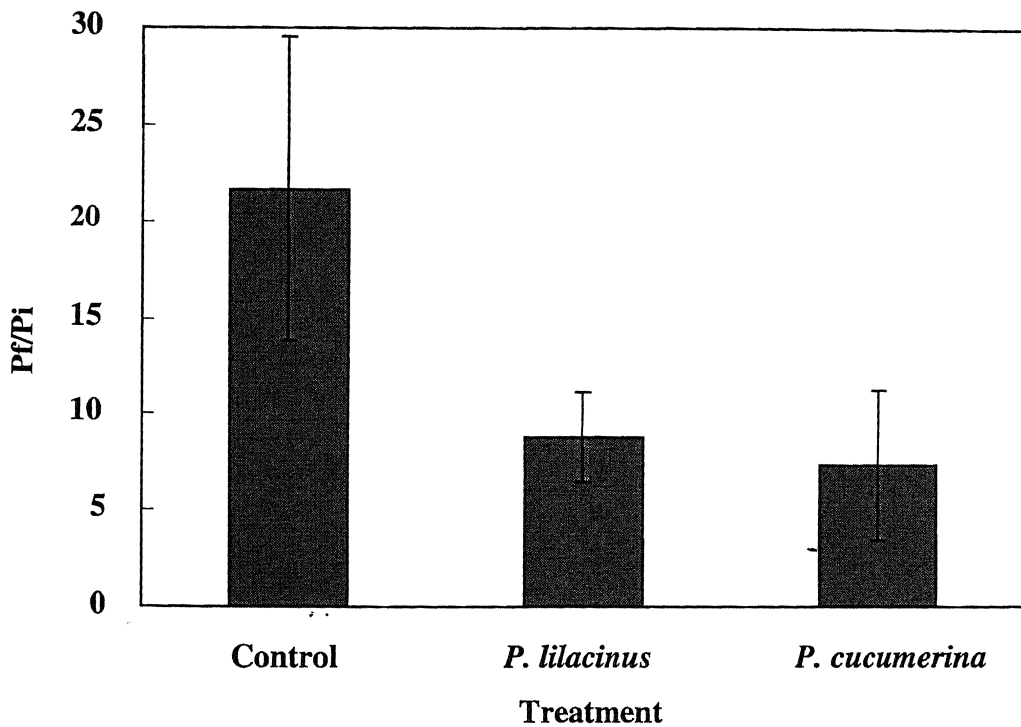
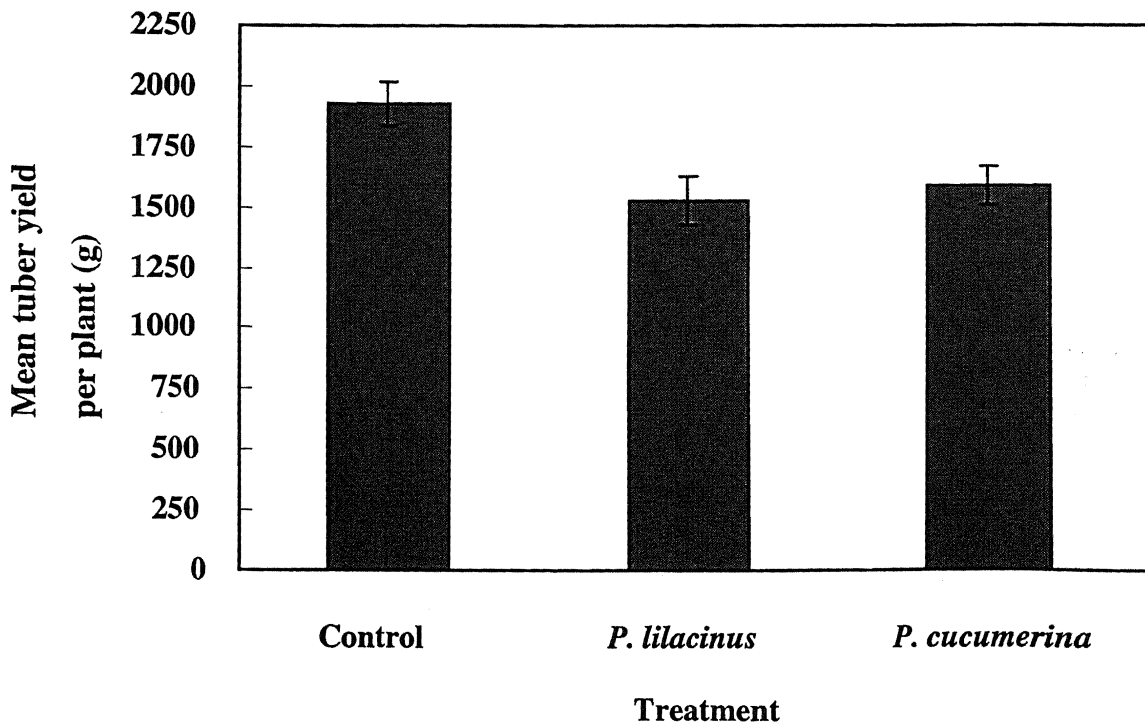


Figure 6.12 Effect of the addition of alginate pellets containing *P. lilacinus* or *P. cucumerina* on the yield of Jersey Royal potatoes in the field. Error bars show the standard error of the mean. N = 13.



6.6 POST-HARVEST APPLICATION OF *VERTICILLIUM CHLAMYDOSPORIUM*

6.6.1 INTRODUCTION

A survey of the incidence of infection of PCN females by nematophagous fungi was carried out in Jersey in May and July 1997 (Appendix 2.2). It was found that *V. chlamydosporium* was present in the soil in May, but had not infected the female PCN. However, females collected in July were found to be infected with *V. chlamydosporium*. *Verticillium chlamydosporium* therefore appears to attack PCN females later on in the season, when the soil temperatures are higher. It was found (section 4.3.2) that *V. chlamydosporium* grew at 20°C but not at 10°C; the soil temperature in Jersey did not rise to 20°C until May (Figure 6.9). *Verticillium chlamydosporium* is a parasite of tropical nematodes, therefore it has a high optimum growth temperature (Irving & Kerry, 1986). Whilst harvesting the field trial it was observed that many of the roots left in the soil after harvest were infested with PCN females. Therefore, *V. chlamydosporium* was tested as a post-harvest treatment because the warmer post-harvest temperatures favour the growth of this fungus, and the nematode is still present in the soil on dying plant material.

6.6.2 METHODS

6.6.2.1 Production of chlamydospores

Milled barley (50 ml) was washed on a 53 µm mesh sieve and mixed with 50 ml of coarse sand (Bourne *et al.*, 1994). The mixture was divided between two

250 ml conical flasks and autoclaved for 30 min. The flasks were cooled before ten plugs of *V. chlamydosporium* (3-4 mm in diameter), taken from the colony edge, were added. The flasks were incubated at 20°C for four weeks and shaken daily for the first few days until the medium was completely colonised. The contents of the flasks were then washed through a 250 µm mesh sieve, into a 53 µm sieve and collected in a tray. Liquid passing through the sieve was then passed through a 10 µm mesh. The milled barley and sand mixture was washed until the liquid ran clear. Chlamydospores caught on the 10 µm mesh sieve were weighed, mixed (1:10) with sand (40-100 mesh, Fisher Scientific UK Ltd., Leicestershire, UK) and refrigerated at 4°C until needed.

6.6.2.2 Post-harvest application of *Verticillium chlamydosporium* to soil previously used in the plunge trial

Eight replicate soil samples (50 g) taken from the control plots, *P. cucumerina* and *P. lilacinus* treated pots from the plunge trial (section 6.2.2) were placed in plastic beakers and covered with parafilm. A further eight replicate samples from each plunge treatments were inoculated with approximately 5000 chlamydospores of *V. chlamydosporium*. The chlamydospores were mixed throughout the soil. Cysts were extracted from the soils (section 2.5) after one months incubation at 20°C. Egg counts were also conducted on eight replicate soil samples before the soils were incubated.

6.6.2.3 Post-harvest application of *Verticillium chlamydosporium* to soil previously used in the field trial

Thirteen replicate soil samples (50 g) from each treatment; control, *P. cucumerina* and *P. lilacinus* treated plots of the field trial (6.5) were placed in plastic beakers. A further thirteen replicates from each treatment were inoculated with approximately 5000 chlamydospores of *V. chlamydosporium* mixed into the soil. Egg counts were also conducted on thirteen further replicate soil samples before the soils were incubated.

6.6.3 RESULTS

The addition of *V. chlamydosporium* chlamydospores to soil samples taken at the end of the plunge trial significantly reduced the number of viable PCN eggs for each of the treatment groups compared to soil samples that were not inoculated with *V. chlamydosporium* chlamydospores ($P < 0.05$, ANOVA). Simply incubating the soil did not significantly reduce the number of PCN eggs compared to the initial counts for the *P. lilacinus* and *P. cucumerina* treated soils, but did for the control soil (Figure 6.13).

When soil sampled at the end of the field trial was treated with chlamydospores, no significant difference ($P < 0.05$, ANOVA) between the egg counts in inoculated and uninoculated soils was shown following incubation (Figure 6.14). Egg counts after incubation, both with and without the addition of chlamydospores, were

significantly lower than those in the field sample before incubation, indicating that incubation alone had an effect on the numbers of eggs of *Globodera* spp.

Figure 6.13 Effect of post-harvest application of *V. chlamydosporium* on PCN egg counts in soil taken from the plunge trial. Egg counts are shown before incubation (■), and after incubation with (▨) and without (□) the addition of *V. chlamydosporium* chlamydospores. Error bars show the standard errors of the means. N = 8.

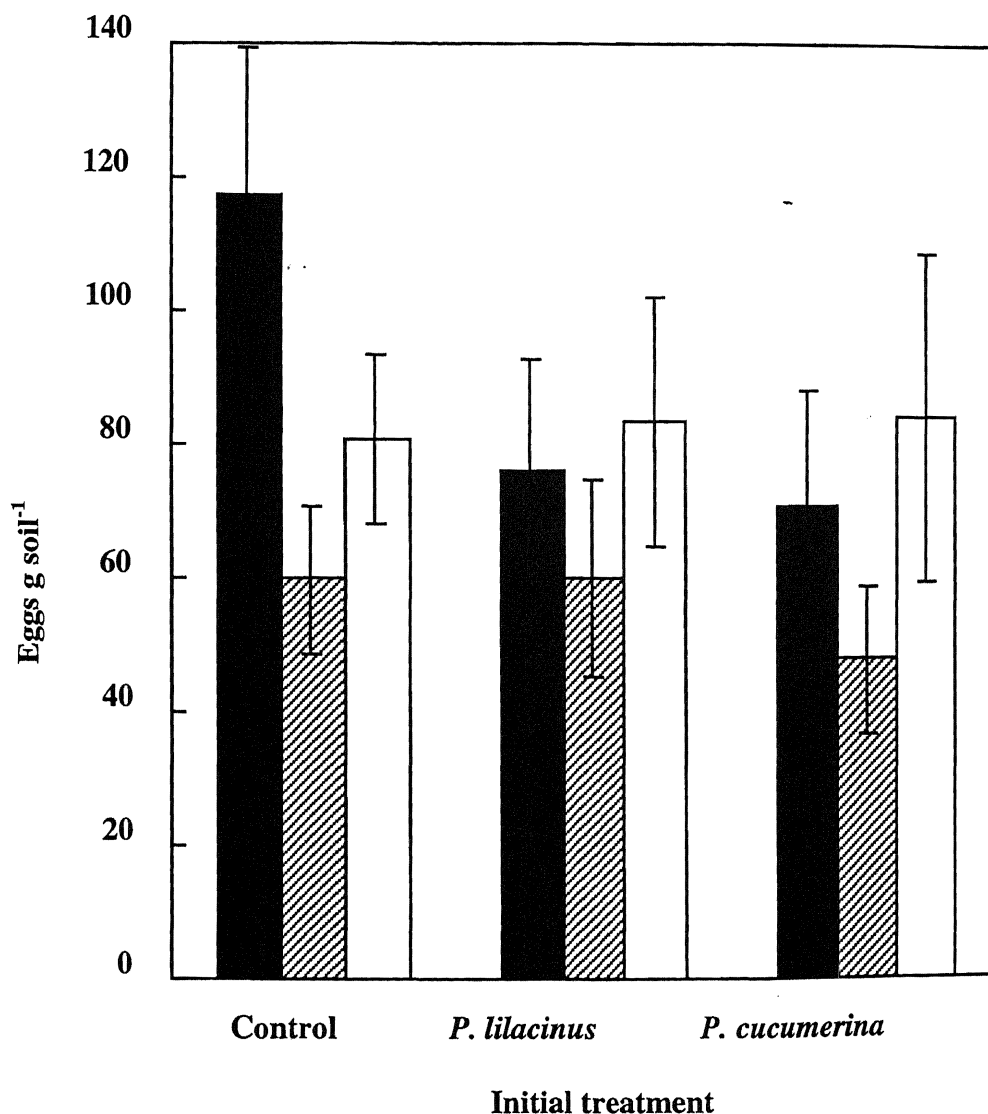
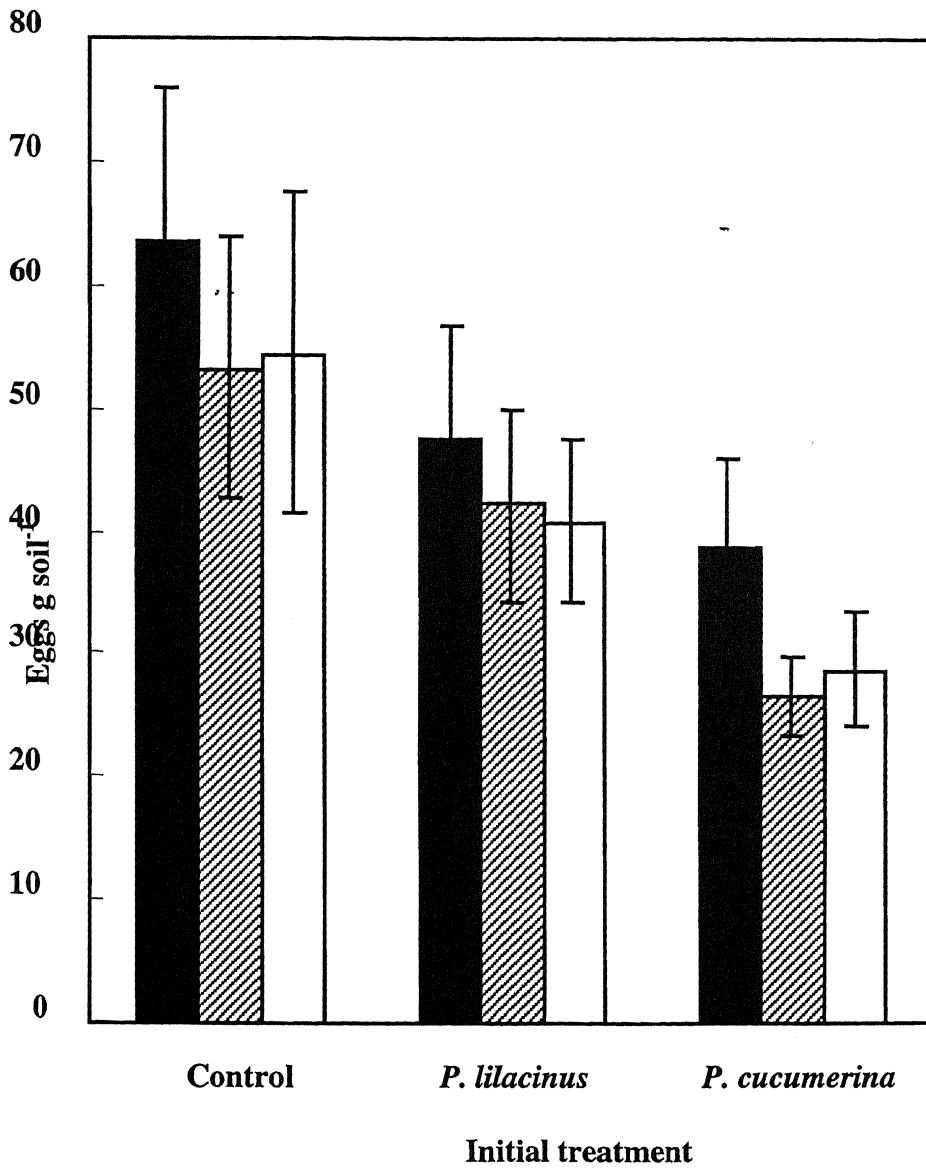


Figure 6.14 Effect of post-harvest application of *V. chlamydosporium* on number of PCN eggs in soil taken from the field trial. Egg counts are shown before incubation (■), and after incubation with (▨) and without (□) the addition of *V. chlamydosporium* chlamydo spores. Error bars show the standard errors of the means. N = 13.



6.7 DISCUSSION

The addition of a single species of fungus appeared to give better control of PCN in the plunge pots than the two species, *P. cucumerina* and *P. lilacinus*, in combination. This may have been due to the two species being antagonistic towards one another as seen in Chapter 4, and consequently inhibiting one another's ability to infect nematodes. Stirling & Smith (1998) found that a combined addition of the nematode trapping fungi *Arthrobotrys dactyloides* and *V. chlamydosporium* formulated into granules gave greater control of *Meloidogyne sp.* than the two fungal species applied separately. Stirling, however, added a double dose of pellets instead of adding half the amount of the normal inoculation dose for both species; thus, the effect may have been due simply to an increased amount of biomass, rather than any synergism between the two fungi.

A trial to determine whether the fungus remained in the soil and was effective in controlling PCN for more than one growing season was not very successful due to two crops being grown consecutively in the first year. Failure to add fertilisers and the use of old seed tubers, due to unavailability of fresh seed tubers at that time of year, resulted in only half the plants growing. Unfavourable climatic conditions later in the season also meant that running two plunge trials in one year was not feasible. At present it is not known if an application of a nematophagous fungus will sustain itself in the field or will need to be re-applied with each crop (Crump, 1998). A self-sustaining biological control agent may not be commercially acceptable. A conventional commercial product, for example chemical

nematicides, would give maximum reduction of the Pf/Pi but have a limited persistence in the soil not extending beyond a single season.

To extrapolate from the plunge trials with 30 g of alginate pellets per pot to field application the dose per pot should be multiplied by a factor of 148118, the number of seed tubers planted per hectare in Jersey (potatoes planted 15 cm apart in rows 45 cm apart). This would give an application rate of 4.4 tonne ha⁻¹. However, this figure only applies if alginate is applied round the tuber of plants, as is commonly practiced with the application of Vydate. Unfortunately, the work has shown that alginate can have an inhibitory effect on tuber yield when the pellets are placed close to the tubers (Figures 6.3, 6.5, 6.7 and 6.12). This could be due to increased microbial activity around the root. If the alginate pellets were to be applied into soil pre-planting, to the top 20 cm (possible plough depth) of soil, the total soil treatment per hectare would be 10,000 m² x 0.2 m = 2000 m³, or equals 2,000,000 litres of soil. In the plunge trial 30 g of pellets were added to each pot containing 4 litres of soil:

$$\frac{2,000,000}{4} = (500,000 \times 30 \text{ g}) = 15,000,000 \text{ g of pellets}$$

which equals 15 tonnes of alginate pellets per hectare .

By increasing the rate of inoculum, the probability of the nematode coming into contact with the fungi is increased. A balance needs to be reached in which the nematode is inoculated with a suitable dose that will reduce the pest population but is also economical to produce and apply. Successful control of nematodes has

been achieved using *P. lilacinus* at the rate of 1-20 tonnes ha⁻¹, but this is too great for wide spread commercial use (Van Driesche & Bellows, 1996). Lower rates of application (0.4 tonnes ha⁻¹) of *P. lilacinus* in augmented form infected fewer *Meloidogyne incognita* eggs in the field (Cabanillas *et al.*, 1989). Spore concentration is also important for the bacterial pathogen *Pasteuria penetrans*. Stirling (1990) found that a dose of 10⁴ spores g soil⁻¹ infected less than 40% of *M. javanica* females, but 10⁵ spores g soil⁻¹ infected 100% of females. As *P. penetrans* is an obligate parasite, it has not yet been successfully cultured for commercial use. Smaller numbers of spores can be used with a nematode tolerant crop with no effect to the yield, and the presence of nematodes will allow the number of *P. penetrans* spores to increase, so that a susceptible crop can be grown the following season. However, *P. penetrans* endospores are only activated when in contact with certain nematodes, the fungi used in this thesis are saprophytic and will grow in the absence of the nematode host. One possible method for reducing the amount of fungal inoculum needed is to formulate the fungus on a nutrient base and add it to the soil before planting. This may allow saprophytic fungi to multiply in the soil before the pest is present. Further tests are required to see if this is a viable option.

Addition of nematophagous fungi formulated in alginate pellets reduced multiplication of PCN in the field by a factor of 3 compared to control plots. The tuber yields, however, were lower for the treated groups than the control group. Reduced tuber yields were also observed in the plunge trial which would indicate the alginate pellets are having an adverse effect on plant growth. It was found in the field trial that at the time of harvest there were no nematodes on the roots in

the area in which the alginate pellets had been applied, but towards the outer edge of the ridge the roots were infected with nematodes. This may suggest that the fungi were neither colonising nor spreading along the roots, but just attacking the nematodes in the immediate vicinity of the applied pellets. Control of PCN may therefore be more successful if the pellets were broadcast throughout the field rather than being applied only under the tuber.

When potato plants were harvested, after 80 days for the plunge or three months for the field trial, the female PCN were still developing on the roots in both field and plunge soil. The different life stages were investigated further in Chapter 7. After harvest the number of eggs per gram of soil may continue to rise as any females left on the roots will continue to develop to the cyst stage, each potentially containing hundreds of eggs. Therefore, the egg counts described in this chapter may be lower than if the cysts were allowed to develop on the roots or if the plants were allowed to develop further.

Addition of *V. chlamydosporium* chlamydospores to the soil after harvest resulted in a reduction in the number of PCN eggs in the soil taken from the plunge trial. Incubation alone did not reduce the numbers of PCN eggs (Figure 6.13). However, when this experiment was repeated using field trial soil, the numbers of PCN eggs decreased during incubation irrespective of whether they had been treated with *V. chlamydosporium* or not (Figure 6.14). Devine *et al.* (1999) found that, with *G. rostochiensis*, the number of viable eggs decreased by up to 15% after four weeks at 20°C due to spontaneous hatch and in-egg mortality. The

differences observed between the plunge trial soil and field trial soil may be due to varying proportions of *G. pallida* and *G. rostochiensis* in the populations. *Globodera rostochiensis* is more likely to hatch spontaneously than *G. pallida*.

7 DETERMINATION OF THE COMPOSITION OF JERSEY PCN POPULATIONS USING BIOCHEMICAL TECHNIQUES

7.1 INTRODUCTION

It has previously been assumed that the PCN population in Jersey consisted almost entirely of *Globodera pallida*. This assumption was possibly based on soil samples taken before biochemical techniques for PCN population identification were widely used (Meadows 1999, pers. com.) and reinforced with the knowledge that the UK populations were becoming dominated by *G. pallida* (Halford *et al.*, 1995).

It is important to know which species of PCN is in the field so that suitable control measures can be used. The restriction of the use of granular nematicides, such as Vydate (the current UK market leader) on Jersey may mean that species selection has not been so great as on the UK mainland (as described in 1.3.3). In addition, the early lifting of the potato crop in Jersey favours multiplication of *G. rostochiensis*, rather than *G. pallida*, as the former hatches earlier and in a single cohort. Most eggs of *G. rostochiensis* have hatched by the sixth week after planting, with the bulk having hatched by week four but *G. pallida* may still be hatching after week ten with the peak of hatching not reached until week six. If

the crop is lifted after twelve weeks, proportionally more *G. rostochiensis* are likely to have completed their life cycle than *G. pallida*.

Cysts recovered by the author from a Jersey soil sample were assessed by personnel at IACR-Rothamsted using isoelectric focusing (IEF) to determine PCN species. The results of the IEF showed that not only was there a mixed population of PCN, but that the Jersey PCN sample produced an extra band on the gel which did not match up with the *G. pallida* or *G. rostochiensis* controls (Figure 7.1). It was also suspected there was a mixed population of PCN in Jersey when the plunge and field trial were harvested, as both white (*G. pallida*) and yellow (*G. rostochiensis*) nematodes were found on the roots (Lane & Trudgill, 1999).

To ascertain the species population make-up of PCN from Jersey soils, biochemical techniques were used, as these are reliable and require less operator skill and experience than standard taxonomic techniques. The two techniques chosen were indirect ELISA (enzyme-linked immunosorbent assay) and RAPD-PCR (random amplified polymorphic DNA - polymerase chain reaction). The ELISA technique was chosen in preference to IEF because two antibodies had been developed at IACR-Rothamsted that only recognise viable eggs of the two PCN species (Curtis *et al.*, 1998) and sample throughput was potentially greater using ELISA. Theoretically, quantification was possible with ELISA and only possible with IEF for a skilled operator.

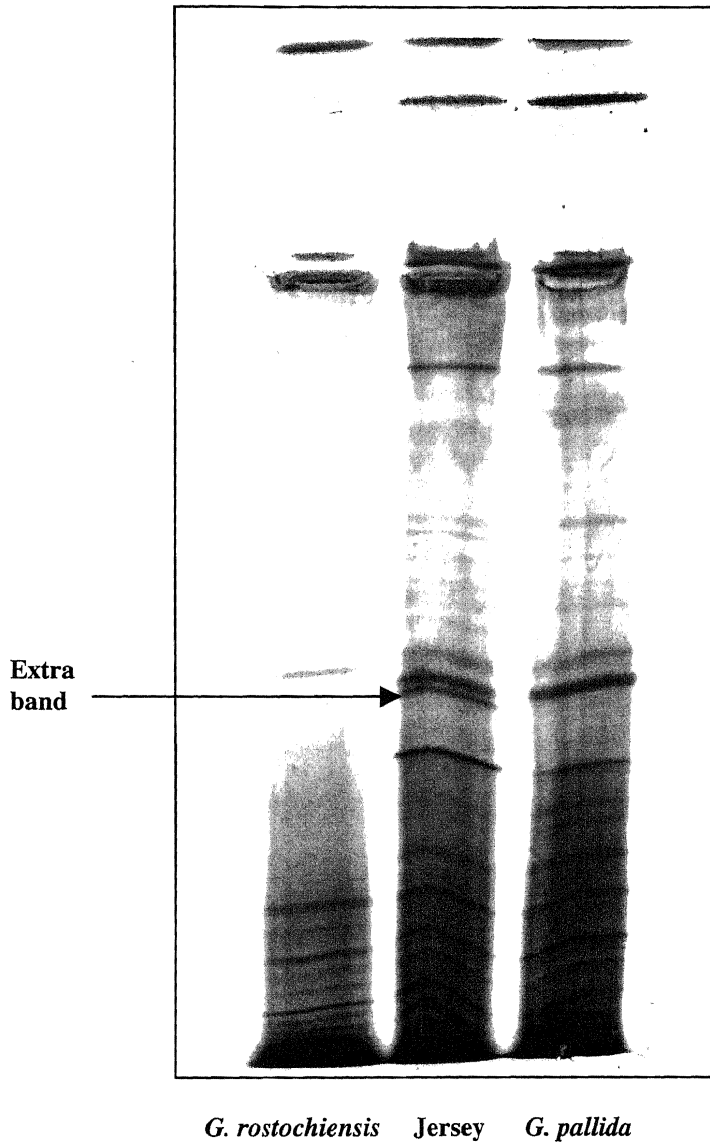


Figure 7.1 IEF gel showing *G. pallida* and *G. rostochiensis* controls with nematodes from Jersey. The Jersey nematode has the same band as both *G. pallida* and *G. rostochiensis*, but also has an extra band.

RAPD-PCR was used as it was an available method able to determine the species of those immature nematodes recovered from the roots after the potatoes were lifted. A hot staining method, which can denature the proteins that are recognised in the IEF technique but, not denature the DNA, was used to locate the nematodes.

The nucleotide primer E19 is consistently used at IACR-Rothamsted for identification of PCN species (Burrows *et al.*, 1996). The primers K4 and K16 are two others which have been used to separate species of the genus *Pratylenchus* (Barker, 1999) but have also been shown to give clear banding patterns for *G. pallida* (Barker 1999, pers. com.).

These techniques were combined in an attempt to determine the ratio of *G. pallida* to *G. rostochiensis* in soil from Jersey at planting and at harvest. The effects of early lifting, as determined by the life stages of the two species found in the roots, and the addition of *P. lilacinus* and *P. cucumerina* on the population composition were investigated.

To determine whether one species of *Globodera* was more susceptible to fungal infection than another, the compositions of the nematode populations in the fungal treated groups from the plunge trial were compared to those of the control populations.

7.2 MATERIALS AND METHODS

7.2.1 STAINING NEMATODES

Roots from potato plants infested with nematodes, that had either been inoculated with 100 g *P. lilacinus* amended alginate pellets or left untreated, were stained to determine whether there was a difference in the number of different life-stages of PCN between treatment groups.

Root samples were taken from all the plants used in the plunge trial (6.4). The whole root from each plant was weighed and a subsample (1 g), randomly selected, was taken. The subsamples were cut into 1 cm sections and put into 20 ml universal bottles in preparation for hot acid staining (Bridge *et al.*, 1982). An adaption of the standard protocol (Bridge *et al.*, 1982) was used (Halford 1999, pers. com.). Hot acid fuschin stain (glycerol, 300 ml; H₂O, 300 ml; lactic acid, 300 ml and acid fuschin, 0.45 g) was poured over the root samples and left for 2-3 min. The stain was poured off the roots and the roots were washed in tap water. Approximately 10 ml of destain (glycerol, 500 ml; H₂O, 500 ml; and lactic acid, 10 ml) was added to each sample. The roots were then macerated using a 'Verso' laboratory mixer emulsifier (Silverson Machine Ltd., Bucks, UK) at full speed for 15 s and the resulting samples kept in destain until needed.

To count the different life stages, the root samples in destain were each made up to 40 ml with water. A 10 ml aliquot was put into a Doncaster counting dish (Doncaster, 1963) and assessed under a light microscope (x250). All nematodes in

the 10 ml sample were counted and the life stages split into four categories; juveniles, males, small females and large females (Figure 7.2).

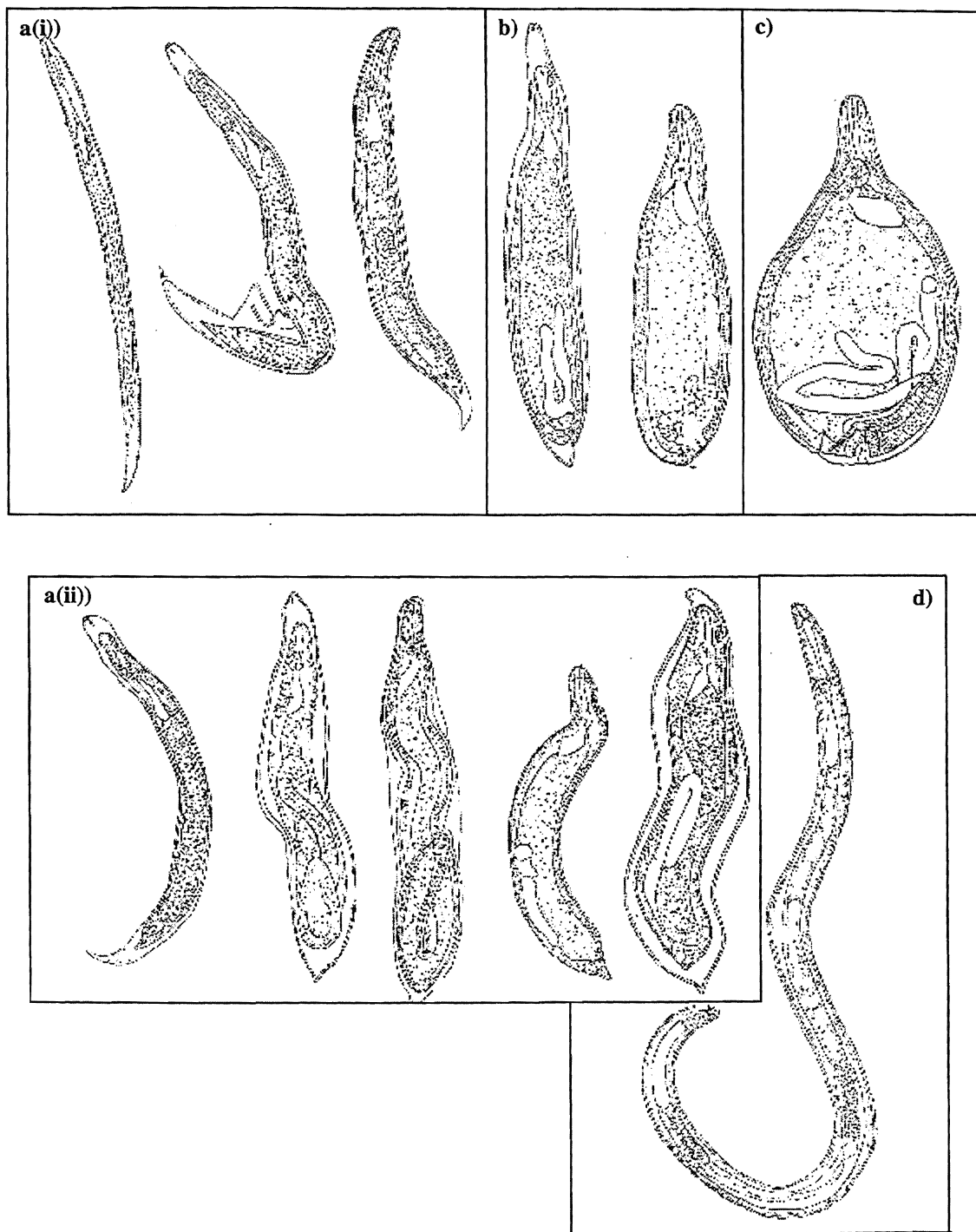


Figure 7.2 Different life stages of PCN. The nematodes were split into four categories, a) juveniles (females (i) and males (ii), b) small females, c) large females and d) males.

7.2.2 ENZYME-LINKED IMMUNOSORBENT ASSAY

7.2.2.1 General description of the indirect enzyme-linked immunosorbent assay

The indirect enzyme-linked immunosorbent assay is based on the antigen, in this case the proteins from the crushed nematodes, adhering to the walls of a polystyrene, 96 well ELISA plate, the walls of which will have been specially treated to aid the binding of the proteins. An antibody is added that has been developed to bind specifically to the targeted antigen and may have been raised in a mammal such as a rat or mouse in response to that antigen. The assay becomes an indirect ELISA when a second antibody is added that specifically recognises the mammal antibodies. After being recovered from the host mammal, the second antibody, unlike the first, has an enzyme conjugated to it (horse radish peroxidase, HRP, in the protocol used for this work). A substrate (hydrogen peroxide) plus a chromogenic donor (tetra methyl benzidine, TMB) are then added which the enzyme cleaves resulting in a colour change in the solution. It should be possible to correlate the colour intensity with the amount of antigen present in the sample through comparison with standards such as known amounts of the target antigen (Figure 7.3).

7.2.2.2 Antibodies used in these studies

Two antibodies that had previously been raised at IACR-Rothamsted, in rats against the two species of PCN as described by Robinson *et al.* (1993b) were

used. Although the antibodies recognise their intended species, it was found that there was a degree of cross-sensitivity (Curtis *et al.*, (1998); Barker *et al.*, (1998)) particularly with the antibody developed for *G. pallida*. Antibodies from the two monoclonal lines were precipitated with ammonium sulphate and a number of aliquots from the processed material were assessed for reactivity. Two aliquots from the processed material were assessed for reactivity. Two aliquots were chosen for this study, R3 (*G. rostochiensis*) and P14 (*G. pallida*).

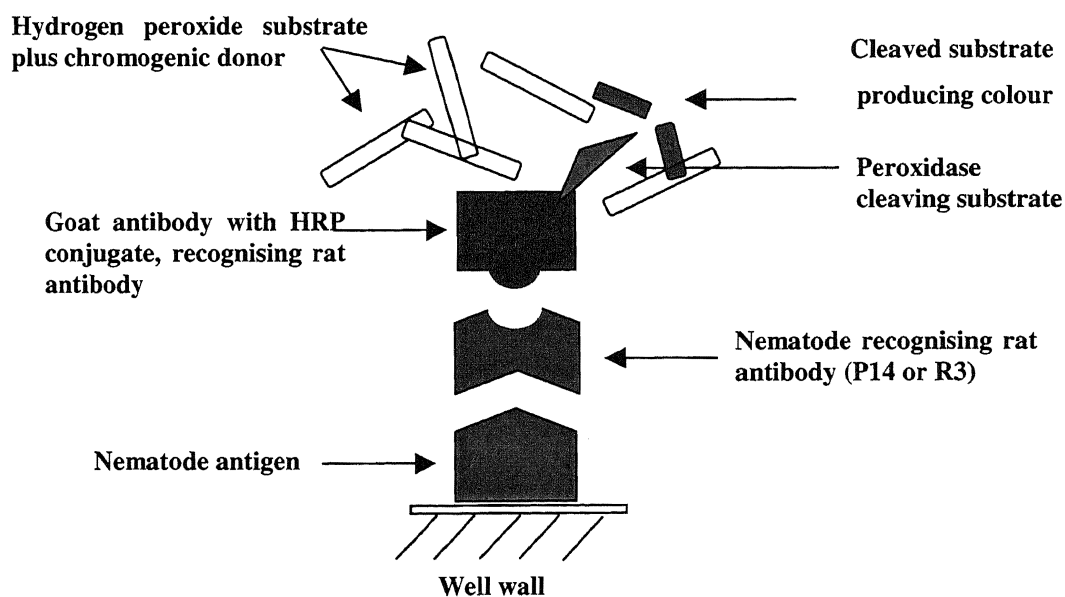


Figure 7.3 Example of an antigen/antibody complex in an indirect ELISA.

7.2.2.3 Protocol for indirect ELISA

Cysts were extracted from the soil samples using a Trudgill fluidising column (as in section 2.5), discarding any that had obvious abnormalities such as obvious parasitism by fungi. Individual cysts were put into micro homogeniser tubes (Biomedix, Pinner, U.K.) with 20 μl phosphate buffer solution (PBS: 1.068 g l^{-1} $\text{Na}_2\text{H}_2\text{PO}_4$, 0.39 g l^{-1} NaH_2PO_4 , 8.5 g l^{-1} NaCl). The cysts were then ground using an electric drill at speeds below 200 rpm. Proteins can quickly become denatured by increased temperature and care was taken at this stage due to heat generated by the friction of the homogenising process. The homogenate were centrifuged at 11,000 rpm for 30 s, to concentrate the homogenate at the base of the tube, then put on ice. A further 200 μl of PBS was added to each sample of homogenate and mixed using a pipette.

The nematode antigen (100 μl) was added to the wells of the first row of an ELISA plate. Two wells per individual nematode (Figure 7.4). Fifty microlitres of PBS were added to the five rows of wells below the top row. A serial dilution of the antigen was carried out by removing 50 μl from the top row and mixing it with the PBS in the second row. This was repeated until the sixth row, from which 50 μl was discarded after mixing leaving a final volume of 50 μl in the well. The plates were kept in the fridge over night for the antigen to bind.

The ELISA plates were developed using an adaptation of a protocol developed at IACR-Rothamsted (Curtis *et al.*, 1998). The wells were washed out 3 times with

PBST (PBS with 3 ml l⁻¹ Tween 80). R3 (*G. rostochiensis* recognising antibody) was diluted 1 in 3000 with PBSTM (PBST with 5% w/v Marvel™ milk powder). P14 (*G. pallida* recognising antibody) was diluted 1 in 400 with PBSTM. The plates were shaken for 2 h on a plate shaker at 75 rpm. The wells were washed out with PBST 3 times and 50µl of goat antibody (Sigma, A9037) diluted in PBSTM (1:5000) was added. The plates were shaken for 40 min before being washed 3 times with PBST. To each well, 80 µl of substrate (1 ml sodium acetate, 9 ml distilled H₂O, 100 µl DMSO and marker, 5 µl H₂O₂) was added. The reaction was stopped after 15 min by the addition of 30 µl sulphuric acid. The plates were read at two wave lengths (450 nm and 620 nm) using a Labsystem Multiscan .

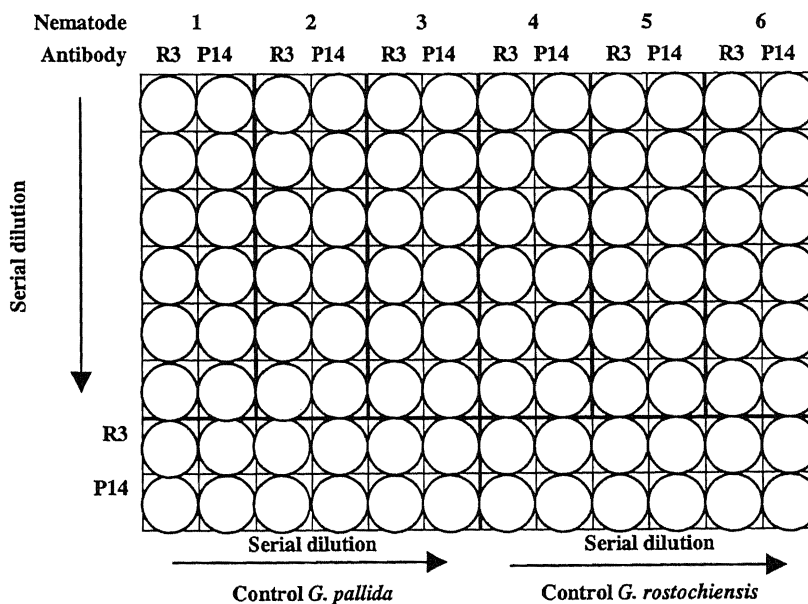


Figure 7.4 Layout of ELISA plate used to determine the number of *G. pallida* and *G. rostochiensis* from Jersey field soil.

7.2.3 RAPD-PCR

7.2.3.1 Outline of RAPD-PCR

The random amplification of polymorphic DNA (RAPD) using polymerase chain reaction (PCR) techniques is a very sensitive method for designating and recognising genetic markers. The sensitivity is due to the short length of the primers (10 nucleotides) which have a greater chance of finding a corresponding match along the length of a piece of DNA than a primer containing more nucleotides. The advantage of the sensitivity is that, in theory, only a single piece of DNA is required for the PCR to be successful. The main disadvantage is its sensitivity in that any nontargeted DNA that is inadvertently included in a reaction is likely to be magnified with the targeted DNA and hence disrupt the intended results.

In principle, the DNA is denatured by heat from double to single strands. The temperature is then reduced and the primer anneals to a single complementary strand and with the help of an enzyme (Taq DNA polymerase) and an excess of single nucleotides, a new complementary strand is formed. The cycle is repeated and there is an exponential increase in the strands of DNA primed by the introduced oligonucleotides. This results in quantities of same length DNA that can be visualised on a gel.

7.2.3.2 DNA extraction

Following a protocol used at IACR-Rothamsted (Halford 1999, pers. com.), different life stages of stained PCN (section 7.2.1) were washed in distilled water and placed into micro homogenisers with 10 µl of 1 x concentration PCR buffer (Promega, M1901) and homogenised using a drill at speeds below 200 rpm. Where possible, all samples were kept on ice. The homogenates were centrifuged at 11,000 rpm for 3 min. The samples were put into sterilised 0.5 ml Eppendorf tubes that already contained 80 µl 6% Chelex-100 resin (BioRad, 143-2832) and 10 µl 1 x PCR buffer. The tubes were incubated in a water bath at 56°C for 30 min and vortexed regularly. Tubes were then incubated at 99°C for 8 min in a PCR machine (OmniGene, Hybrid), vortexed and centrifuged for 2-3 min at 13000 rpm. The supernatant was stored at -20°C until needed.

7.2.3.3 Polymerase chain reaction

Three, ten nucleotide primers (10 mer oligonucleotides) were used, E19 (ACGGCGTATG), K4 (CCGCCCAAAC) and K16 (GAGCGTCGAA).

A stock solution of PCR reagent was made that contained per 2 µl of DNA product: 36.35 µl sterile distilled water, 5 µl 10 x concentration of reaction buffer (Taq DNA polymerase storage buffer, Promega), 3 µl (25 mM solution) MgCl₂, 3 µl primer (50 ng µl⁻¹ solution either E19, K16 or K4), 0.25 µl dNTP. The DNA product, stock solution and 4 µl of Taq polymerase (Promega, M2868) were

added to Eppendorf tubes, then the solution was overlaid with a drop of light white mineral oil (Sigma, 8042-47-5). Tubes were put into the PCR machine which was programmed to heat the samples as shown in Table 7.1. An outline of the samples processed is shown in Tables 7.2 and 7.3.

Table 7.1 Program of cycles for RAPD-PCR.

Stage	Step	Temp(°C) / Time
1 Number of cycles = 1	1	92 / 2 min
	2	55 / 1 min
	3	72 / 1 min
2 Number of cycles = 38	1	92 / 1 min
	2	35 / 1 min
	3	72 / 1 min
3 Number of cycles = 1	1	92 / 1 min
	2	35 / 1 min
	3	55 / 1 min

Table 7.2 Outline of samples processed with the RAPD-PCR techniques using the primers K16 and K4. Plants were previously treated with 100 g of *P. lilacinus* amended alginate pellets (100 g Pl).

PCR reaction	Treatment	Lanes	PCN sampled	Primer
1	100 g Pl	1-2	Juvenile	K16
		3-4	Small female (a)	
		5-6	Large female	
		7-8	Male	
		9-10	Small female (b)	
		11-12	Positive control (<i>G. pallida</i>)	
		13-14	Positive control (<i>G. rostochiensis</i>)	
		15-16	Negative control	
2	100 g Pl	1-2	Juvenile	K4
		3-4	Small female (a)	
		5-6	Large female	
		7-8	Male	
		9-10	Small female (b)	
		11-12	Positive control (<i>G. pallida</i>)	
		13-14	Positive control (<i>G. rostochiensis</i>)	
		15-16	Negative control	

Table 7.3 Outline of samples processed with the RAPD-PCR techniques using the primer E19. Plants were previously treated with 15 g of *P. lilacinus* amended alginate pellets (15 g Pl), 100 g of *P. lilacinus* amended alginate pellets (100 g Pl) or left untreated (control).

PCR reaction	Treatment	Lanes	PCN sampled	Primer
3	15 g Pl	1-2 3-4 5-6 7-8 9-10 11-12 13-14	Large female (a) Large female (b) Large female (c) Large female (d) Juvenile Positive control (<i>G. pallida</i>) Negative control	E19
4	Control	1-2 3-4 5-6 7-8 9-10 11-12 13-14 15-16 17-18 19-20 21-22 23-24 25-26 27-28	Male (a) Male (b) Male (c) Large female (a) Large female (b) Large female (c) Large female (d) Large female (e) Large female (f) Large female (g) Large female (h) Positive control (<i>G. pallida</i>) Positive control (female 1b) Negative control	E19
5	100g Pl	1-2 3-4 5-6 7-8 9-10 11-12 13-14 15-16 17-18 19-20	Large female (a) Large female (b) Large female (c) Large female (h) Large female (f) Male (a) Male (b) Male (c) Positive control (<i>G. pallida</i>) Negative control	E19
6	100g Pl	1-2 3-4 5-6 7-8 9-10 11-12 13-14 15-16 17-18 19-20 21-22 23-24 25-26	Large female (a) Large female (b) Large female (c) Small female (a) Small female (b) Small female (c) Juvenile (a) Juvenile (b) Juvenile (c) Male Positive control (<i>G. pallida</i>) Positive control (<i>G. rostochiensis</i>) Negative control	E19
7	15 g Pl	1-2 3-4 5-6 7-8	Positive PCN control Large female (1d) Juvenile Negative control	E19

7.2.3.4 Agarose gel electrophoresis

To visualise the PCR products, a 0.5% TBE agarose gel was made by dissolving 1 g of agarose gel in 0.5 x Tris-borate (TBE) (Tris base, 13 g; boric acid, 5.5 g and 0.5 M EDTA (pH 8.0), 4 ml). The UV-sensitive dye ethidium bromide (4 µl per 100 ml of TBE) was added to the cooled agarose solution before it was poured into the chamber to set.

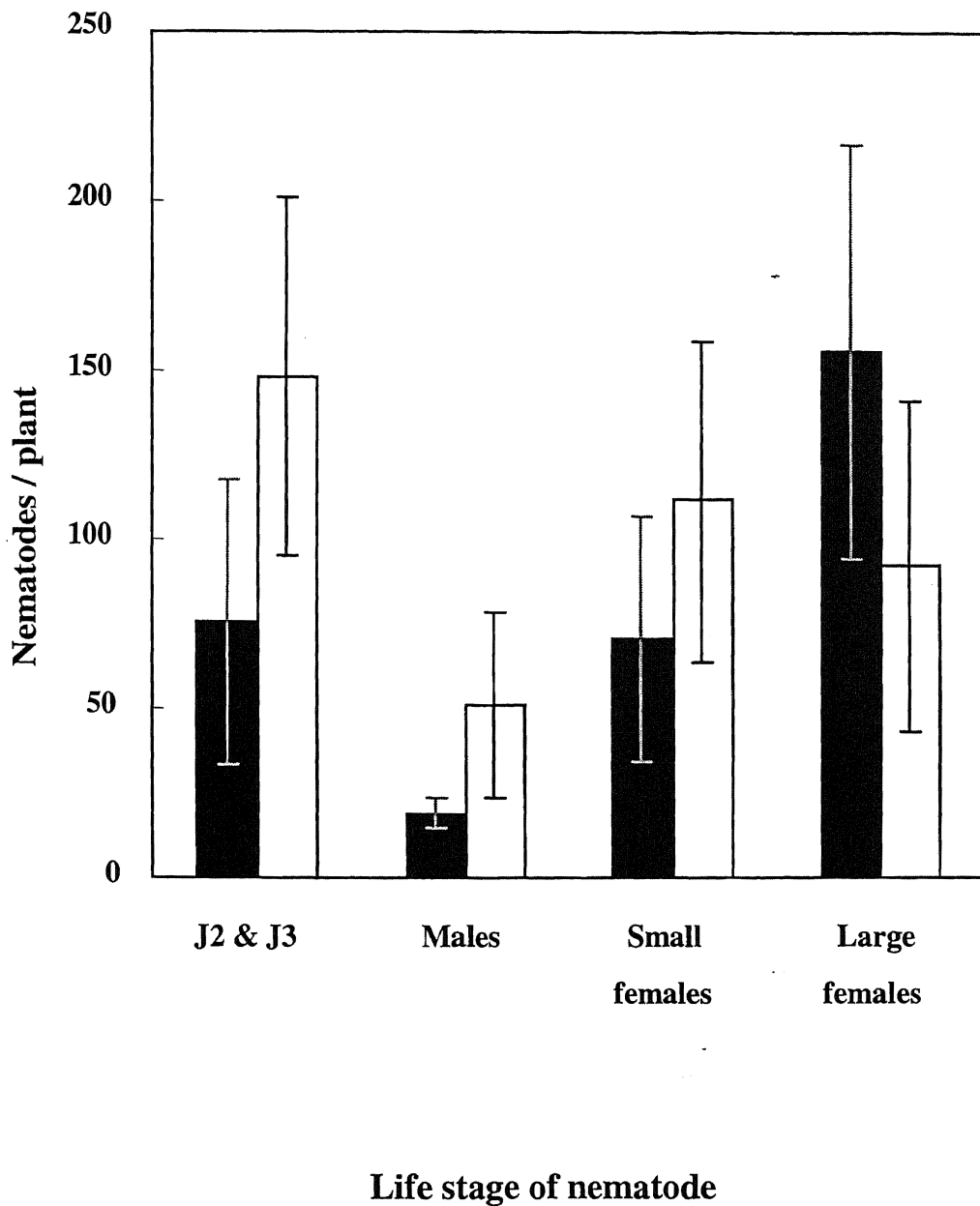
The wells of the gel were loaded with a mixture of 6 µl of the unknown DNA PCR products, 2 µl Blue/Orange 6 x loading dye (Promega) and 4 µl sterile distilled water. Other controls were also run on the gel and these consisted of 4µl 100 bp DNA ladder (Gibco BRL), a negative control containing no DNA and a positive control which contained known PCN DNA. The gel was run for 45 min at 90 volts. The bands on the gel were visualised using UV light.

7.3 RESULTS

7.3.1 LIFE STAGES OF PCN IN ROOTS

Staining of nematodes in the root samples revealed more juveniles, males and small females in the *P. lilacinus* treated group than in the control group (Figure 7.5). There were, more large females in the control group than in the *P. lilacinus* treated group, however these differences were not statistically significant ($P < 0.05$, t-test).

Figure 7.5 Proportion of PCN at different life stages in stained roots taken from the plunge experiment. PCN populations from untreated controls (■) were compared to those on plants treated with 100g of *P. lilacinus* (□) amended alginate pellets. Error bars show standard deviations. N = 5.



7.3.2 ELISA

It was difficult to determine whether applying the nematophagous fungi had an effect on the distribution of PCN species in the plunge trial (Table 7.4). For most samples there were more unknowns than positive identifications, which would indicate that the antibody is cross reacting. No *G. pallida* were identified in the samples from pots treated with *P. lilacinus* for two successive seasons.

7.3.3 RESULTS FROM RAPD-PCR

Work at IACR-Rothamsted had shown it was possible to derive PCR products from nematodes that had been stained using the hot acid fuchsin method as outlined in the methods (Halford 1999, pers. com.). However, the work was very preliminary and the results variable.

The reactions processed using the E19 primer, as outlined in Table 7.3 in the methods section (7.2.3), gave poor results, in part due to contamination, but there were indications this was due to there being insufficient, undamaged DNA. After each set of reactions, fresh nematodes were recovered and although the whole process was repeated from the beginning and new chemicals were used each time, the results continued to be unclear.

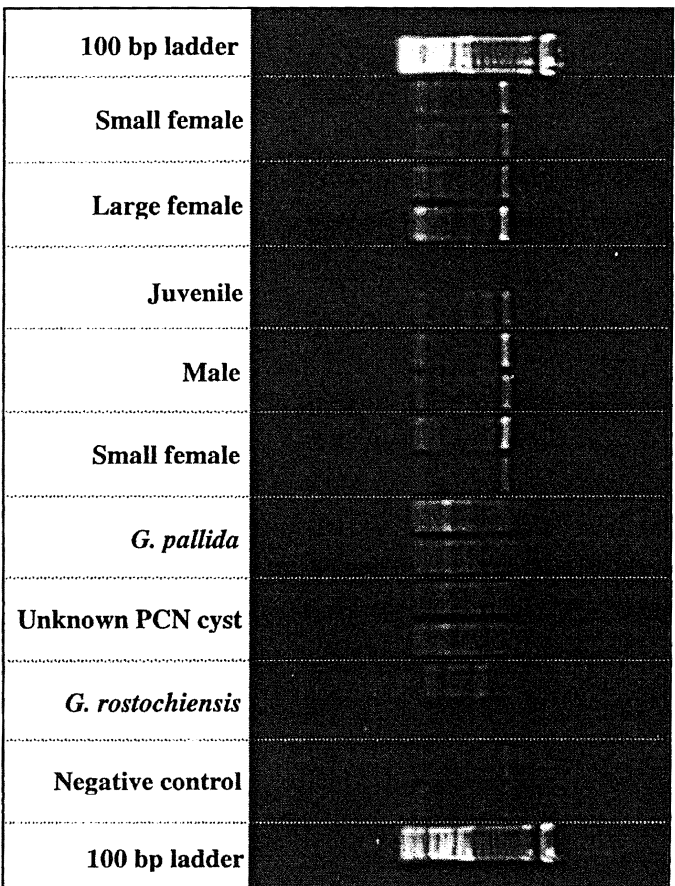
The primers K16 and K4 gave much clearer banding patterns (Figure 7.6) but there continued to be a problem with suspected contamination as can be seen in

the lanes containing negative controls. Unfortunately, the banding patterns for the positive controls are very indistinct and do not represent typical patterns for these species for these primers (Barker 2000, pers. com.). The banding patterns of the different life stages from the K16 PCR reactions, are very similar and may represent the same species but it is not possible to discern which species due to the poor controls.

Sample details				Proportion of nematodes identified		
Treatment	Dose	No. of seasons	Sampled	<i>G. rostochiensis</i>	<i>G. pallida</i>	Unknown
Control	None	1	planting	2	5	5
Control**	None	1	harvest	4	11	9
<i>P. cucumerina</i>	30 g	1	planting	1	1	10
<i>P. cucumerina</i>	30 g	1	harvest	5	3	4
<i>P. lilacinus</i>	30 g	1	planting	1	1	10
<i>P. lilacinus</i>	30 g	1	harvest	1	4	7
<i>P. lilacinus</i>	100 g	1	planting	1	6	5
<i>P. lilacinus</i>	100 g	1	harvest	4	5	3
Control	None	2	planting	5	1	6
Control	None	2	harvest	6	2	4
<i>P. lilacinus</i>	30 g	2	planting	2	0	10
<i>P. lilacinus</i>	30 g	2	harvest	2	0	10
<i>P. cucumerina</i> *	30 g	2	planting	1	0	5
<i>P. cucumerina</i>	30 g	2	harvest	3	1	8

Table 7.4 Determination of the proportion of *G. rostochiensis* and *G. pallida* in selected pots from the plunge trial, using indirect ELISA. Twelve cysts (except *, 6 cysts **, 24 cysts) were extracted from soils taken at planting and at harvest. Unknowns either with OD >100 or very similar readings for P14 and R3.

a)



b)

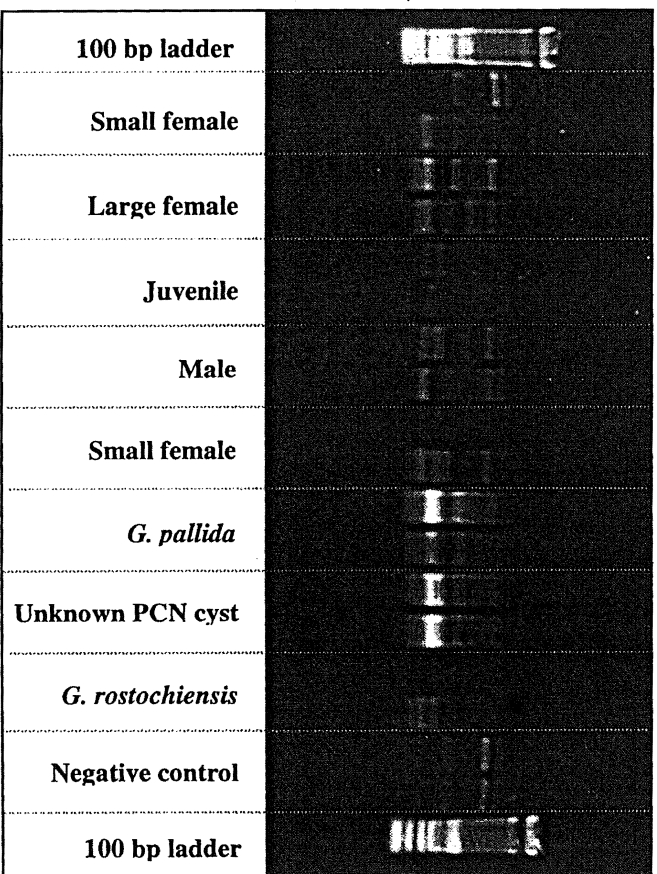


Figure 7.6 RAPD-PCR showing DNA products extracted from stained PCN at different life stages within potato roots using different primers a) K16 and b) K4.

7.4 DISCUSSION

The higher proportion of juveniles, males and small females determined by staining in the *P. lilacinus* treated group compared to the control group may have been due to the alginate or fungus containing a compound that caused the juveniles to hatch. Seaweed, a component of alginate pellets, has been reported to reduce the fecundity of plant parasitic nematodes (Whapham *et al.*, 1994; Wu *et al.*, 1997). However, seaweed extract has been observed to increase the number of hatched PCN juveniles (Sheridan, 2000, pers. com.).

The greater number of large females in the control group compared to the *P. lilacinus* treated group may be due to the fungus parasitising the females and preventing development from small to large.

The results from the indirect ELISA (Table 7.4) show that there is a mixed population of PCN in at least one field in Jersey. Out of the 174 cysts tested 38 were identified as *G. rostochiensis*, 40 as *G. pallida* and 96 were not identifiable. Therefore, it appears the proportions of *G. pallida* to *G. rostochiensis* were similar. The large number of unknowns (optical density >100. Table 7.4) may be due to the fungal infection of the nematodes, as Curtis *et al.* (1998) found PCN cysts exposed to nematophagous fungi did not react to the antibodies. This was because the eggs were no longer alive and, therefore, did not possess intact, recognisable sites on the antigen. These antibodies could therefore potentially be used to determine the amount of fungal infection in a population of PCN eggs

with suitable positive and negative controls. The indeterminate results from the ELISA readings, could also be attributed to the antibodies cross reacting with both species of *Globodera*. For some of the cysts there was enough antigen to give a optical density reading greater than 100, but both antibodies, R3 and P14, recognised the antigen almost equally.

Due to the high numbers of unknowns from the ELISA results, it was difficult to determine whether the population composition of PCN was shifting from one species to another following treatment with the nematophagous fungi. A larger number of cysts would need to be tested to determine whether there is a real change in the population.

Overall, the RAPD-PCR results were inconclusive due to contamination as indicated by the negative controls (Figure 7.6). However, the bands evident in the positive control for the K16 primer set of reactions are faint and indicate the level of contamination was low in this case. With the K16 primer, the banding pattern of the unknown samples closely match those of *G. pallida* (Halford 2000, pers. com.). This implies that the positive controls have either degenerated or become contaminated hence the cloudy and indeterminate banding.

With the PCR reactions using the K4 primer, the replication and banding is poor. In addition to contamination, this may be due to low levels of original DNA. With very low levels of DNA, PCR reactions can produce spurious banding patterns due to the primers trying to replicate with themselves.

The nematodes used for the PCR were stained in the roots using acid fuchsin stain to enable location and recovery before being used for the PCR reaction. This is the first time it has been reported that DNA from stained nematodes can be used for PCR. The poor results may be due to the staining technique disrupting the strands of DNA by breaking the single strands into small sections. This may either be due to the heat or acid breaking the DNA's hydrogen bonds.

Tests were carried out to determine the minimum exposure time needed for the nematodes within the root to become stained. It was found that after one minute, the nematodes were stained sufficiently to allow for identification. Unfortunately, there was not enough time to complete the studies and ascertain if the DNA recovered from the samples treated with the adjusted protocol, improved the results. Different temperatures and stains also need to be investigated to determine whether useful DNA can be extracted for successful PCR reactions. Longer oligonucleotides may help to reduce the low level DNA contamination as they are more specific to the target DNA (Fullando *et al.*, 1999). Finally, the PCR program could be adjusted to perhaps increase annealing time, for example.

8 GENERAL DISCUSSION

8.1 FACTORS GOVERNING THE DEVELOPMENT OF PCN POPULATIONS IN JERSEY

8.1.1 ECONOMIC AND COMMERCIAL REQUIREMENTS

In Jersey, potatoes are monocropped which can potentially lead to very high levels of PCN building up in the soil. Rotation cannot be used as a method of control in Jersey, as it is on the mainland, because the high value of the land is such that farmers cannot afford to grow crops such as cereals that have low cash returns. Therefore, chemical control is currently the main means of controlling PCN. However, there is considerable political pressure to reduce chemical inputs and move towards a more organic approach to farming, with the intention for the island of Jersey to eventually grow only organic produce.

8.1.2 EFFECTS OF NEMATICIDES, NATURAL CONTROL AND TIMING OF HARVEST

A number of factors should be taken into account when considering the population dynamics of PCN in Jersey. These are: the two species of *Globodera* now known to be present in Jersey; the early lifting dates for Jersey Royal

potatoes; the possible presence of natural control in some fields; and the application of nematicides.

At present, the use of nematicides is only permitted in fields with over 7 eggs g⁻¹ soil, and this accounts for approximately 30% of all fields in which potatoes are grown. Therefore, approximately 70% of potato fields are not treated with nematicides, although these are still cropped annually. The PCN populations in some of these fields are not increasing, which indicates there is some form of natural control. A survey of nematophagous fungi in Jersey found that the three most commonly occurring species, *Paecilomyces lilacinus*, *Plectosphaerella cucumerina* and *Verticillium chlamydosporium*, were not present in all of the fields sampled (Appendix 2.2). The presence of these fungi in some of the fields not treated with nematicides could be one explanation as to why numbers of PCN do not increase after each crop of potatoes.

The ELISA results and the observations of cyst colour made by the author, clearly indicate the presence of both species of *Globodera* in Jersey. In the fields where nematophagous fungi are absent and nematicides are not used, the effect of early lifting of the crop is likely to alter the ratio of species towards *G. rostochiensis*. Although both *G. rostochiensis* and *G. pallida* were found on and in the roots of the potatoes lifted, a greater number of the *G. rostochiensis* found may be the offspring of nematodes hatched in the same season. This may be due to *G. rostochiensis* hatching earlier and in a shorter period in response to the growing crop than *G. pallida*.

However, due to the slower response of *G. pallida* and longer period of hatch, the effects of granular nematicides on the population of this species are less marked. The half life of the active ingredients of the two market leaders (Temik and Vydate) is approximately three weeks, which is longer than the peak hatching period for *G. rostochiensis* but two to three weeks shorter than the time taken for peak hatch of *G. pallida*. Therefore, the application of nematicides can skew the ratio of the two species of PCN towards *G. pallida*.

The early lifting dates in Jersey may have a significant effect on PCN populations where the crop is lifted up to two months ahead of its natural senescence. In both the plunge and field trial it was found that developing females were still present on the roots at harvest. This is compared to the majority of crops in the UK, which may be grown for up to five months at which point the plants are beginning to senesce naturally. Because of the early lifting, the crop can, to some extent, act as a trap crop, but due to its duration will mainly trap *G. pallida*, as most of the *G. rostochiensis* population will have completed its life cycle.

These different factors have a conflicting effect on a mixed population and predicting the outcome of a particular management decision is not a simple task.

8.2 APPLICATION OF NEMATOPHAGOUS FUNGI FOR CONTROL OF PCN IN JERSEY

8.2.1 FORMULATION

Studies have shown that alginate pellets are a useful method for entrapping fungal spores to prevent them from germinating until rehydrated, or to encapsulate fungal biomass which will produce spores later when added to the soil. A balance of nutrients is needed within the alginate pellets. The fungi must be able to survive within the inoculum until the female nematodes are present on the roots, but the nutrient source must not be so high that fungi remain in the saprophytic phase of their life cycle and fail to parasitise the nematodes. A high level nutrient provider, such as milled barley, may increase the level of competition from other microorganisms (Stirling & Smith, 1998). Therefore, if a fungus that can form resting structures, such as *V. chlamydosporium*, is added, high nutrient formulation may be a disadvantage. Further research is needed to develop the optimum level of nutrients within the alginate pellets.

An alternative to alginate pellets is a soil drench of conidia, one advantage of which is that the conidia can be applied in a nutrient poor medium. This may increase the pathogenicity towards PCN of that application because the fungus has not been encouraged into its saprophytic phase. The malt coated Terra-Green[®], could be used to culture the fungus until conidia are produced, at which point, the conidia can be harvested. A second advantage of a drench is that it can be applied

to a growing crop. Further tests would be needed to determine whether this is a viable option.

8.2.2 ANTAGONISM AND AGROCHEMICALS

Fungal antagonism within the soil needs to be considered when selecting a fungal species for biological control. This work found that a combination of nematophagous fungi may not be as effective as a single species, as the different species may have some inhibitory effect on the growth of each other. If more than one species of fungus is required in a single field application, compatibility tests should be undertaken.

Our studies indicate that an IPM strategy could be implemented in Jersey in which the nematophagous fungi *P. lilacinus* and *V. chlamydosporium*, are used with tuber applied fungicides. The ability of the fungi to function in the presence of certain fungicides is a valuable asset, particularly when convincing a farming community to use a non-established method of disease control. Also of benefit, an additional field operation is not necessary for the application of the biocontrol agent.

Paecilomyces lilacinus was also shown to reduce the incidence of *R. solani* without the aid of a fungicide. This fungus is potentially very useful as a dual pest control agent for use in organic farming or within an IPM strategy.

8.2.3 FUNGAL SPECIES

Fungal species isolated for use in a biological control may be more effective if they are isolated from the same geographical region in which the target pest is found as they may be adapted to infect the target pest. The biological control agent will also be able to survive the local climate and soil conditions. The mode of parasitism of the nematophagous fungi can vary depending on the region from which they have been isolated. For example the distribution of nematode trapping fungi is determined by the pH of the soil more than any other factor, such as soil moisture, nematode density, predators or organic matter (Gray, 1985). Therefore, local isolation and production of biological agents is one way of ensuring optimum control of the pest by the control agent. The isolates used in the studies described in this thesis were all taken from Jersey.

The phenotypic variation found between the monoconidial isolates, in addition to potentially helping prevent resistance build up in the target pest, may indicate a level of flexibility in adapting to other factors such as a variation in soil environments across and between fields. The variation between monoconidial isolates is also important when considering the potential genotypic variation of the PCN populations the fungus may be used against. If a combination of polyclonal isolates is used, then genotypic as well as phenotypic variation would be apparent in an application of a fungus, further improving the long term efficacy of the fungus.

There have been some reports that *P. lilacinus* may be pathogenic to man (Castro *et al.*, 1990). However, this fungus appears to be an opportunistic parasite and tends to infect only immuno-compromised patients. *Paecilomyces lilacinus* has been commercialised in Australia for controlling plant parasitic nematodes as Paecil™, also called Bioact™, and Nemachek, and toxicity tests for this product have found it is non toxic but caution is needed when using these products. *Paecilomyces lilacinus* can produce very large numbers of conidia which can form airborne dust that is easily inhaled and may cause an allergic reaction (Gumowski *et al.*, 1991). To prevent this from happening, the fungus can be augmented to encapsulate the conidia.

At present the mode of action for fungal infection of the nematode is not known. The fungus is known to infect the female stage of the life cycle; this was confirmed when comparing *P. lilacinus* treated plants to untreated plants (Figure 7.5). It would appear that alginate pellets containing *P. lilacinus* increased hatch compared to the controls, but then attacked the young female PCN when developing on the root, to prevent the development of mature females. Further work is needed to determine whether the fungi are invading the nematodes via the mouth, having colonised the plant endophytically, or are penetrating the nematode through its cuticle, possibly through ectophytic colonisation of the potato roots. By studying stained roots under a light microscope, the author observed all three fungi colonising the roots as ectophytes (data not shown). Techniques such as transmission electron microscopy or confocal microscopy may be able to determine more conclusively whether the fungi are endophytes.

These studies did not show any difference in the susceptibility of the two species of PCN to control by nematophagous fungi studied. The long term effect of these biological control agents would be to reduce the population overall but not to change the ratio between the species. However, the results are inconclusive and further work needs to be done to confirm this.

8.2.4 RECOMMENDATION FOR CHANGES IN CROP MANAGEMENT PRACTICES

The results given in the thesis indicate that these fungi could be used for commercial control of PCN in Jersey, possibly replacing or augmenting chemical nematicides and some fungicides. A suitable regime for the control of PCN in Jersey, using the augmented nematophagous fungi developed in this thesis, would be to broadcast the alginate pellets throughout the soil, to ensure even distribution and prevent the possible phytotoxic effect of the alginate pellets. This would be followed by a soil drench of *V. chlamydosporium* to prevent females developing on the root, applied post-harvest, when soil temperatures are above the growth threshold for that fungus.

The application of a biological control agent has to be justified economically in terms of increased tuber yield over a period of years. The fixed cost for the control of nematodes using either biological control or chemical nematicides is the cost of the tractor and man hours for application of the product. The variable cost is that of the product applied. The economic threshold will be reached when the application of the biological control agent is paid for by increased return of yield.

With the use of nematophagous fungi the cost could decrease over the years as the fungi become established in the soil. However, more data is needed on the long term effectiveness of the fungi in field soil over several years.

The disadvantage of using fungal biocontrol in comparison to nematicides is that nematicides will reduce nematode populations during the growing season so yield will not be lowered, but treatment with fungi may not show yield increase until the second season. To minimise yield losses, the nematophagous fungi could be applied to fields with low PCN egg counts to prevent a build up of PCN the following year. If a field has a high nematode infestation, granular nematicides could be applied in combination with the fungi. If the population was predominantly *G. pallida*, the nematicide would have an effect on the initial hatching juveniles but break down after a few weeks, whereas the fungi will remain saprophytic in the soil until the females are present and then attack them. By adding the fungus with the nematicide, the fungus has a chance to work in the first year and may remain present in the soil until the next year.

8.3 RECOMENDATIONS FOR FUTURE WORK

A recurrent problem throughout this thesis and common within the field of biological control (and nematology) is the variation in final egg or nematode counts within treatment groups. For many of the results described within this thesis, there are large differences between experimental treatments that have not

been statistically significant. Some of the work should be repeated but with a greater number of replicates.

As mentioned in chapter seven (section 7.4) alginate pellets have been found to cause premature hatch in PCN. If further studies could demonstrate that this occurs with no crop present, the pellets could be applied sometime before cropping thereby inducing hatch when there is no host plant present. However, there are a number of factors to be considered, such as soil temperature and season. Regardless of obvious environmental factors such as temperature fluctuation and soil water content, PCN appear to preferentially hatch in the spring rather than at any other time of year.

Field scale studies are needed to validate the efficacy of the three fungi against PCN when used in a commercial situation in conjunction with other pesticides. These studies would need to be extended over several seasons to assess long term changes in PCN and fungal populations.

Studies to look at the efficacy of the fungi against different populations of each species of PCN both in Jersey and in other parts of the world such as South America, the area in which PCN originated, needs to be conducted. This is because there are a large number of recognised pathotypes and isolates of PCN (Bendezu, 1997), which may be affected differently by different isolates of fungi and the right isolate for the targetted PCN population would need to be found.

The ability to predict the response of a PCN population within a particular field would be very useful as a management tool but, as described earlier, the factors to be considered have a complex effect. Further studies to look at any selective effects of a biological control on a mixed PCN population would also be needed.

In summary, the work outlined in this thesis has demonstrated that there is potential for a commercially viable biological control agent that can be easily incorporated into current farming practices.

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APPENDICES

APPENDIX 1 PUBLICATIONS

VARIATION AND CHANGES WITHIN SPECIES OF NEMATOPHAGOUS FUNGI

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Abstract - *IOBC Working Group*, International Organisation for Biological Control, Wageningen, The Netherlands. December, 1996.

The fungi that have been studied for use as biological control agents of nematodes are subject to much variation in growth rates, morphological appearance, spore production, and pathogenicity towards nematodes. Not only do these variations exist between geographically different isolates of the same fungal species, but also between different single spore isolates taken from the same culture. Additionally, these fungi can change with time, depending on the conditions in which they are kept. This partly explains some of the problems and inconsistencies that we have experienced in working with such fungi. However, by understanding more about these variations, and exposing the fungi to certain selective pressures, it may be possible to improve our strains, and make them more stable.

POTENTIAL FOR BIOLOGICAL CONTROL OF POTATO CYST NEMATODES IN
JERSEY

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Abstract – *Nematology*, Association of Applied Biologists, London, UK,
December, 1997.

Jersey Royal potatoes are an important industry for Jersey with an export value of nearly £28 million in 1996. Fields are intensively cropped, occasionally twice per annum, so there is no opportunity for crop rotation to control potato cyst nematodes (PCN). Control of these pests in Jersey has traditionally relied on the use of nematicides, but with increased awareness of the deleterious effects of nematicides there is a desire to find alternative control measures. Due to the high value but small scale nature of the industry, the intensity of the cropping and the climate, the use of a fungal biological control agent, integrated with other control measures, looks promising.

Three nematophagous fungi have been studied as potential biocontrol agents, *Acremonium sp.*, *Paecilomyces lilacinus* and *Verticillium chlamydosporium*. The survival of these fungi when applied to the field will depend upon interactions of the nematophagous fungi with agrochemicals and with other soil fungi. *In vitro* tests have shown that fungicides used to control Black scurf and Stem canker caused by *Rhizoctonia solani* reduce the growth rate and alter the morphology of the nematophagous fungi studied. It is known that the incidence of *R. solani* on

plants is reduced as a consequence of competition with other fungi (Chand & Logan, 1984. Trans. Brit. Mycol. Soc. **83**, 107-112). The nematophagous fungi were therefore tested against *R. solani*, to determine whether they could inhibit the growth of this plant pathogen, removing the need to apply fungicides. *Paecilomyces lilacinus* was an antagonist towards *R. solani* and could possibly be used as a biocontrol agent for both PCN and *R. solani*. *Verticillium chlamydosporium* inhibited *R. solani* and *Acremonium sp.* at 20°C but not at 10°C, showing that competition between the fungi is affected by temperature. *Acremonium sp.* was found to be a poor competitor when grown in the presence of the other fungi; this will have to be taken into account if this species is developed further as a biological control agent for PCN.

BIOLOGICAL CONTROL OF POTATO CYST NEMATODES IN JERSEY

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Abstract – IOBC Working Group, International Organisation for Biological Control, Bonn, Germany, February, 1998.

The survival of the nematophagous fungi *Acremonium sp.*, *Paecilomyces lilacinus* and *Verticillium chlamydosporium*, when applied to the field will depend upon interactions with agrochemicals and the interactions with abiotic and biotic soil factors. *In vitro* tests have shown that fungicides used to control Black scurf and Stem canker caused by *Rhizoctonia solani* reduce the growth rate and alter the morphology of the nematophagous fungi studied. It is known that the incidence of *R. solani* on plants is reduced as a consequence of competition with other fungi (Chand & Logan, 1984. Trans. Brit. Mycol. Soc. **83**, 107-112). The nematophagous fungi were therefore tested against *R. solani*, to determine whether they could inhibit the growth of this plant pathogen, removing the need to apply fungicides. *Paecilomyces lilacinus* was an antagonist towards *R. solani* and could possibly be used as a biocontrol agent for both potato cyst nematodes (PCN) and *R. solani*. *Verticillium chlamydosporium* inhibited *R. solani* and *Acremonium sp.* at 20°C but not at 10°C, showing that competition between the fungi is affected by temperature. *Acremonium sp.* was found to be a poor competitor when grown in the presence of the other fungi and may affect its potential as a biological control agent for PCN.

ANTAGONISM BETWEEN AGROCHEMICALS, PLANT PATHOGENIC FUNGI AND
NEMATOPHAGOUS FUNGI.

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**Abstract – *The future of fungi in the control of weeds, pests and diseases*,
British Mycological Society, Southampton, UK. April, 1998.**

The nematophagous fungi *Acremonium sp.*, *Paecilomyces lilacinus* and *Verticillium chlamydosporium* are potential biological control agents for the potato cyst nematode (*Globodera sp.*). The survival of these fungi when applied to the field will depend upon interactions of the nematophagous fungi with agrochemicals and other soil fungi. *In vitro* tests have shown that fungicides used to control Black scurf and Stem canker caused by *Rhizoctonia solani* reduce the growth rate and alter the morphology of the nematophagous fungi studied. It is known that the incidence of *R. solani* on plants is reduced as a consequence of competition with other fungi (Chand & Logan, 1984. *Trans. Brit. Mycol. Soc.* **83**, 107-112). Competition between the nematophagous fungi and *R. solani* was investigated by growing the four species in pairs on plates. *P. lilacinus* was antagonistic towards *R. solani* and could possibly be used as a biological control agent for both PCN and *R. solani*, thus reducing the need for fungicide application and increasing the likelihood that a fungal biological control agent would persist in the soil. *V. chlamydosporium* inhibited *R. solani* and *Acremonium sp.* at 20°C but not at 10°C, showing that competition between soil fungi is affected by temperature. *Acremonium sp.* was found to be a poor competitor when grown in

the presence of the other fungi, which may compromise its potential as a biological control agent for PCN.

DEVELOPMENT OF AN IMMUNOASSAY FOR THE IDENTIFICATION AND
QUANTIFICATION OF *GLOBODERA PALLIDA* AND *G. ROSTOCHIENSIS* USING
THE 34 KD ANTIGEN

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Abstract – *Nematology*, Association of Applied Biologists, London, UK,
December, 1998.

Two monoclonal antibodies which can differentiate *G. pallida* from *G. rostochiensis* by recognising the two diagnostic proteins with pIs of 5.7 and 5.9 respectively, both of which have a molecular weight of 34kD, are being used to develop a quantitative assay. The monoclonal antibody which recognised *G. pallida* showed much greater variation in replicate tests with different batches of *G. pallida* cysts than did the monoclonal antibody which recognised *G. rostochiensis*. Results obtained using single cysts were far more variable than when 5 or more cysts were processed simultaneously but the variation observed between single cysts did not correlate to cyst size. Tests that used BSA, milk powder or extracts of other nematodes species to block non-specific binding sites did not reduce the variation. Factors such as soaking time and storage temperature following extraction also affected the variability of the assay.

Small amounts of cross-reactivity by the monoclonal antibodies makes the identification and accurate quantification of mixed potato cyst nematode samples difficult. A method is being developed which quantifies each of the nematode

species by probing extracts of mixed samples by each of the two monoclonal antibodies. A set of standard mixtures of antigen of the two species of potato cyst nematodes was made and the concentration of antigen for each nematode species plotted against optical density for each monoclonal antibody. From these plots of standard mixtures, planes of equivalence can be constructed. At a given optical density, two lines can be constructed along two planes formed by a set of standard mixtures of the two potato cyst nematode species. The lines can be described by equations which, when solved simultaneously, will give x and y coordinates that can be compared to the standards. From this it is possible to identify and quantify each of the nematodes present in the sample.

APPENDIX 2 SUPPLEMENTARY DATA

APPENDIX 2.1 EFFECT OF PRE-TREATMENT OF ALGINATE PELLETS

Table A2.1 The effect of pre-treatment of alginate pellets on numbers of *G. rostochiensis* eggs (section 5.3.2.3).

Eggs g ⁻¹ soil	Pellet pre-treatment			
	Replicate	Pellets	Crushed	Soaked
1	318	102.1	248.9	
2	193.7	45.7	342.7	
3	149.5	33.1	343.8	
4	148	67.1	476	
5	155.1	63.9	125.4	
6	334.8	84.7	279.6	
7	140.7	461.2	275.2	
8	332.	10.6	141.7	
9	232.4	492.8	83.8	

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Pellets	9	2004.494	222.7215	7098.566
Crushed	9	1361.156	151.2395	34893.14
Soaked	9	2317.334	257.4816	15546.92

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	52815.94	2	26407.97	1.376882	0.271591	3.402832
Within Groups	460309.1	24	19179.54			
Total	513125	26				

APPENDIX 2.2 NEMATODE SURVEY

Figure A2.1 Nematophagous fungi recovered from nematodes and soil* in fields in Jersey.

