POTENTIAL OF BIOCONTROL AGENTS AND COMPATIBLE CULTURAL PRACTICES FOR ROOT-KNOT NEMATODE MANAGEMENT IN TOMATO

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DECLARATION

This thesis is my original work and has not been submitted for a degree in any other university.

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DEDICATION

To my beloved late mother, Editha Mhagama, who passed away few days before I submitted this thesis. She was eager to see me get what I have been working so hard for. Her prayers to me contributed to the success of this work. God Rest Her Soul in Eternal Peace, Amen.

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TABLE OF CONTENTS

DEDICATION iii

AKNOWLEDGEMENTS iv

TABLE OF CONTENTS v

LIST OF TABLES xii

LISTS OF FIGURES xiv

LIST OF APPENDICES xv

LIST OF ABBREVIATIONS xvi

ABSTRACT xvii

CHAPTER 1 1

GENERAL INTRODUCTION 1

1. 1 Introduction 1

1. 2 Objectives 6

1. 2. 1 Overall objective 6

1. 2. 2 Specific Objectives 6

CHAPTER 2 8

LITERATURE REVIEW 8

2. 1 Tomato production in Kenya 8

2. 2 Root-knot nematodes 9

2. 2. 1 Life cycle of root-knot nematode 9

2. 2. 2 Symptoms of root-knot on plants 10

2. 3 Management of root-knot nematodes 11

2. 3. 1 Chemical methods 12

2. 3. 2 Cultural methods 12

2. 3. 2. 1 Organic amendments 12

2. 3. 2. 2 Crop rotation 14

2. 3. 2. 3 Other methods 16

2. 3. 3 Biological control 17

2. 3. 3. 1 Bacteria 18

2. 3. 3. 2 Fungi 19

2. 3. 4 Integrating fungi and organic amendments in rotation system for the control of root-knot nematodes 23

CHAPTER 3 26

GENERAL MATERIALS AND METHODS 26

3. 1 Introduction 26

3. 2 Media used 26

3. 2. 1 Laboratory media 26

3. 2. 2 Soils 27

3. 3 Production of Pochonia chlamydosporia 27

3. 3. 1 Production of *Pochonia chlamydosporia* for *in vitro* experiments 27

3. 3. 2 Production of *Pochonia chlamydosporia* for glasshouse and field experiments. 28

3. 4 Preparation of root-knot nematode inoculum 29

3. 4. 1 Glasshouse experiments 29

3. 4. 2 *In vitro* experiments 30

3. 5 Preparation of organic materials and their extracts 30

3. 5. 1 Preparation of organic materials 30

3. 5. 2 Preparation of organic materials 31

3. 6 Data collection 31

3. 6. 1 Percentage eggs parasitized (infected) by fungus 31

3. 6. 2 The number of fungal propagules in soil and roots 32

3. 6. 3 Root galling index 33

3. 6. 4 Extraction of root-knot nematode juveniles from the soil and roots 33

3. 6. 5 Plant shoot weight 34

3. 7 Statistical analysis 34

CHAPTER 4 35

EFFICACY OF DIFFERENT FUNGAL ISOLATES IN THE CONTROL OF ROOT-KNOT NEMATODES 35

4. 1 INTRODUCTION 35

4. 2 MATERIALS AND METHODS 37

4. 2. 1 Source and production of *Paecilomyces lilacinus* inoculum 37

4. 2. 2 Parasitic activity of different fungal isolates on root-knot nematode eggs 38

4. 2. 3 Effect of soil sterilization on growth and efficacy of fungal isolates 38

4. 2. 4 Statistical analysis 40

4. 3 RESULTS 40

4. 3. 1 Parasitic activity of different fungal isolates on root-knot nematode eggs 40

4. 3.2 Effect of soil sterilization on growth and efficacy of fungal isolates on egg parasitism 41

4. 4 DISCUSSION 43

CHAPTER 5 48

EVALUATION OF LOCALLY AVAILABLE ORGANIC MATERIALS FOR USE IN PRODUCTION OF *Pochonia chlamydosporia* 48

5. 1 INTRODUCTION 48

5. 2 MATERIALS AND METHODS 50

5. 2. 1 Evaluation of organic substrates for the production of *Pochonia chlamydosporia* chlamydospores 50

5. 2. 2 Effect of extract from organic materials on chlamydospore production 51

5. 2. 3 Effect of different extracts from organic materials on growth of different isolates of *Pochonia chlamydosporia* 52

5. 2. 4 Effect of different concentrations of extracts from different organic materials on growth of *Pochonia chlamydosporia* 52

5. 2. 5 Statistical analysis 53

5. 3 RESULTS 53

5. 3. 1 Evaluation of organic materials for the production of *Pochonia chlamydosporia* chlamydospores 53

5. 3. 2 Effect of extracts from organic materials on chlamydospore production 54

5. 3. 3 Effect of extract from different organic materials on growth of different isolates of *Pochonia chlamydosporia* 56

5. 3. 4 Effect of different concentrations of extracts from organic materials on growth of *Pochonia chlamydosporia* 57

5. 4 DISCUSSION 60

CHAPTER 6 66

EFFECT OF ORGANIC AMENDMENTS ON THE ACTIVITY OF *Pochonia chlamydosporia* AGAINST ROOT-KNOT NEMATODES 66

6. 1. INTRODUCTION 66

6. 2 MATERIALS AND METHODS 69

6. 2 1 Efficacy of organic materials on growth of *P. chlamydosporia* and suppression of root-knot nematodes 69

6. 2. 2 Effect of different incubation periods of *Pochonia chlamydosporia* and maize stover in the management of RKN in tomato 70

6. 2. 3 Effect of time of incubation and media on the parasitic activity of *Pochonia chlamydosporia* 70

6. 2. 4 Effect of extracts from organic materials on parasitism of root-knot nematodes eggs by *Pochonia chlamydosporia* 71

6. 2. 5 Effect of different concentrations of extract from organic materials on the parasitism of *Pochonia chlamydosporia* on root-knot nematode eggs 72

6. 2. 6 Statistical analysis 73

6. 3 RESULTS 73

6. 3. 1 Effect of organic materials on growth of *Pochonia chlamydosporia* and activity against effect on root-knot nematodes 73

6. 3. 2 Effect of different incubation periods of *Pochonia chlamydosporia* and maize stover in the management of RKN in tomato 76

6. 3. 3 Effect of time of incubation (24 and 48 h), extracts from organic materials and media on the parasitic activity of *Pochonia chlamydosporia* 79

6. 3. 4 Effect of extracts from organic materials on root-knot nematode egg parasitism by *Pochonia chlamydosporia* 81

6. 3. 5 Effect of different concentrations of extracts from organic materials on parasitic activity of *Pochonia chlamydosporia* on root-knot nematode eggs.. 82

6. 4 DISCUSSION 84

CHAPTER 7 89

EFFECT OF SOIL TEMPERATURE, PH AND CARBON:NITROGEN RATIO ON GROWTH AND INFECTIVITY OF *Pochonia chlamydosporia* ON ROOT-KNOT NEMATODE EGGS 89

7. 1 INTRODUCTION 89

7. 2 MATERIALS AND METHODS 91

7. 2. 1 Effect of exposing organic materials in various temperatures on growth and parasitic activity of *Pochonia chlamydosporia* 91

7. 2. 2 Effect of pH on the infectivity of *Pochonia chlamydosporia* on eggs of root-knot nematodes 92

7. 2. 3 Effect of carbon and nitrogen ratios on the efficacy of *Pochonia chlamydosporia* to parasitize root-knot nematode eggs *in vitro* 93

7. 2. 4 Statistical analysis 94

7. 3 RESULTS 94

7. 3. 1 Effect of exposing organic materials in various temperatures on growth and parasitic activity of *Pochonia chlamydosporia* 94

7. 3. 2 Effect of pH on the parasitic activity of *Pochonia chlamydosporia* to root-knot nematode eggs 96

7. 3. 3 Effect of carbon and nitrogen ratios on the parasitic activity of *Pochonia chlamydosporia* to root-knot nematode eggs *in vitro* 97

7. 4 DISCUSSION 98

CHAPTER 8 102

INTEGRATED MANAGEMENT OF ROOT-KNOT NEMATODE USING BIOLOGICAL AGENTS, ORGANIC MATERIALS AND CROP ROTATION 102

8. 1 INTRODUCTION 102

8. 2 MATERIALS AND METHODS 105

8. 2. 1 Selection of commonly found plant species that support fungal abundance. 105

8. 2. 2 Integrated management of root-knot nematode 106

8. 2. 2. 1 Glasshouse experiment 106

8. 2. 2. 2 Field experiments 107

8. 2. 3 Statistical analysis 108

8. 3 RESULTS 108

8. 3. 1 Growth and survival of *Pochonia chlamydosporia* in the soil and rhizosphere of different plants 108

8. 3. 2 Integrated management of root-knot nematodes 109

8. 3. 2. 1 Glasshouse experiment 109

8. 3. 2. 2 Field experiments 114

8. 3. 2. 2. 1 Field trial one 114

8. 3. 2. 2. 2 Field trial two 118

8. 4 DISCUSSION 121

CHAPTER 9 127

CONCLUSIONS AND RECOMMENDATIONS 127

9.1 Introduction 127

9. 2 Conclusions 127

9. 3 Recommendation for future work 129

LITERATURE CITED 130

APPENDICES 149

LIST OF TABLES

Table 4. 1. Survival of isolates of *Pochonia chlamydosporia* and *Paecilomyces lilacinus* in sterile and non-sterile soil and their effects on second stage juveniles (J2) and galling index in tomato 42

Table 5. 1. Number and percentage viability of chlamydospores from different organic substrates. 54

Table 5. 2. Effects of extracts from organic materials on chlamydospore production and percent viability of isolates of *Pochonia chlamydosporia*. 57

Table 5. 3. Numbers of chlamydospores produced from different concentrations of extracts from organic substrates. 59

Table 6. 1. Effect of organic materials on colony forming units of *Pochonia chlamydosporia* in the soil, galling index and second stage juveniles/200cm^3 soil (J2) associated with tomato. 75

Table 6. 2. Effect of time of incubation of maize stover and *Pochonia chlamydosporia* before planting of tomato on the population of fungus (CFU), dry shoot weight and number of second stage juveniles (J2). 77

Table 6. 3. Effect of time of incubation, type of organic materials and media on percent egg infected by *Pochonia chlamydosporia*. 81

Table 6. 4. Effect of extracts from organic materials and their pH on root-knot nematodes eggs infected by *Pochonia chlamydosporia*. 82

Table 6. 5. Effect of varying concentrations of extracts from organic materials on root-knot nematode egg infected by *Pochonia chlamydosporia*. 83

Table 7. 1. Effect of decomposing organic materials at various temperatures on the population density of *Pochonia chlamydosporia*. 95

Table 7. 2. Effect of decomposing organic materials at different temperatures on the percent egg infected by *Pochonia chlamydosporia*. 96

Table 7. 3. Effects of carbon:nitrogen ratio on parasitic activity of *Pochonia chlamydosporia* to root-knot nematode eggs. 98

Table 8. 1. Treatments for integrated management of root-knot nematode in two seasons.. 106

Table 8. 2. Colonisation of *Pochonia chlamydosporia* in the soil and in roots of six different crops at 30 and 90 days after application. 109

Table 8. 3. Survival of applied *Pochonia chlamydosporia* and its effects on second stage juveniles (J2), galling index and dry shoot weight of tomato. 110
Table 8. 4. Effect of crop rotation, *Pochonia chlamydosporia* and maize stover on root-knot nematode population, galling index, dry-shoot weight and fungal propagules in the second season for the glasshouse experiment. 113
Table 8. 5. Effect of application of *Pochonia chlamydosporia* on fungal propagules (CFU) and second stage juveniles (J2) in the roots, number and yield of tomato.......... 114
Table 8. 6. Effect of application of *Pochonia chlamydosporia* and rotation crops on fungal propagules (CFU) and nematode population (J2) in the soil.............................. 116
Table 8. 7. Effect of rotation crop, *Pochonia chlamydosporia* and maize stover on root-knot nematode population, galling index, fungal propagules, number and yield of tomato in the second season in trial one of the field. 117
Table 8. 8. Effect of application of *Pochonia chlamydosporia* on colony forming units (CFU) and nematode population (J2) in the roots, number and yield of tomato............ 118
Table 8. 9. Effect of combining *Pochonia chlamydosporia* and rotation crops on the fungal propagules (CFU) and nematode populations (J2) in the soil. 119
Table 8. 10. Effect of crop rotation, *Pochonia chlamydosporia* and maize stover on root-knot nematode population (J2), galling index, fungal propagules (CFU), yield (number and weight) of tomato in the second season in trial two of the field. 120

LISTS OF FIGURES

Fig. 4. 1. Percentage of eggs parasitism by the six isolates of *Pochonia chlamydosporia* (Pc) and one isolate of *Paecilomyces lilacinus* (Pl). 41

Fig. 4. 2. Dry shoot weight of tomato, after application of *Pochonia chlamydosporia* (Pc) and *Paecilomyces lilacinus* (Pl) in sterile and non-sterile soils. 43

Fig. 5. 1. Numbers of chlamydospores produced from different extracts of various organic substrates. 55

Fig. 5. 2. Effect of organic substrates on colony diameter of isolates of *Pochonia chlamydosporia* 56

Fig. 5. 3. Colony diameter of *Pochonia chlamydosporia* on agar amended with extracts from organic materials of different concentrations. 58

Fig. 5. 4. Percentage viability of chlamydospores of *Pochonia chlamydosporia* on xtracts from organic materials at different concentrations. 59

Fig. 6. 1. Effect of organic materials and *Pochonia chlamydosporia* on dry shoot weight of tomato 74

Fig. 6. 2. Effect of incubating maize stover and *Pochonia chlamydosporia* on root galling of tomato 78

Fig. 6. 3. Effect of incubating maize stover together with *Pochonia chlamydosporia* on the root weight of tomato. 78

Fig. 6. 4. Effect of duration of exposing root-knot nematode eggs to *Pochonia chlamydosporia* on percent egg infection. 79

Fig. 6. 5. Influence of water and yeast extract media on the percent eggs infected by *Pochonia chlamydosporia*. 80

Fig. 6. 6. Effect of amending growth medium with extracts on infectivity of *Pochonia chlamydosporia* to root-knot nematode eggs. 80

Fig. 7. 1. Effect of different pH on parasitic activity of *Pochonia chlamydosporia* to root-knot nematode eggs. 97

Fig. 8. 1.Effect of maize and tomato on numbers of root-knot nematodes (J2) and fungal propagules (CFU) in the soil. 111

Fig. 8. 2. Effect of fungus on root galling of tomato 115

LIST OF APPENDICES

Appendix 1. The content of different media 149

Appendix 2. Physical and chemical properties of the soil collected from Kabete Field Station. 150

Appendix 3. Nutrient analysis for 15 organic materials used in the study 151

Appendix 4. Component and amount used to make different buffer mixtures used for pH.. 152

Appendix 5. Galling index key (From Coyne *et al.*, 2007) 153

LIST OF ABBREVIATIONS

ANOVA: analysis of variance

CAVS: College of Agriculture and Veterinary Sciences

CFU: colony forming unit

CMA: corn meal agar

C:N: carbon: nitrogen ratio

RCBD: randomized complete block design

CRD: complete randomized design

FAO: food and agriculture organization

J2: second stage juvenile(s)

LSD: least significant difference

Mi: *Meloidogyne* spp.

mM: milli Molar

NaOCl: sodium hypochlorite

NPK: nitrogen, phosphorus, and potassium

OA: organic amendment/material (s)

PDA: potato dextrose agar

PPN: plant parasitic nematode (s)

RKN: root-knot nematode (s)

RRes: Rothamsted research station

Sp: specie

Spp.: species

UK: United Kingdom

USA: United state of America

VFN: virus, fungus and nematodes

W/W: weight by weight

YEM: yeast extract medium

ABSTRACT

Root-knot nematodes (*Meloidogyne* spp.) are among the major constraint to profitable tomato production in Kenya. This study was conducted to determine the efficacy of biocontrol agents, organic amendments, crop rotation and an integrated management package of the nematodes in tomato. Six isolates of *Pochonia chlamydosporia* and one isolate of *Paecilomyces lilacinus* were screened against root-knot nematodes *in vitro*, under glasshouse and field conditions. The isolates selected after the initial evaluation were tested against root-knot nematodes in combination with organic materials. The organic materials namely bean straw, cabbage leaves, *Crotalaria ochroleuca*, cow, goat and chicken manure, filtermud, maize stover, maize cobs, *Mucuna pruriens*, rice husk, sawdust, *Tagetes minuta* and *Tithonia diversifolia* were tested for ability to support growth and production of chlamydospores of fungal isolate 10. The isolate was applied in a rotation cycle involving maize and tomato in the first season which was followed by only tomato in the second season. Maize stover was also incorporated in the second season as a soil amendment. Among the fungal isolates screened, isolates 10 and 392 of *Pochonia chlamydosporia* were the most potent in parasitizing eggs of root-knot nematodes. At 30 days, the significant ($P<0.05$) higher fungal propagules was from isolates 10 in sterile soil (1.5×10^3) and 392 in non sterile soil (6×10^3) than from isolate 10, Pl-20K and Pl-plus ($<8.2\times10^2$) in non sterile soil. The highest shoot weight (8.5g/plant) was recorded in tomato plants grown in sterile soil to which isolate 392 was applied. The evaluation of locally available materials for the production of viable chlamydospores showed that, *Mucuna pruriens*, maizecobs, *Tithonia diversifolia*, *Tagetes minuta*, goat manure and bean straw are promising substrates with comparable efficacy to rice (standard). For instance, chlamydospore counts were between 9 to 43×10^6/g of organic

substrate having 50-62% viability while rice had 7.8×10^6 chlamydospores/g with 48% viability. Addition of organic materials and the fungus significantly increased the dry shoot weight compared to treatments without the fungus. Among the organic materials used, maizestover and *Tithonia diversifolia* had 64-65% higher shoot weight than the control. Incubation of organic material and fungus for 30 days increased the fungal propagules of *Pochonia chlamydosporia* in the soil and rhizosphere compared to application at planting. A higher percentage of nematode egg infection was recorded on extracts from rice (53%), sawdust (42%), filtermud (38%), ricehusk (37%), *Crotalaria ochroleuca* (26%) and maizecobs (22%) than the other substrates. The incubation of *Crotalaria ochroleuca*, maize cobs and sawdust at 15, 20 or 25°C, significantly increased the percentage of egg infection and the number of fungal propagules in the soil. Under glasshouse conditions, 28 and 58% reduction in numbers of second stage juveniles was recorded when the fungus was applied to tomato and maize in the first season, respectively. A combined application of the fungus and maize stover resulted in 50% decrease in numbers of second-stage juveniles in tomato roots. Application of the fungus in a rotation cycle involving maize in the first season and tomato in the second season led significant a decline in root galling indices from 1.7 to 1.1. In addition, combining crop rotation with the fungus resulted in an increase in yield of tomato by 39 to 63%.

CHAPTER 1

GENERAL INTRODUCTION

1. 1 Introduction

Tomato (*Solanum lycopersicum*) is one of the most widely grown and consumed vegetables in Eastern and Southern Africa (Nono-Womdim *et al.*, 2002). The crop is grown mainly for cash income especially in the peri-urban areas with good access to markets. In East and Southern Africa, tomato production is dominated by small-scale farmers who produce mainly for fresh consumption and local markets. It is widely cultivated in this region but the yield does not exceed 20t/ha (Sikora and Fernandez, 2005) which is generally low compared to the global average yield of 36t/ha (FAS/USDA, 2003). The main challenges that are a hindrance to optimum production are nutrient deficiencies, diseases and pests (Seliga and Shattuck, 1995). Most of the soils in the tropics are either less fertile or have nutrients that are not available to crops. Diseases caused by fungi (mainly late blight due to *Phytophthora infestans*, damping off by *Rhizoctonia solani* and *Fusarium* wilt by *Fusarium oxysporum*) are a major threat to tomato production (Shen and Welbaum, 1999). Other diseases include those caused by bacteria such as bacterial wilt (*Ralstonia solanacearum*) (Fatmi and Schaad, 2002) and due to viruses like tomato sported wilt (Rubio *et al.*, 2003).

The major pests that affect tomato production are insects and nematodes. The destructive insects are cut worm (*Noctuidae*), stink bugs (*Pentatomidae*), tomato horn worm (*Manduca sexta=Protaparce sexta*) and flea beetles (*Phllotreta* sp.) (Zalom, 2002). Various nematodes affect tomato production including *Meloidogyne* spp., *Rotylenchulus* spp., *Pratylenchus* spp.. *Tylenchus* spp. and *Tylenchorhynchus* spp. (Gaur and Meher, 1994). Of these, *Meloidogyne*

spp. (root-knot nematodes) are rated as the most devastating pest, especially where the crop is intensively cultivated on a continuous basis (Webster and Dunphy, 1987).

Root-knot nematodes (RKN) affect many crops as they adapt to wide environmental conditions (Evans and Rowe, 1998). It has been reported that the annual crop losses on a worldwide scale in different crops due to plant-parasitic nematodes (PPN), is about US $ 100 billion, of which 70% are specifically due to RKN (Sasser and Freckman, 1987; Kerry, 2001). They are found in a range of environments, parasitize many different plants and are widely distributed throughout the tropical and subtropical regions (Hunt *et al.*, 2005). They reproduce and feed within plant roots and induce small to large galls, the presence of which disrupts the physiology of the plant, thereby reducing crop yield and product quality (Karssen and Moens, 2006). In tomato, an infected crop produces fewer fruits and the life span of the crop is also reduced due to early senescence caused by inefficient water and nutrients uptake. These combinations of factors make RKN of great economic importance.

Efforts to control RKN in tomato are restricted as the crop is mostly produced by small-scale farmers who have little capital to invest in production and management systems. In most developed countries, the use of nematicides to control RKN is effective and widespread (Kerry, 2001). Nematicides have been one of the methods relied upon to control nematodes in general, but in tropical countries, such as those in Africa where subsistence farming is practised by peasant farmers, it is difficult or impractical to use nematicides due to financial constraints and/or their unavailability. However, in Kenya, commercial farmers producing high-value crops such as flowers and vegetables use synthetic nematicides. The use of most nematicides has now been banned as a result of their potential environmental and health risks (Kratochvil *et al.*, 2004; Kiewnick and Sikora, 2006). Moreover, vegetable growers are

restricted in their use of nematicides due to the risks of residues in harvested crops (Atkins *et al.*, 2003a). In such situations, it is important to assess other ways of controlling RKN using easily available materials and environmentally safe methods.

Methods like resistant cultivars, crop rotation, organic amendments (OA), and nematode antagonists have been applied in an effort to reduce losses caused by RKN, but with limited success. This is caused by the nature of RKN which have wide host ranges and a rapid reproductive rate (Kerry, 2001). In various countries, varieties of tomato resistant to nematode infection have been used with success in research studies and by commercial tomato growers (McSorley *et al.*, 1999; Nono-Womdim *et al.*, 2002; Cortada *et al.*, 2008). However, most of the resistant varieties fail to meet consumer demands because of a short shelf life, difficulties in storage and handling, and are therefore not readily accepted in the market.

Crop rotation has been one of the successful methods for the management of RKN (Desaeger and Rao, 2000; Widmer *et al.*, 2002; Warnke *et al.*, 2008). In addition, the strategy is becoming increasingly popular due to the current awareness of the environmental hazards posed by the use of nematicides (Kratochvil *et al.*, 2004). According to Wang *et al.* (2002). crop rotation with non-host crops like sorghum and onions has proved effective in the management of RKN in vegetable crops. However, for the rotation to be effective, a long rotation time is required. In addition, crop rotation is limited by the shortage of arable land and in situations where the range of preferred crops is dictated by the market (Scurrah *et al.*. 2005). For the method to be effective, potential rotation crops for nematode management need to be evaluated for host status and the length of time required for the crops to effectively

suppress nematodes. Such factors require research to reduce rotation time in the control of RKN in vegetable producing systems through integration with other management practices.

Application of organic amendments such as *Tagetes* sp. and chicken manure has been used in the management of plant-parasitic nematodes (Desaeger and Rao, 2000; Kimpinski *et al.*, 2003). The efficacy of organic amendments depends on the chemical composition (Chavarria-Carvajal *et al.*, 2001). Decomposition of the organic amendments results in decrease in the number of nematode due to the release of chemical nematicides (Akhtar and Mahmood, 1994). According to Kimenju *et al.* (2004), the use of chicken and cow manure, and residues of velvetbean, marigold or neem as soil organic amendments gave acceptable results in the management of RKN, with chicken manure proving to be the most effective. Addition of OA supports the population and survival of soil micro organisms including biological agents in the soil (Huang *et al.*, 2006). It also improves soil fertility, water holding capacity and soil structure (Akhtar and Malik, 2000). However, for the method to be effective, large quantities are needed which may be impractical or too expensive. Alternatively, these OA could be by products that are cheap.

Biological control may be used in combination with OA and crop rotation in order to improve control levels with no harmful effects to the environment and human health (Kerry, 2001). Most of the research on the application of biological control methods has been conducted outside Africa. Efforts need to be made to either identify appropriate local biological agents or to introduce agents from outside and then test for efficacy and survival under local conditions. Fungal biological control agents such as *Paecilomyces lilacinus*, *Pochonia chlamydosporia*, *Trichoderma* spp., *Monographella cucumerina* (*Plectosphaerella cucumerina)* and bacteria such as *Pasteuria penetrans*, *Agrobacterium radiobacter*, *Bacillus*

subtilis and *Pseudomonas* spp. have shown to have potential for biocontrol (Atkins *et al.*, 2003a). Among these, nematophagous fungi namely *Pochonia chlamydosporia* and *Paecilomyces lilacinus* have shown promising results in controlling cyst and RKN in crops in Europe and South America (Atkins *et al.*, 2003a; Atkins *et al.*, 2005). *Pochonia chlamydosporia* and *P. lilacinus* are parasites of eggs and females of sedentary plant-parasitic nematodes. As the efficiency of these fungi depends on edaphic conditions such as the physical nature of the soil, their efficiency needs to be tested under local conditions (Meyer and Roberts, 2002). Conditions like temperature, moisture and soil nutrients affect the efficiency of these fungi in controlling RKN (Sayre and Walter, 1991).

Biological control is not a replacement for nematicides but it can be a useful management tool, especially in small-scale farms. According to Viaene *et al.* (2006), use of antagonistic micro-organisms in management of plant-parasitic nematodes is partially effective. Due to this, it should be applied in combination with other practices in order to maintain nematode populations below economic threshold levels. Use of more sustainable control measures such as disease-free planting material, soil amendments and biological agents, is only partially effective, so that the methods cannot be used individually (Kerry, 2001). This underscores the need for research to look for a practicable method in terms of cost and ease of application to small-scale tomato growers.

In general, non-chemical methods are not fully effective in controlling RKN. This emphasises the need to develop pragmatic strategies based on sustainable integrated pest management packages to underpin RKN in agricultural soil. The approach of controlling plant-parasitic nematodes by sustainable integrated management practices has been recommended by many researchers (Gaur and Meher, 1994; Verdejo-Lucas *et al.*, 2003; Sikora and Fernandez,

2005). One of the integrated practices to control RKN in tomato recommended by Gaur and Meher (1994) was the application of nematicide (ebuphos), fertilizer (NPK) and manure (farm-yard manure). In Cuba, control of RKN in vegetable production is based on integrated methods where biological agents like *P. chlamydosporia*, soil amendments (cattle manure) and poor host crops, such as bean and cabbage, are used with great success (Atkins *et al.*, 2003b). Introduction of integrated means of nematode management can be of benefit to small-scale growers but it requires local evaluation of control strategies and technical support.

The current work aims to study other management practices such as nematophagous fungi, organic amendments and crop rotation which are integrated with the aim of managing RKN on tomato production in Kenya.

1.2 Objectives

1.2.1 Overall objective

To evaluate the efficacy of biocontrol agents in combination with crop rotation and organic materials in the control of RKN in tomato in a small-scale production context.

1.2.2 Specific Objectives

1. To determine the efficacy of different isolates of *Pochonia chlamydosporia* and *Paecilomyces lilacinus* in the control of root-knot nematodes (RKN)
2. To screen locally available organic materials as substrates in the production of *P. chlamydosporia* inoculum

3. To evaluate the efficacy of the most effective isolates of *P. chlamydosporia* and *P. lilacinus* in the presence of organic materials in RKN management
4. To assess the effect of time of application of organic materials and *P. chlamydosporia* in the management of RKN in tomato
5. To assess the potential of combining *P. chlamydosporia* with crop rotation and organic substrates in RKN management in tomato
6. To determine the impact of edaphic factors such as soil temperature, pH, nitrogen, and carbon on the nematicidal activity of the selected fungal isolate on RKN eggs

CHAPTER 2
LITERATURE REVIEW

2. 1 Tomato production in Kenya

Tomato is among the most popular crops in small and commercial gardens. In Kenya, tomato is grown under rain-fed and irrigated conditions, 23% of the total land used for vegetable production being dedicated to tomato production. According to the Ministry of Agriculture (2005) tomato is ranked third after kale and cabbage in terms of the area under production. The average annual production of tomato in Kenya was 330,000 tonnes in 2007 (FAO, 2007). Production covers almost all areas of the country with the main growers being the small scale farmers (Anderson, 2000).

Tomato is consumed in many different ways including salads, sauces, and making stews with meat, fish and other vegetables like okra. They provide useful quantities of vitamins A and C to the body. Also, tomato production is a source of income as the crop can be sold fresh or processed. Most of the tomato growers in Kenya produce tomatoes which are sold in the local markets (Otipa, 2002). Optimal tomato production is hindered by many factors like low soil nutrients and pests with RKN being one of the major pests (Seliga and Shattuck, 1995). Other pests including arthropods, weeds and diseases (Clark *et al.*, 1998) .

Worldwide, RKN is believed to be responsible for the largest proportion of tomato yield losses estimated at about 21% (Sasser and Freckman, 1987; Guzman-Plazola *et al.*, 2006). However, in tropical countries such as Kenya, yield losses are larger because the environmental conditions favour rapid multiplication of RKN. Sustained efforts in managing RKN are therefore required in order to minimise losses.

2. 2 Root-knot nematodes

Root-knot nematodes belong to the genus *Melodogyne* in the order *Tylenchida* and have an obligate, sedentary endoparasitic, lifestyle (Dhandaydham *et al.*, 2008). The genus contains one of the nematode pests of great economic importance because of their wide host range and capacity to survive and proliferate under different environmental conditions. RKN affect many crops and are reported to be one of the leading problems to vegetable growers (Atkins *et al.*, 2003a; Atkins *et al.*, 2003b). They are favoured by warm temperatures that are prevalent in the tropical and subtropical regions (Hunt *et al.*, 2005). However, some species are able to adapt to local climatic conditions and may be found in temperate climates (Sikora and Fernandez, 2005). The most widely distributed species are *M. incognita*, *M. javanica*, *M. arenaria* and *M. hapla*, among the 97 (Hunt 2009 personal comm.) known species in the genus *Meloidogyne*. Others are less economically important but they may still cause significant yield losses on some crops (Manzanilla-Lopez *et al.*, 2004). For example, in the tropics and warmer climates, *M. incognita*, *M. javanica* and *M. arenaria* are the most important, while in temperate regions *M. hapla*, *M. chitwoodi* and *M. fallax* are prevalent (Wesemael, 2007).

2. 2. 1 Life cycle of root-knot nematode

The basic life cycle of RKN is not much different from that of other nematodes. The eggs are retained within a gelatinous matrix in which they are embedded outside the roots or inside the galls where the infective second-stage juveniles (J2) hatch (Wright and Perry, 2006). When the J2 enter plant roots, they establish a feeding site of specialised giant cells and develop and moult into third-stage juveniles (J3) and then into fourth-stage juveniles (J4) which moult

either to adult males or females (Karssen and Moens, 2006). Many RKN, including those that are of major economic importance, are parthenogenetic and males are not necessary in order for females to produce viable eggs (Decraemer and Hunt, 2006). Males may migrate from the roots while females are sedentary and remain within the root tissues (Hunt *et al.*, 2005). The female lays eggs outside the gall in a gelatinous matrix, on the root surface (Karssen and Moens, 2006). According to Wesemael (2007) a female can lay between 30 and 80 eggs per day. In the soil only the eggs, J2 and males can be found while the females and other juvenile stages remain inside the roots. The life cycle may be completed in about 25 days depending on the host, climatic conditions and nematode species. According to Canto-Sáenz (1985), at temperatures of 27°C, the probability of having more generations is high as the life cycle is rapid. On crops like tomato, which has a cycle of about four months to maturity, RKN will have about four generations, an important consideration when applying management methods.

2. 2. 2 Symptoms of root-knot on plants

As a result of gall formation, these nematodes are commonly known as root-knot nematodes (Whitehead, 1967). Root-knot nematodes induce many symptoms that are visible to the naked eye while some can only be seen through the microscope. The J2 enters the roots by penetrating at the tips in the elongation zone. They move between plant cells until they find a suitable location to establish a feeding site (Wesemael, 2007). This follows the formation of the giant cells and the nematodes becomes stationary (Weerasinghe *et al.*, 2005; Dhandaydham *et al.*, 2008). Infected roots form galls in response to nematode invasion which differ in size depending on the plant species and can be confused with nodule on leguminous

plants (Manzanilla-Lopez *et al.*, 2004). For example, infected cucurbits form large galls while the galls formed on chillies are much smaller (Dasgupta and Gaur, 1986).

During dry or hot conditions, broad-leaved plants are known to wilt, become yellow and show stunted growth due to reduced root growth, which inhibits water, nutrient uptake and translocation (Kratochvil *et al.*, 2004). Infected seedlings may die and if they survive they bear few fruits resulting in significant yield losses (Sikora and Fernandez, 2005). The quality of crops is adversely affected, especially in root and tuber crops such as carrots and potatoes, which lose quality (misshapen), making them unacceptable to consumers (Schomaker and Been, 2006). Nematodes are patchily distributed in soil and so crop growth appears uneven in fields infested by the nematodes (Dasgupta and Gaur, 1986). The deterioration of the underground part of the crop is closely related to the expected yield loss. In general, symptoms due to RKN may be used to estimate potential yield. But it is difficult because the symptoms are non-specific.

2. 3 Management of root-knot nematodes

Management means minimizing RKN populations to levels that cause no economic damage to the crop. Methods such as the use of organic amendments, crop rotations and biological control have been developed and sometimes used in integration, with the aim of restraining nematode populations below the economic threshold. Other methods which include chemical and cultural strategies are also used by farmers.

2. 3. 1 Chemical methods

Chemical nematicides are fast acting and occur in the form of either fumigants or non-fumigants. The use of chemicals to control nematodes has been found to be an effective measure and generates a good profit for growers of high value crops (Wesemael, 2007). In addition, many different types of PPN can be controlled quickly offering an opportunity for the farmers to maximize production (Halbrendt and LaMondia, 2004). Despite the positive results on RKN, nematicides often have a negative impact on the environment and pose risks to human health (Saha *et al.*, 2007). In addition, many small growers (majority of the tomato farmers in Kenya) are unable to purchase nematicides because of the cost.

2. 3. 2 Cultural methods

There are different cultural practices for controlling PPN which include organic amendments, crop rotation and others such as flooding, soil solarisation and resistant cultivars. These methods can easily be used by small scale farmers as they are cheaper and relatively easy to apply compared to nematicides. However, these methods are not fully effective and should be used in integration with other methods.

2. 3. 2. 1 Organic amendments

Organic amendments are incorporated into the soil as various waste materials from agricultural processing and as manures (Halbrendt and LaMondia, 2004). These materials have been used for a long time with the aim of improving chemical and physical characteristics of the soil (Akhtar and Malik, 2000). However, the realisation has grown that

organic amendments have more than one function in the soil. According to Karssen and Moens (2006), organic amendments improve the properties of the soil and reduce RKN populations through the decomposition process. Control of RKN by organic amendments is a cultural method but may play a major role in biological control in enhancing the growth and populations of biocontrol agents in soil (Viaene *et al.*, 2006).

The populations and activities of biological agents, which include nematophagous fungi, bacteria, predacious nematodes and mites, may increase with application of organic matter (Webster and Dunphy, 1987; McSorley *et al.*, 2006). These effects call for research on what local organic amendments can be integrated with biological agents without adversely affecting their efficacy. Organic amendments can, therefore, be an additional method in the RKN management systems.

Application of some green manures has shown encouraging results in reducing nematode infestations. It reduces the RKN activity and also has the indirect effects of improving plant health and changing plant root physiology which makes the plant more resistant to RKN infection (Whitehead, 1998). Also, the decay of green plant materials in soil results in a decreased effect of nematodes on plants. This is because it may enhance the production of breakdown products that have nematicidal effects (Viaene *et al.*, 2006). For instance, OA such as marigolds (*Tagetes* spp.) and castor have nematicidal effects and when applied to soil can help to control nematodes (Stirling, 1991).

Decomposition of OA may result in the release of volatile substances which suppress RKN population but in order for OA to be effective, large amounts are needed which increases the transport costs if the materials are not locally available (Wesemael, 2007). It has also been noted that the application of large amounts of OA in the soil increased the yield of susceptible

crops (Whitehead, 1998) . However, this method can only be recommended for small areas and in fields which are very close to a source of the OA (Viaene *et al.*, 2006).

Research shows that organic amendments which have high chitin content are more effective in reducing the number of PPN nematodes due to the release of toxic compounds like phenols and ammonia during the decomposition process (Stirling, 1991). Moreover, organic materials with high chitin content have a high capacity to stimulate the growth of nematode antagonistic organisms (Sikora and Fernandez, 2005). However, the activity of some biocontrol agents is reduced by the addition of soil amendments (Akhtar, 1997). To increase their effectiveness, OA need to be applied together with nitrogen (N), phosphorus (P) and potassium (K) which improve its function (Whitehead, 1998).

2. 3. 2. 2 Crop rotation

This is the practice of growing different types of crops for each season on the same piece of land. Growing different types or cultivars of crops which are poor hosts or resistant to RKN, such as grasses and cereals, each season helps to reduce the build-up of RKN populations, (Webster and Dunphy, 1987). Rotational crops can be planted for one to three consecutive seasons. For example, planting of marigold which produce nematoxins that prevent nematodes from feeding on the roots and hence starving to death (Whitehead, 1998). A good rotational crop is expected to reduce nematode populations and increase yields of the following susceptible crop (Halbrendt and LaMondia, 2004). However, sometimes it is difficult to find a suitable rotation crop due to the wide host range of RKN which exist as many species and races (Wesemael, 2007). The problem is compounded by the fact that alternative crops have to be accepted by farmers. This method is advised more for annual

crops than perennial crops that become difficult to remove after they have become established (Halbrendt and LaMondia, 2004).

Rotation of non-host crops depends on the cultivar grown, environment and RKN species of that area. For example, it has been noticed that groundnuts is one of the non-host crops for *M. javanica* and *M. incognita* but it is susceptible to *M. arenaria* and *M. hapla* (Whitehead, 1998). A study conducted in Kenya to find the appropriate rotational crops in controlling RKN in beans showed that maize and *Crotalaria* sp. reduced RKN populations whereas planting of *Sesbania* before a bean crop increased damage by RKN (Desaeger and Rao, 2000). This is because maize is a poor host to RKN while *Sesbania* is a good host. It is also advised that, when implementing rotations, weeds should be controlled because many are hosts of RKN (Wesemael, 2007). Crop rotation also has positive effects on soil properties and may reduce other soil pathogens and increase crop yield (Struik and Bonciarelli, 1997).

Despite the many success stories of crop rotation, more studies are needed in order to establish the correct rotational system and crops so as to reduce RKN population on vegetable growing areas of Sub-Saharan Africa. The major problem in using rotation alone to control RKN is the fact that the nematode's ability to hatch depends on temperature and moisture and is not triggered by the presence of host plants (Halbrendt and LaMondia, 2004). Also for the method to be effective, it needs long time rotation before planting of the host crop. This makes it difficult to be practiced in areas where the productive land is small and when the crop is a main source of income. For this method to be effective it needs to be integrated with other management methods.

2. 3. 2. 3 Other methods

Flooding is done by leaving standing water on the land for up to four months (Whitehead, 1998). Flooding reduces oxygen supply, changes the pH and releases toxic substances to the soil (Viaene *et al.*, 2006). This reduces PPN because some of the nematodes cannot survive for a long time in flooded land (Bridge *et al.*, 2005). This method is good for crops such as lowland rice which are grown on flooded soil (Halbrendt and LaMondia, 2004). However, flooding is not 100% efficient and is limited to areas where water is plentiful (Bridge *et al.*, 2005). It also needs levelled fields and at least 12 weeks inundation to be effective (Karssen and Moens, 2006; Viaene *et al.*, 2006).

Soil solarisation involves the covering of soil using clear polyethylene sheets for some time in order to use the heat from the sun to raise the soil temperature and so kill nematodes (Halbrendt and LaMondia, 2004). This method is effective in areas having long periods of sunshine and high temperatures (Karssen and Moens, 2006). The method is known to raise the temperature of the covered moist soil to 45°C, which is high enough to kill nematodes (Sikora *et al.*, 2005). However, the heating effects decrease with increasing depth of soil and this makes the method not 100% effective (Whitehead, 1998).

Resistant cultivars are plants that permit nematode access and a varying level of parasitism but not reproduction (Hunt *et al.*, 2005). Selection and development of resistant cultivars needs money and takes a long time as the cultivars are selected together with other agronomic factors (Starr and Roberts, 2004). RKN resistant cultivars of tomato have the *Mi*-gene introduced which works against *M. arenaria, M. incognita* and *M. javanica* but not *M. hapla* (Sikora and Fernandez, 2005). However, tomato cultivars bearing the *Mi*-gene and are resistant to *M. incognita*, their resistance is said to breakdown at temperatures of 28°C and

above (Cortada *et al.*, 2008). Hence for a resistant cultivar to be effective, selection should be done locally and it should not be planted continuously in order to reduce the build up of virulent (resistance-breaking) strains (Whitehead, 1998).

2. 3. 3 Biological control

Biological control has been given many definitions from different scientists but most definitions have more or less the same meaning. One of the current definitions is the management of plant diseases and pests with the aid of another living organism (Viaene *et al.*, 2006). It is aimed at reducing diseases and pests to a level which is less detrimental (Pembroke, 2002). The control organisms include a wide range of invertebrates and microbial pathogens, particularly fungi, bacteria and viruses (Kerry and Hominick, 2002). In order for the biological control method to be successful it depends on many things, including soil environmental factors (Whitehead, 1998).

It has been noted that there are many biological agents that can be used in the management of diseases and pests but there are also many factors which limit its effective utilisation. Few organisms have been developed and those that have been developed are not widely available for use (Kerry, 1987). Also, biological control agents cannot provide full management on their own and must be used with other compatible control methods, so market for their development are small (Meyer and Roberts, 2002). They may be specific to the target pests or diseases and there are often difficulties in mass production and application. Their activity is influenced by many interactions with the additional problem in some countries of obtaining permission before they can be released. Before releasing of an organism as a biological agent, all of the above limiting factors need to be evaluated for the specific local conditions.

In Sub-Saharan Africa few or no studies have been done on biological agents that can control RKN to know if they can also do well in local environments. Soil conditions, such as temperature, pH and soil nutrients, have effects on the growth, sporulation and activities of the fungal biological agents in the management of RKN (Kerry, 1987). Researches on the interaction of biological agents and environment where fungi and bacteria will be used in the control of PPN are needed. In PPN, biological control is aimed at reducing nematode populations through parasitism, predation, antagonism and competition (Viaene *et al.*, 2006). In some soils this happens naturally and in others by introduction (Zhang *et al.*, 2006). There are many beneficial micro-organisms which attack PPN but most research has been done using fungi and bacteria (Viaene *et al.*, 2006), some of which have been tested to the field level and development of commercial products (Meyer and Roberts, 2002). This is due to the easy handling of fungi and bacteria in terms of mass production and storage compared to other organisms. These biological control agents have been applied in the control of RKN and resulted in decreased infestations of RKN in susceptible crops (Whitehead, 1998), although not by 100%, which therefore emphasises the need for them to be integrated with other methods. Wesemael (2007) suggested that the positive effects of the biological control method in sustainable management will depend on the other methods with which it is integrated.

2. 3. 3. 1 Bacteria

Several reports are available on the effectiveness of bacterial in the management of plant parasitic nematodes (Chen and Dickson, 2004; Kariuki *et al.*, 2006; Kariuki and Dickson, 2007). These bacteria that control nematodes have been divided into two main groups

depending on their mode of action against nematodes. There are bacteria such as species of *Bacillus*, *Clostridium*, *Pseudomonas* and *Azotobacter* which release substances which are toxic, antibiotic or inhibitory and which have effects on nematodes (Dhawan and Kamra, 1995). *Bacillus thuringiensis* is used against insect pests but has also been found to suppress *M. javanica* and prevent formation of galls by *M. incognita* juveniles on tomato roots (Jonathan *et al.*, 2000). Another group includes species of *Pasteuria* which parasitize nematodes, affecting their reproductive tissues and hence, reducing fecundity (Stirling, 1991). Among the species of *Pasteuria*, most research has been done using *P. penetrans* because of its potential as a biocontrol agent and the fact that it is found in many countries (Viaene *et al.*, 2006). The efficiency of these bacteria has been tested against nematodes on many crops in glasshouses and proved to be useful (Chen and Dickson, 2004). Other advantages of *P. penetrans* include its ability to withstand heat, desiccation and the fact that it can survive for more than two years in soil (Dhawan and Kamra, 1995). However, usage of *P. penetrans* is hindered by the lack of an appropriate technique for its mass production as different media have been tested with limited success (Viaene *et al.*, 2006).

2. 3. 3. 2 Fungi

Beneficial fungi have been used worldwide in the control of diseases and pests. It has been noted that by the year 1999 about 40 fungal products have been developed for the control of different crop diseases (Whipps and Lumsden, 2001). The products are made in different forms such as powders and suspensions. The fungi which are used in controlling plant parasitic nematodes are grouped as nematophagous fungi (Stirling, 1991). The nematophagous fungi consist of about 150 species and some have been tested for their

efficacy in controlling PPN under glasshouse and field conditions (Whipps and Lumsden, 2001). The way in which these fungi act allows them to be grouped into trapping fungi, endoparasites of vermiform nematodes, parasites of sedentary females and eggs, fungi producing antibiotic substances and vesicular arbuscular mycorrhizal fungi (Chen and Dickson, 2004). A good example of egg and female parasites are the *Pochonia* spp. and *Paecilomyces* spp. (Stirling, 1991). They colonise the eggmasses of RKN, develop mycelium and destroy nematode eggs (Chen and Dickson, 2004). They penetrate RKN eggs using specialised infection structures called appressoria (Stirling and Mankau, 1979). The penetration is facilitated by mechanical and enzymatic actions (Chen and Dickson, 2004).

Most of the *Pochonia* spp. attack nematode eggs, but they differ in efficacy with *P. chlamydosporia* being more effective (Kerry, 2001). *Pochonia chlamydosporia var. catenulata* is an example of a good *P. chlamydosporia* and has a biological product developed to control RKN (Kiewnick and Sikora, 2006). This is one of the major nematode antagonistic fungi and has resulted in the natural reduction of *H. avenae* in Europe (Chen and Dickson, 2004). Isolates of *Pochonia* spp. differ markedly in their growth and sporulation *in vitro* (Kerry, 2001), virulence, saprophytic competitiveness and rhizosphere competence (Kerry and Leij, 1992). Such differences between isolates of the same species of micro-organism are common and there is a need for simple laboratory-based screening methods to select the most promising isolates for further testing in Sub-Saharan Africa. According to Atkins *et al.* (2003b), many research efforts on *P. chlamydosporia* have been done in Europe and South America.

The efficacy of *P. chlamydosporia* as a biological control agent for RKN is affected by three key factors: the amount of fungus in the rhizosphere, the rate of development of eggs in the

egg masses and the size of the galls in which the female nematodes develop (Kerry and Leij, 1992). In large galls, female RKN may produce egg masses which remain within the gall and are not exposed to parasitism by *P. chlamydosporia,* which is confined to the rhizosphere (Kerry and Bourne, 2002).

Pochonia chlamydosporia is said to be effective when the RKN populations are small and in such instances egg infestation of up to 80% has been recorded (Whitehead, 1998). However, if the RKN population is high, the effectiveness can be delayed until the second season and requires integration with other control measures (Leij de *et al.*, 1993). This fungus is believed to be non-pathogenic to plants, higher animals and humans and can be integrated with other non chemical control measures (Chen and Dickson, 2004). However, *P. chlamydosporia* is unlikely to be useful in situations where a grower would normally apply a nematicide.

Paecilomyces lilacinus is found in warmer areas and uses organic substrates as a source of nutrition, providing an opportunity for its use in tropical and sub-tropical areas (Karssen and Moens, 2006). It is widely tested and has shown success as a biological control agent in the management of nematodes and consequent increase in yield (Chen and Dickson, 2004). The fungus can grow in a wide range of conditions, including temperatures of 10-30°C and pH of 3.5-8.5, and it can also tolerate some toxic chemicals (Alam and Jairajpuri, 1990). Like *P. chlamydosporia, P. lilacinus* also attacks eggs on the root surface, resulting in partial reduction of nematode populations (Van Damme *et al.*, 2005). Also, it is believed that even juveniles hatched from eggs and exposed to *P. llilacinus* have a reduced capacity to infect tomato roots (Whitehead, 1998). Kilama *et al.* (2007) reported that *P. lilacinus* controls the females and juveniles of *Radopholus similis* which is a migratory endoparasite of banana.

According to Mendoza *et al.* (2007), this is one of the fungi where many of the field tests have been conducted and has been registered for commercial products.

Research reported by Chen and Dickson (2004), shows variation of virulence among isolates where one *P. lilacinus* strain can suppress *M incognita* and *M. arenaria* but not *M. javanica.* Despite its efficiency in the control of RKN, there is a concern as to whether *P. lilacinus* has an effect on humans due to its appearance inside the human eye and sinuses (Anastasiadis *et al.*, 2008).

The number of *P. chlamydosporia* and *P. lilacinus* in the soil does not reflect its efficiency in managing RKN because isolates differ in terms of their ability to colonise the soil and also manage nematodes. Some isolates can be abundant in the soil but have less effect in infecting RKN eggs. This is because the isolates are not capable of colonising the rhizosphere (Kerry, 1987). According to Kerry and Bourne (2002), tomato crop favours rhizosphere colonisation of these fungi, but the size of the galls produced by its roots are large, a fact that most of the eggs remain within the galls and escape from being parasitized by the fungus. In the application of these fungi, screening for locally grown plants which can be rotated in tomato production and are poor hosts to RKN and easily colonised by *P. chlamydosporia* or *P. lilacinus* is required. Application of fungi to poor host plants will make the fungi more effective as the size of the nematode galls are small on such hosts and most of the egg masses are exposed on the root surface where the fungi can easily infect.

Biological control of RKN using *P. chamydosporia* and *P. lilacinus* is affected by the life stage of the RKN. These biological agents infect the eggs of RKN which are exposed on the plant rhizosphere. Hence, if the condition for the J2 to hatch from the egg is favourable the life cycle goes fast and the efficacy of *P. chlamydosporia* and *P. lilacinus* will be low. One of

the factors which favours rapid egg hatch is temperature (Kerry, 1987). For example, in areas where the temperature is above 30°C, 40% of the eggs escape from being parasitized by fungi (Kerry and Leij, 1992). The efficiency of *P. chlamydosporia* and *P. lilacinus* at local temperatures needs to be tested before they are released to farmers.

Other soil factors like pH, soil major nutrients such as nitrogen (N), phosphorus (P) and potassium (K) also need to be tested in order to know their impact on the efficacy of these agents in the environment. The importance of integrated management of RKN to vegetable growers makes for an urgent need for research to test *P. chlamydosporia* and *P. lilacinus* in Kenya and later in other countries in Southern and Eastern part of Africa.

2. 3. 4 Integrating fungi and organic amendments in rotation system for the control of root-knot nematodes

Pochonia chlamydosporia and *P. lilacinus* are facultative parasites of RKN eggs and they have a saprotrophic phase outside their host (Morton *et al.*, 2004). The addition of organic amendments to the soil increases the abundance of these fungi. Research reported by Sikora *et al.* (2000) shows that integrating OA with good biological control agents increased the levels of nematode control. According to Jaffee (2004) the efficacy of the fungi to infect RKN eggs depends on the type of organic materials applied.

Testing chickpea pod waste and farmyard manure together with *P. chlamydosporia* and *P. lilacinus* showed that chickpea pod waste increased fungal growth and decreased RKN populations (Dhawan *et al.*, 2004). However, incorporation of organic materials with a high nutrient content resulted in the suppression of fungal enzymes that help in infecting nematode eggs (Viaene *et al.*, 2006). The presence of easily metabolised nutrients from OA results in

the fungi having a low efficacy in the management of RKN, even in the presence of large numbers of fungal propagules (Jaffee, 2002). Research on the appropriate time for applying OA and the fungus therefore needs to be done. This study will examine whether the time of incubation of OA and fungus before planting of tomato has effects on the management of RKN and will indicate the appropriate time for planting when OA and fungus are used to control RKN.

Organic amendments on their own reduce RKN infestations (Akhtar and Malik, 2000). It has also been noted that application of OA already colonized by biological agents, especially facultative agents, is more effective in establishing nematophagous fungi in soil (Kerry, 1987). However, the efficiency of this mixture in controlling RKN is not well understood. It has been noted that, application of the substrate alone decreased nematode populations to the same extent as the substrate colonized by the agent and there is no clear evidence of biological control (Akhtar and Malik, 2000). Appropriate treatments, as well as an untreated control, should be included if the organism is added with a substrate.

This study will include treatments with substrate alone and a mixture of substrates and biological agents so as to allow separation of the effects of the substrates on the activities of the biological agents. It is advised that the test organism, and any OA, should be applied at practical application rates; 0.1 percent w/w soil is equivalent to 2.5 tons/ha and should represent a maximum dose (Kerry, 1987). Also, tests should always be performed in a non-sterilized soil with a natural resident soil micro flora. In conducting this research, biological agents and OA will be tested under glasshouse and field conditions in order to obtain results which will represent the actual field conditions for small scale tomato growers.

Crop rotation with poor host crop to RKN is one of the methods that can easily be integrated with nematophagous fungi in order to shorten rotation period. One of the rotation systems reported by Kerry and Bourne (2002) for the control of RKN is susceptible host - poor host-poor host - resistant or non-host-susceptible crop. Application of the fungus before the first or second poor host may permit the shortening of this rotation or the replacement of the resistant or one non-host crop, without increasing damage by nematodes to the susceptible crops. It is hoped that by combining the non-host plant and the biological control agent, the result will be achievement of more effective and consistent control.

CHAPTER 3

GENERAL MATERIALS AND METHODS

3. 1 Introduction

The materials and methods in this chapter comprise the general information covering various experiments in the study. Information that is specific to individual experiments is appropriately placed in the relevant chapter. The laboratory experiments were conducted at the College of Agriculture and Veterinary Sciences (CAVS), University of Nairobi and at Rothamsted Research Institute (RRes), UK. All glasshouse and field experiments were conducted at CAVS, University of Nairobi.

3. 2 Media used

3. 2. 1 Laboratory media

Media used in this study to culture the nematophagous fungi were corn meal agar (CMA), potato dextrose agar (PDA), sorbose agar with antibiotics and a semi-selective medium for the isolation of *P. chlamydosporia*. These media were prepared using the procedures described by Kerry and Bourne (2002). Other media, such as rice broth, water agar with antibiotics, dilution medium (0.05% water agar), and medium for *P. lilacinus* were prepared using the procedures described by Atkins *et al.* (2003a). The formulations of each medium are shown in Appendix 1.

3. 2. 2 Soils

The soil used in the glasshouse experiments was collected from Kabete field station and taken to Soil Science Department, Kabete, Nairobi for analysis of its physical and chemical properties using procedure and techniques outlined by Okalebo *et al.* (2002) and the results are shown in Appendix 2. The sterile soil used in glasshouse was sterilised at 121°C for 30 minutes in autoclavable plastic bags, left to cool and aerated for 14 days before use. The potting medium for all glasshouse experiments was made by mixing soil and sand at a ratio of 3:1.

3. 3 Production of *Pochonia chlamydosporia*

A total of six *P. chlamydosporia* isolates, namely accessions 10, 144, 147, 126, 177 and 392 were used either separately or in combination for the experiments. The cultures were stored as freeze dried material which was activated by soaking for less than 30min. in sterile distilled water. The cultures were placed on PDA in Petri dishes, allowed to grow for 7 days at 25°C and thereafter kept at 4°C in a refrigerator and taken out when needed.

3. 3. 1 Production of *Pochonia chlamydosporia* for *in vitro* experiments

Inoculum for laboratory experiments was produced using PDA or CMA media by subcuturing from 7 days old culture prepared as described in 3. 3. The fungus was grown on PDA for 14 days or on CMA for 21 days for conidia and chlamydospores productivity respectively. Depending on medium used, 5ml sterile distilled water was added to Petri dishes after 14 and 21 days and conidia or chlamydospores were harvested by rolling the

surface of the cultures with L-shaped glass rod. The conidial suspension was passed through 20μm sieve. The sieve was then rinsed with 10ml sterile distilled water. The conidia in the filtrate were counted using a haemacytometer and compound microscope at 20× magnification. The suspension was diluted to 5.5×10^4 conidia ml^{-1} (Esteves, 2007). The chlamydospores suspension was sieved through 53μm and passed to 15 μm where chlamydospores were collected. The chlamydospores were counted and the suspension was diluted to 5000 chlamydospores /ml.

3. 3. 2 Production of *Pochonia chlamydosporia* for glasshouse and field experiments

Rice was used as a substrate for the mass production of *P. chlamydosporia* inoculum that was used in the glasshouse and field experiments. The procedures described by Hidalgo-Diaz (2003) were followed. About one kilogram of rice was washed using tap water to remove fine particles and then air dried at room condition. After drying, 300ml of tap water was added, mixed and pre-cooked for 12 minutes at 121°C. From precooked rice, 100g was weighed and placed in a 500ml capacity conical flask. The mouth of the conical flasks was sealed with a cotton wool plug and covered with aluminium foil before being autoclaved at 121°C. After cooling, the rice was inoculated using three *P. chlamydosporia* plugs taken from the edge of an actively growing seven-day-old culture. The cotton plug and aluminium foil on top were returned, sealed with Parafilm and later incubated at 25°C in darkness. Three days after incubation, the conical flasks were shaken and returned to the incubator. Twenty one days after incubation, the colonised rice was thoroughly mixed and one gram was suspended in nine millilitre water agar (0.05%). Chlamydospores were counted using a Neubauer haemocytometer under a compound microscope (20× magnification). From the counts, the

total number of chlamydospores per gram of rice was estimated. After counting the number of chlamydospores per gram of rice, one ml of the suspension was diluted to 10^{-3} or 10^{-4}, depending on the number of chlamydospores, so as to make it easier for testing their viability. About 0.2ml containing 100-500 chlamydospores, was cultured in each of four Petri dishes containing sorbose agar with antibiotics. The Petri dishes were incubated for 48 hours at 25°C in darkness and the number of germinated chlamydospores was counted under a dissecting microscope at magnification of 50×. The numbers of spores that germinated were recorded and percentage viability from about 100 chlamydospores determined. The remaining colonized rice was used to harvest chlamydospore inoculum used in subsequent experiments. Harvesting was done using a series of different sieves sizes; 250, 53 and 20 and 15µm nested onto each other. The base of the 15µm sieve was blotted from below using tissues and chlamydospores on top of the sieve were collected by scrapping them off the sieve.

3. 4 Preparation of root-knot nematode inoculum

3. 4. 1 Glasshouse experiments

Different crops that are hosts of RKN, like tomato and spinach, were grown in sterile soil in pots in the glasshouse and inoculated with 3000 RKN eggs and juveniles per plant. Infected roots were taken from the glasshouse and used to obtain the RKN inoculum. The root maceration method used to extract the nematodes eggs and juveniles as described by Coyne *et al.* (2007). Roots were gently washed with tap water cut into one centimetre long pieces. The roots were weighed and water was added at ratio of one gram of root to 20ml water and 0.5% sodium hypochlorite (NaOCl) into a domestic blender (Blendmaster™ manufactured by Hamilton Beach®) and blended for 15s at high speed (Hooper *et al.*, 2005). The materials

were sieved using 250µm, 53µm and collected on 45µm sieves. Using a dissecting microscope the egg and second stage juveniles (J2) were enumerated to estimate the concentration per one millilitre. The egg and juvenile suspension was stored at 4°C before using.

3. 4. 2 *In vitro* experiments

The eggmasses were hand picked from the roots of six week old tomato plants. Picking was done under a dissecting microscope 50× magnification. The eggmasses were kept in an Eppendorf tube at 4°C before use. The eggs were disaggregated from the eggmasses by putting eggmasses and water in a 50ml skirted centrifuge tube (Sterilin) and 0.5% commercial bleach (sodium hypochlorite) was added to double the amount of the water containing the eggmasses in the tube. The mixture was homogenised using vortex Whirli Mixer (Fisher brand) for one minute and sieved on 125µm to trap soil and other debris followed by 20µm pore sieves. The eggs collected on the 20µm pore sieve were rinsed using 400ml sterile distilled water before being transferred onto Sterilin tubes.

3. 5 Preparation of organic materials and their extracts

3. 5. 1 Preparation of organic materials

The organic materials were either collected from the field station either after harvesting (maize stover, maize cobs, cabbage leaves, ricehusk and bean straw) or as weeds (*Tagetes minuta, Tithonia diversifolia* and *Mucuna pruriens*), bought from the market (*Crotalaria ochroleuca*) and collected from processing areas (sawdust and filtermud). Also the animal

manures were collected from the university livestock unit (cow manure) and from neighbours (chicken and goat manure). They were sun-dried until constant weight was achieved. Fresh materials were sun-dried and then ground to make a powder that was used in the subsequent experiments. Sub samples for each OA were taken and analysed for nitrogen, carbon, phosphorus and potassium (Appendix 3) by Department of Soil Science, Rothamsted Research Institute.

3. 5. 2 Preparation of organic materials

Extracts from organic materials were obtained by soaking 25g of the materials in a litre of sterile distilled water and kept at $4^{o}C$ temperature overnight. The solid materials were filtered off using 250µm, 45µm sieves and cheese cloth before use. After filtering with cheese cloth, the filtrates were passed through two different size filter papers (Whatman®) starting with number 1 followed by number 42. The extracts were sterilised using a 0.20µm pore size sterile Nalgene Disposable Filter Units under vacuum.

3. 6 Data collection

3. 6. 1 Percentage eggs parasitized (infected) by fungus

Assessment of the eggs infected by the fungus was done after incubation of eggs and fungus either in Petri dishes containing water agar plus antibiotics, or in liquid media. The eggs were examined using a dissecting microscope at 50× magnification and calculated as,

$$\%Parasitized = \frac{Parasitized}{Parasitized + Not\text{-}parasitized.} \times 100$$

3. 6. 2 The number of fungal propagules in soil and roots

The soil samples were taken from each treatment for the assessment of the number of fungal propagules (CFU). One gram of moist soil from each replication was suspended in nine millilitre sterile distilled water agar (0.05%) and 0.2ml from each 10^{-2} and 10^{-3} dilutions of each treatment were added to semi-selective medium for *P. chlamydosporia*, and to *Paecilomyces* medium for *P. lilacinus* (Appendix 1) and then incubated at 25°C for 14 (*P. chlamydosporia*) and seven (*P. lilacinus*) days. The colonies that emerged after incubation were counted. Then, using a compound microscope (50× magnification), the colonies were observed and compared with control samples. The remaining moist soil samples were weighed and oven dried at 90°C for 24 hours to get the dry weight of the soil. The total number of fungal propagules in one gram of the dry soil was calculated using the method described by Kerry and Bourne (2002).

For the root CFU, the roots were gently washed with tap water and cut into one centimetre long pieces. Using nine millilitre sterile distilled water agar (0.05%), one gram of roots was crushed using a pestle and mortar. From the crushed roots, one millilitre was diluted in nine millilitre sterile distilled water in a universal bottle to dilutions of 10^{-2} and 10^{-3}. From dilutions 10^{-2} and 10^{-3} , 0.2ml was cultured on Petri dishes containing semi-selective (*P. chlamydosporia*) and *Paecilomyces* (*P. lilacinus*) media and incubated at 25°C for 14 (*P. chlamydosporia*) and seven (*P. lilacinus)* days, after which colonies were counted and the counts used to calculate CFU/g roots.

3. 6. 3 Root galling index

Roots of pot-grown plants were washed gently and the galls were assessed using the rating chart illustrated by Coyne *et al.* (Coyne *et al.*, 2007). The ratings were from one (no galling damage) to five (severe galling) (Appendix 5).

3. 6. 4 Extraction of root-knot nematode juveniles from the soil and roots

The representative soil samples from each treatment were used to extract RKN juveniles (J2) using a modified Baermann technique as described by Hooper *et al.* (2005). Two tissue papers were placed on the sieve which was placed in a plastic dish. About 200ml of soil was placed on the tissue and evenly spread on the sieve. Water was added to the dish to moisten the soil and the extraction filters were covered using another dish for 24-48 h. After this time, the sieves containing the soil were removed and excess water in the dish was reduced to 10ml by passing the extract through a small sieve of 38µm aperture sieve. Using one millilitre extract, RKN J2 were counted in a counting dish under a dissecting microscope.

Second stage juveniles in the roots were extracted using the maceration technique (Hooper *et al.*, 2005). The roots were chopped into small pieces of one centimetre length and then five gram was blended into 100ml water incubated for 24-48 h (Hooper *et al.*, 2005) following procedures and techniques used in 3. 4. 1. The nematodes were concentrated to 10ml using sieve of 38µm mesh and counted as for the soil samples.

3. 6. 5 Plant shoot weight

Tomato plants were uprooted and the shoot cut off at the soil line. The fresh mass was taken before placing the shoots in paper bags. The bags were placed in an oven at 70°C until constant mass were achieved about 72 h before the weight was recorded.

3. 7 Statistical analysis

All the data were checked for normal distribution before subjecting them to analysis of variance (ANOVA) using Genstat package version 11. Data on CFU were log10 (x+1) transformed while percentages were either transformed using Arcsine of square root or Logit ((%infection+0.5)/(100.5-%infection)). The means were compared using least significance difference (LSD) at P=0.05.

CHAPTER 4

EFFICACY OF DIFFERENT FUNGAL ISOLATES IN THE CONTROL OF ROOT-KNOT NEMATODES

4.1 INTRODUCTION

Use of fungi that infect nematode eggs and females of root-knot and cyst nematodes has been gaining popularity as the need for alternatives or supplements to chemical nematicides increase. Studies done using *Pochonia chlamydosporia (Verticillium chlamydosporia)* and *Paecilomyces lilacinus* have shown promising results in the control of root-knot nematodes (RKN) in countries like Cuba, Philippines, Australia, USA and South Africa (Atkins *et al.*, 2003a; Atkins *et al.*, 2005; Kiewnick and Sikora, 2006). The two fungi, *P. chlamydosporia* and *P. lilacinus*, parasitize nematode eggs through contact between the vegetative mycelia and the eggshell, which lead to development of appresoria that adhere to the eggshell (Kerry and Bourne, 1996; Kiewnick, 2006). An infection peg is developed that penetrates the eggshell thus destroying the embryo (Kerry, 2000).

The two nematophagous fungi are known to have many species with *P. lilacinus* being more common in warmer climate (Karssen and Moens, 2006; Esteves, 2007). However, not all isolates of the same species have the same capacity to infect RKN eggs. Success depends on host susceptibility and origin of the fungus (Leij de and Kerry, 1991). A number of studies have been done on the virulence of different isolates of *P. lilacinus* with strain 251 being the most effective (Holland *et al.*, 1999; Kiewnick and Sikora, 2006; Anastasiadis *et al.*, 2008). According to Kerry (2000), isolates of *P. lilacinus* from different nematode eggs in the same soil have considerable variation in their virulence against nematodes. To determine the

efficacy of these isolates from different habitats, screening has to be done based on different parameters.

These fungi are facultative parasites of RKN eggs and also live as saprophytes in the absence of nematodes (Verdejo-Lucas *et al.*, 2003). Scarcity of nematodes in the soil causes the fungus to form chlamydospores which germinate when favourable conditions resume (Kerry and Bourne, 1996). Growth and ability of the fungus to colonise and parasitize RKN eggs in the soil varies depending on the type and condition of the soil (Leij de *et al.*, 1993). Glasshouse experiments have shown that the fungus may colonise the soil but not control the nematodes (Jaffee, 2004). It has been reported that roots infested by RKN harbour large numbers of *P. chlamydosporia* and *P. lilacinus* growing in their rhizosphere (Kerry, 2000). However, the efficacy in control of RKN by *P. lilacinus* and *P. chlamydosporia* is much less on nematode susceptible crops due to the formation of large galls which 'shield' the egg masses inside the galls thus hindering infection by the fungi (Atkins *et al.*, 2003a).

Excluding South Africa where *P. lilacinus* has been commercialized, little work on these fungi have been done in other countries like in Sub Saharan Africa (Bourne *et al.*, 2004). Researchers in Africa need to focus on making biological control a more viable management option to farmers. Before the introduction of the commercial products, more research needs to focus on locally isolated biological agents, their efficacy in nematode control, survival and growth under different environmental conditions. Nematophagous fungi such as *P. chlamydosporia*, which is being used in other tropical countries like Brazil in the control of RKN, need to be tested under local conditions and compared with effective strains of the fungus like *P. lilacinus*. In order to come up with good recommendations on the efficacy of these biological agents, they have to undergo tests (Kerry and Bourne, 1996). Currently,

management of RKN relies on application of organic amendments, rotation with poor host crops, sanitation, destruction of residual crops and chemical nematicides (Nono-Womdim *et al.*, 2002; Kimenju *et al.*, 2004). Most of these methods are not highly effective due to the reason that they are either used singly or the application rates are low. Discovery of effective biological agents will increase the range of management combinations needed for integrated management of RKN, especially to small scale farmers. In this study, *P. chlamydosporia* and *P. lilacinus* isolates were tested against RKN in vegetable production system with an aim of using them in East and Southern Africa. Therefore, the specific objectives of this study were;

- To determine the parasitic activity of different isolates of *P. chlamydosporia* and *P. lilacinus* against root-knot nematodes eggs *in vitro*
- To determine the effect of soil sterilization on the efficacy of isolates of *P. chlamydosporia* and *P. lilacinus* against root-knot nematode

4. 2 MATERIALS AND METHODS

4. 2. 1 Source and production of *Paecilomyces lilacinus* inoculum

Two isolates of *P. lilacinus*, one a Kenyan isolate with accession Pl-20K and the other a commercial product, Pl-Plus® were evaluated. The commercial product Pl-Plus® was from Biological Control Products SA (PTY) Ltd., South Africa. Inoculum of *P. lilacinus* isolate 20K was produced by sub-culturing a seven-day-old culture on PDA in Petri dishes and incubated for 14 days at 25°C. After incubation the conidia were harvested using 5ml sterile distilled water and L-shaped glass rod. The conidia were counted using a haemocytometer under a compound microscope 20 × as described in section 3 .3. 1.

4. 2. 2 Parasitic activity of different fungal isolates on root-knot nematode eggs

An *in vitro* fungal parasitism experiment was conducted using the procedure described by Kerry and Bourne (2002). A total of six isolates of *P. chlamydosporia* (10, 126, 144, 147, 177 and 392) and one isolate of *P. lilacinus* were screened for their efficacy on RKN eggs. The experiment had one control where no fungus was applied to the eggs making a total of eight treatments. The treatments were arranged in a completely randomised design (CRD) and replicated four times. The fungal isolates that were cultured in Petri dishes on water agar amended with antibiotics for 72 h (Appendix 1) were used to test for their parasitic activity against RKN eggs. Root-knot nematodes eggs were extracted and prepared from the infected tomato roots using the root maceration method described by Coyne *et al.* (2007). The egg suspension was then sieved through a cheese cloth to remove plant debris. About 0.2ml of RKN egg suspension, containing about 500 eggs, was spread evenly on the fungal colonies and incubated at 25°C for 72 h. Data on parasitized eggs (infected by the fungus) and non-parasitized eggs (not infected by fungus) were recorded and used to estimate the percentage of eggs parasitized as described in section 3. 6. 1. This experiment was repeated using the procedure above but with three replications.

4. 2. 3 Effect of soil sterilization on growth and efficacy of fungal isolates

The experiment was conducted using 500g soil capacity pots in a glasshouse. Two isolates of *P. chlamydosporia* were selected from the *in vitro* screening experiment outlined above. The isolates tested were *P. chlamydosporia* isolates 392 and 10, and two isolates of *P. lilacinus* (one from the above experiment and the second the commercial isolate Pl-Plus). A control without any fungus was included and the treatments were in sterilised and non-sterilised soil

making a total of ten treatments. Treatments were arranged in a CRD and replicated five times. Pots were filled with soil that was collected from the field and sterilised for 30 minutes at 121°C. Pots filled with non-sterilised soil from the same field were used as control treatments for each fungal isolate. Using polyethylene plastic bags, the soil for each treatment was mixed with the appropriate fungal isolate at the rate of 5000 chlamydospores per gram of soil (Kerry and Bourne, 2002) while PI-Plus was applied at the rate of 2kg/ha. One pre-germinated tomato seedling was planted in each pot. Two weeks after planting, RKN eggs and juveniles that were extracted from infected tomato roots using root maceration method as described by Coyne *et al.* (2007), counted and diluted were introduced to each pot with 3000 eggs or second stage juveniles. The pots were watered regularly to maintain soil moisture and NPK fertiliser (17:17:17) was applied at 10g/pot. One month (30 days) after application of the fungus, soil sampling was done to estimate the fungal propagules in the soil.

The experiment was terminated after 90 days when the plants were uprooted and assessed for galling index using a scale of 1-5 as described by Coyne *et al.* (2007). The plant shoots, cut at soil level, were packed in paper bags and oven dried at 70°C for 48 h before data on dry shoot weight was recorded. Also, the soil samples were taken from each treatment for assessment of the number of fungal propagules (CFU) and J2 in the soil. The number of fungal propagules was assessed by plating 10^{-2} and 10^{-3} dilutions on semi-selective medium (Appendix 1) in Petri dishes and incubated for 14 days before the counts on the colony and the estimate of the number of fungal propagules was done as described by Kerry and Bourne (2002). The RKN J2 were extracted using the modified Baermann technique as described by Hooper *et al.* (2005). The details of techniques and procedures used in the assessments were described in 3. 6.

4. 2. 4 Statistical analysis

The data were checked for normal distribution where by CFU and second stage juveniles counts were Log_{10} transformed while percent parasitism were Arcsine transformed. The data were subjected to ANOVA using Genstat package version 11 and the means were compared using LSD.

4. 3 RESULTS

4. 3. 1 Parasitic activity of different fungal isolates on root-knot nematode eggs

Percentage parasitism among the isolates of *P. chlamydosporia* and one *P. lilacinus*-20K significantly differed ($P<0.001$) among isolates. *Pochonia chlamydosporia* isolates 392 and 10 had a significantly higher percent egg infection compared to isolates 126, 144, 147, 177 of *P. chlamydosporia* and the isolate of *P. lilacinus*. The differences in egg parasitism among the other isolates of *P. chlamydosporia* (126, 144, 147 and 177) were not different from each other and from the isolate of *P. lilacinus* (Fig. 4.1). Findings from the repeated experiment also showed significant differences ($P\leq0.001$) among the fungal isolates. Isolates 10 and 392 were more effective than isolates 126, 144 and 177 of *P. chlamydosporia* and *P. lilacinus* (Fig. 4.1). However, the percentage of eggs parasitized by isolates 147 and 10 of *P. chlamyodosporia* were not significantly different ($P<0.05$) from each other in the repeated experiment (Fig. 4.1).

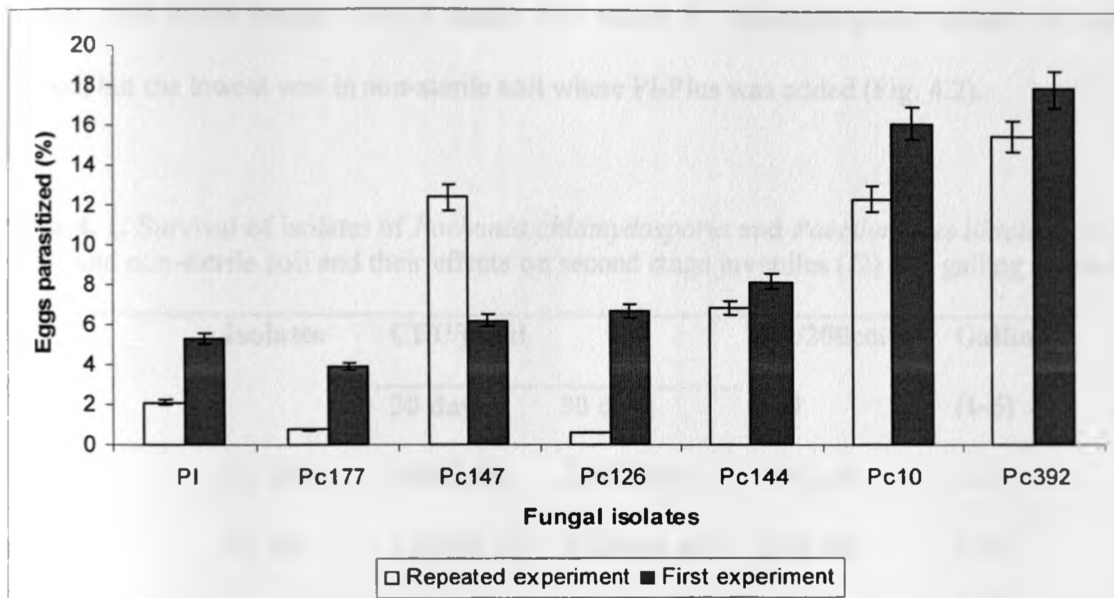


Fig. 4. 1. Percentage of eggs parasitism by the six isolates of *Pochonia chlamydosporia* (Pc) and one isolate of *Paecilomyces lilacinus* (Pl).
Means were compared using LSD=5.267 for the first experiment and LSD=7.005 for repeated experiment. The I bars signify 5% error.

4. 3.2 Effect of soil sterilization on growth and efficacy of fungal isolates on egg parasitism

The four different fungal isolates in sterile and non-sterile soils showed a significant ($P<0.05$) effect on fungal propagules at 30 days and numbers of J2, galling index (Table 4.1) and dry shoot weight of the plants (Fig. 4.2) at 90 days. The fungal propagules counted at 30 days after soil infestation was highest for *P. chlamydosporia* isolate 10 in sterile soil and for *P. chlamydosporia* isolate 392 in non-sterile soil. However, the *P. lilacinus*-20K was a slow coloniser with no soil fungal propagules found at 30 days, but an increase in fungal propagules was recorded after 90 days. Application of Pl-plus and *P. chlamydosporia* isolate 392 in sterile and non sterile soil decreased the numbers of J2. *Pochonia chlamydosporia* isolate 10 effectively reduced J2 in non-sterile soil only. Application of Pl-plus in non-sterile soil increased galling index same as the control treatments. Among the isolates tested, the

highest plant shoot weight was in sterile soil where *P. chlamydosporia* isolate 392 was applied, but the lowest was in non-sterile soil where Pl-Plus was added (Fig. 4.2).

Table 4. 1. Survival of isolates of *Pochonia chlamydosporia* and *Paecilomyces lilacinus* in sterile and non-sterile soil and their effects on second stage juveniles (J2) and galling index in tomato.

Soil	Isolates	CFU/g soil		J2 /200cm^3	Galling
		30 days	90 days	soil	(1-5)
Sterilised	Pc 392	986(2.93)	2,852(3.43)	14(1.18)	2.67
	Pc 10	1,509(3.17)	33,294(4.45)	42(1.48)	2.67
	Pl-20K	0(0.00)	789,059(5.89)	44(1.64)	2.67
	Pl-plus	605(2.78)	13,465(3.96)	8(0.86)	3.00
	Control	0(0.00)	0(0.00)	68(1.83)	3.33
Non-sterilised	Pc 392	6,122(3.78)	1,796(3.18)	16(1.22)	2.67
	Pc 10	406(1.86)	5,542(3.71)	12(1.05)	3.00
	Pl-20K	818(2.06)	5,315(3.68)	49(1.66)	2.33
	Pl-plus	825(2.06)	2,012(2.32)	17(1.08)	3.33
	Control	0(0.00)	0(0.00)	30(1.49)	4.00
LSD		1.62	1.16	0.46	0.94

Values in brackets are Log_{10} transformed means used in comparison with LSD

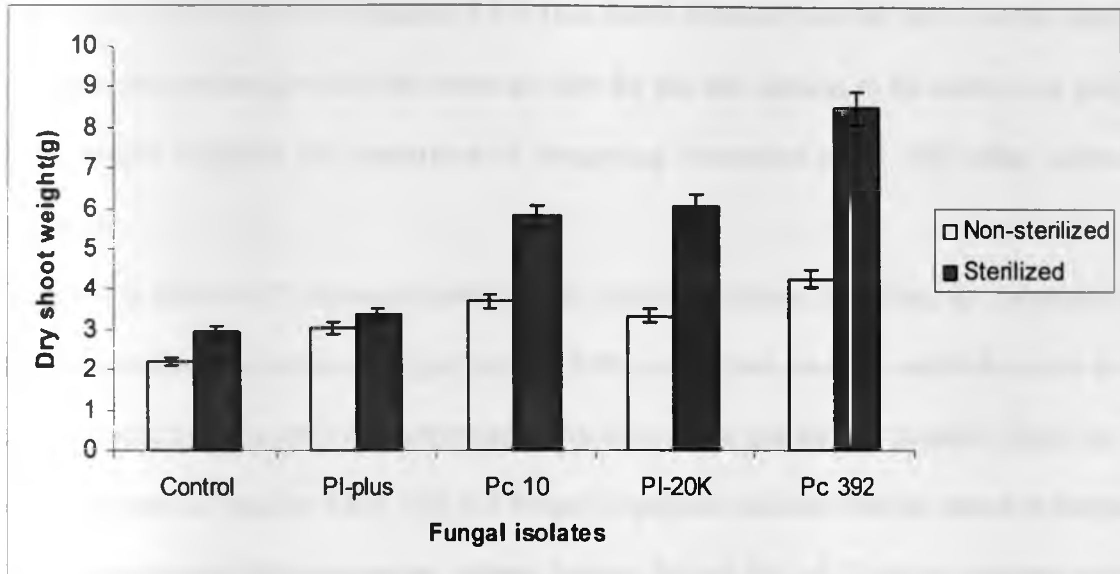


Fig. 4. 2. Dry shoot weight of tomato, after application of *Pochonia chlamydosporia* (Pc) and *Paecilomyces lilacinus* (Pl) in sterile and non-sterile soils.
Data were compared using LSD 1.40. I bars signify 5% error.

4. 4 DISCUSSION

This study has established that different isolates of *P. chlamydosporia* exhibit differences in parasitism of RKN eggs. Differences in efficacy of the isolates were recorded even among isolates from the same species. The differences between isolates in parasitizing RKN corroborates with findings documented by other scientists (Godoy *et al.*, 1982; Leij de and Kerry, 1991; Esteves, 2007). *Pochonia chlamyodosporia* isolates 10 and 392 have consistently shown high efficacy in comparison to other isolates. This implies that the two *P. chlamydosporia* isolates, which are effective in infecting RKN eggs, could be a good choice to be used as a strategy in the management of RKN. The reason for isolate 10 and 392 of *P. chlamydosporia* performing better than the others could be associated with their origin, since the two were isolated from *Meloidogyne* eggs and therefore perhaps better adapted to this genus. According to Morton *et al.* (2003), isolates obtained from *Meloidogyne* eggs have

been found to be more active against RKN than those isolated from the soil or other hosts. However, the percentage infection is not as high for the two isolates to be used on its own. These results suggests the importance of integrating biocontrol agent with other control measures.

Comparison between *P. chlamydosporia* and *P. lilacinus* isolates, including the commercial product revealed that different fungal isolates differ in the time taken to establish in the soil and the capacity to control soil nematodes. This shows that not all egg parasitic fungi have the same potential against RKN. The soil fungal propagules indicate that the speed of fungus to colonise the soil differs between isolates. Isolates 10 and 392 of *P. chlamydosporia* were able to colonise the soil within a shorter period (30 days) of time compared to the other isolates which took 90 days. Isolate 10 has shown that it can colonise the soil for longer period of time than the others, thereby demonstrating that this isolate can be stable in the soil for a longer period and probably suppress nematodes faster than the others. Isolate 10 of *P. chlamydosporia* could be a good competitor against other soil antagonistic micro-organisms. The high ability of isolate 10 to colonise soil can be due to the reason given by Mauchline *et al.* (2004) who suggested that a fast-growing isolate is capable of utilising a wider range of energy resources by competing with other soil micro-organisms, thus leading to high fungal population density. The slow speed of the fungus to colonise the soil does not mean it will not establish and control nematodes but this result implies that it may delay the attack of early nematode infection. Therefore, the results suggest that the isolates which are slow colonisers should be applied to the soil much earlier than the planting time. Van Damme *et al.* (2005) noted differences in the capacity of different *P. chamydosporia* isolates to colonise the soil

and recommend that adequate time should be allowed for the fungus to establish in the soil to effectively parasitize the first generation nematodes.

In addition to soil colonisation, the study found that isolates 10 and 392 were more effective in reducing the number of J2 along with Pl-Plus. The efficacy of *P. lilacinus* has been reported by Kiewnick and Sikora (2006) who found 90% reduction in *M. hapla* after application of isolate 251 in tomato crop. This implies that *P. chlamydosporia* isolates 10 and 392 can potentially be as effective as Pl-Plus, a commercially available product from South Africa, in reducing soil nematodes. The efficacy of isolates 10 and 392 might be attributed to favourable tropical environmental conditions. Also, the use of isolate 392 of *P. chlamydosporia* increased the dry shoot weight of the tomato plants. The ability of isolate 392 to colonise the soil fast and reduce the number of soil nematodes can be reflected in enhanced early growth by the plants which led to high dry shoot weight. This concurred with the results reported by Holland *et al.* (2003) who applied egg parasitic fungus *P. lilacinus* in tobacco and significantly reduced galling and increased yield. As with fungal propagules, the shoot weight was higher in sterile soil than in non-sterile soil. According to Egli *et al.* (2006) sterilisation of soil led to increased pH and made the soil alkaline. This means that, perhaps sterilisation helped to neutralise the soil pH and increase the availability of the nutrient limited by low pH. In addition, the high shoot weight in sterile soil could result from the fact that sterile soil being free from other micro-organisms, including those which normally compete with the fungus.

For most of fungal isolates used in this study have indicated sterile soil is more easily colonised compared to non-sterile soil. This is because there was no competition with other soil micro-organisms in sterile soil compared to the non-sterile soil where various

antagonistic micro-organisms may be present. This is supported by Mauchline *et al.* (2004) findings, that isolates of *P. chlamydosporia* differ in their ability to compete with saprophytes in the rhizosphere of different soil types.

The study has found that most fungal isolates were able to reduce the root galling in sterile and non-sterile soil conditions. This could be due to the ability of *P. chlamydosporia* and *P. lilacinus* to colonize the eggs and thereby rendering them non-viable and effectively reducing the populations of a second generation of nematodes. In the process, the plant may not escape to form galls caused by the infection of first generation nematodes. The reduction in root galling for the plants grown in soil treated with the fungus compared to plants from non-treated soil was earlier reported by Kiewnick and Sikora (2006) when isolate 251 of *P. lilacinus* was applied into the soil to control RKN in tomato plants.

The study has recorded higher dry shoot weight when tomato is grown in sterile soil than non sterile soil. The high shoot weight in sterile soil might have attributed to the activity of large population of fungus in sterile soil at termination of the experiment that generally reduced root galling and increased dry shoot weight. According to Wang *et al.* (2004) most nematophagous fungi work better in sterile soils than in non-sterile soils. And one of the reason that this fungus cannot work better on non-sterile soil is the higher nematode density and competition from other micro-organisms (Chen and Dickson, 2004). This result implies that isolate 392 of *P. chlamydosporia* is the best of the tested isolates and can give higher shoot weight in all soil conditions than other isolates.

The isolates showed different behaviour on different parameters used in this study (soil J2 RKN counts, galling index and shoot weight) to assess the efficacy in managing RKN. For example, the isolate 392 of *P. chlamydosporia* has shown promising results on fungal

propagules at 30 days, 90 days and plant dry shoot weight. Also *P. lilacinus*-20K had no fungal propagules at 30 days but high at 90 days. The ability of *P. chlamydosporia* and *P. lilacinus* isolates to vary in the capacity to manage RKN has been supported by Gaspard *et al.* (1990).

These results suggest that *P. chlamydosporia* isolates 10 and 392 should be tested in the field because they have shown promising results in non-sterile soil when tested under glasshouse condition. Also, these isolates had an efficacy that was comparable to the Pl-plus which is a commercial product.

CHAPTER 5

EVALUATION OF LOCALLY AVAILABLE ORGANIC MATERIALS FOR USE IN PRODUCTION OF *Pochonia chlamydosporia*

5.1 INTRODUCTION

Pochonia chlamydosporia synonym: *Verticillium chlamydosporium* Goddard is one of the biological control agents which have shown success in the management of RKN (Verdejo-Lucas *et al.*, 2003). In addition, it has been established that the fungus is widespread in subtropical and tropical soils (Stirling *et al.*, 1998). *Pochonia chlamydosporia* is a facultative parasite on eggs and females of root-knot and cyst nematodes (Atkins *et al.*, 2003a). The fungus exists as a saprophyte on organic materials and can survive even when there are no nematode eggs, which makes it easy to produce *in vitro* (Verdejo-Lucas *et al.*, 2003; Esteves, 2007). In the absence of a suitable host, the fungus forms chlamydospores which are multicellular structures as a survival mechanism (Atkins *et al.*, 2003a). The fungus also produces conidia which can be used for *in vitro* experiments and forms a white cottony growth on potato dextrose agar (PDA) and corn meal agar (CMA) (Kerry and Bourne, 2002; Esteves, 2007).

Biological control agents are widely spread in cultivated soils but their development into commercial products has been very slow (Akhtar and Malik, 2000). For example, there are only few published researches on *P. chlamydosporia* that have been conducted in East African countries (Karanja *et al.*, 2004). This has also brought a limitation on the ways in which it could be disseminated for use by farmers. Therefore, this biological agent has hardly been used by farmers in Sub-Saharan Africa. This has partly been due to lack of suitable

methods and scanty information on economically sustainable methods for mass production. In many places, production is done using cereal grains like rice, wheat and maize (Leij de and Kerry, 1991; Kerry and Bourne, 2002; Atkins *et al.*, 2003a), but, these grains are also a valuable and major food source for the people in this region. This raises the argument that one reason for the limited use of biological control agents in the region has been due to the expense incurred in mass production (Stirling *et al.*, 1998).

Introducing the use of *P. chlamydosporia* as a biological control agent to poor resource, small scale farmers depend on research on mass production using locally available substrates. It's presumed that use of locally available cheap substrates for mass production of *P. chlamydosporia* will therefore increase its utilisation. The method for the production of biological fungi have been developed and practised in some countries (Lopez-Llorca *et al.*, 1999). Some of the useful substrates in the production of biocontrol fungi are plant wastes (Lopez-Llorca *et al.*, 1999). However, production of biological agents needs to be done with care, particularly with respect to quality of the product. Quality helps to increase product performance, guarantee product safety, normalize manufacturing expenses and decrease the risks of supply failure (Jenkins and Grzywacz, 2000).

Organic materials such as maize stover and cobs are one of the locally available materials in many places in Kenya. They are incorporated in the soil for the management of soil fertility and as animal feeds. They have potential to be one of the substrates that can be used to produce chlamydospores at a lower cost. However, different substrates differ in their suitability for spore production depending on the nutrient content of the substrate (Liu and Chen, 2003). Differences in the state and nutrient content of the materials which lead to differences in growth rate and sporulation (Akhtar, 1997). According to Liu and Chen (2003),

some carbon sources inhibit or allow growth when they are in either solid or liquid condition. The need for tests of different substrates in order to identify a good substrate is therefore necessary.

The overall objective of this study was to develop a cost effective method of producing *P. chlamydosporia.* The specific objectives of this study were:

- To determine the suitability of different organic materials and their extracts as substrates for the production and viability of chlamydospores of different isolates of *P. chlamydosporia*
- To determine the effect of different concentrations of extracts from effective organic materials on growth of isolates 10 of *P. chlamydosporia*

5. 2 MATERIALS AND METHODS

5. 2. 1 Evaluation of organic substrates for the production of *Pochonia chlamydosporia* chlamydospores

The experiment was carried out in the laboratory with the aim of determining the most effective organic materials that supports fungal growth and production of viable chlamydospores. Fourteen organic materials that included green manures (cabbage leaves, *Crotalaria ochroleuca, Tagetes minuta, Mucuna pruriens,* and *Tithonia deversifolia*), animal manures (cow, goat and chicken) and crop residues (maize stover, maize cobs and bean straw) and industrial by-products (sawdust, rice bran and filtermud) were evaluated. Rice was included as a control. The treatments were arranged in a CRD with three replications. Isolate 10 of *P. chlamydosporia* which was selected in section 4. 4 was used in further experiments.

The organic materials (50g) were pulverized as described in section 3. 5. 1 and mixed with sand in 500 ml capacity flasks at a ratio of 1:1 w/w. The substrates were autoclaved at 121°C for 30 min. After cooling, the mixtures were inoculated with 3 fungal plugs of 7.5mm diameter cut out from 7-day-old cultures. The mixtures were incubated at 25°C in darkness for 21 days after which the number of chlamydospores was estimated and their viability determined following the procedure described in section 3. 3.

5. 2. 2 Effect of extract from organic materials on chlamydospore production

The experiment was conducted in the laboratory with the aim of determining the ability of extracts from 15 substrates to support growth of *P. chlamydosporia* and chlamydospore production. The experiment had 15 treatments (extracts from 14 organic substrates and rice) arranged in a CRD and each treatment was replicated three times. After preparing the extracts (see section 3. 5. 2), the solutions were incorporated into agar (non nutrient agar) by autoclaving at 121°C for 20 min. and pouring into Petri dishes (9cm diameter). Three 7.5mm plugs of 7-day-old cultures of *P. chlamydosporia* isolate 10 were added and incubated at 25°C in darkness for 21 days. After incubation, 9ml of sterile water was used to harvest chlamydospores from the media by scraping the surface of the colony with an L-shaped glass rod. The chlamydospore counts per ml of suspension were determined as described in section 3. 3.

5. 2. 3 Effect of different extracts from organic materials on growth of different isolates of *Pochonia chlamydosporia*

The organic materials selected (*T. minuta*, maize stover, cabbage and rice (control)) were used in the laboratory experiment and each mixed with water at 25g per litre of water. The extracts from organic materials (section 3. 5. 2), media preparation and inoculation was done using methods and procedures described in section 5. 2. 2. Three effective isolates (10, 147 and 392) of *P. chlamydosporia,* selected from two experiments in chapter 4 were used in this experiment. The media amended with extracts from organic materials were inoculated with 7-day-old cultures of the fungus. The Petri dishes (9cm) were sealed and incubated at 25°C in darkness for 21 days. Data on fungal growth and chlamydospore production were taken as described above.

5. 2. 4 Effect of different concentrations of extracts from different organic materials on growth of *Pochonia chlamydosporia*

One *in vitro* experiment was conducted using four organic material extracts which were among the best in the production of viable chlamydospores of *P. chlamydosporia* in sections 5.2.1&2. The materials were *T. minuta*, cabbage, maize stover and rice (control). The materials were prepared and extracted as in section 3. 5. 1. The extracts, made as described in section 3. 5. 2, were diluted with water to 75%, 50% and 25% organic substrate concentrations. Each concentration was used to make agar media as described in section 5. 2. 2 and plated on 9cm diameter Petri dishes. Three 7.5mm diameter plugs of 7-day-old *P. chlamydosporia* isolate 10 were inoculated in each Petri dish. The Petri dishes were sealed and incubated at 25°C in darkness for 21 days. After 21 days, data on diameter of the fungal

colonies were measured and the chlamydospores formed were harvested, counted and tested for viability as described in section 3. 3.

5. 2. 5 Statistical analysis

All data were checked for normal distribution and, when required, data on chlamydospores and percent viability were Log_{10} and Arcsine transformed, respectively, before subjecting them to analysis of variance (ANOVA). The means were compared using LSD.

5. 3 RESULTS

5. 3. 1 Evaluation of organic materials for the production of *Pochonia chlamydosporia* chlamydospores

The differences in numbers of chlamydospores were significant ($P<0.001$) among the organic substrates tested (Table 5.1). *Mucuna pruriens* and maize cobs had higher counts compared to counts from rice. Most of the substrates were as good as rice, which was used as a standard, while no chlamydospores were produced in cabbage, cow manure and sawdust (Table 5.1). The viability of chlamydospores from different OA substrates were significantly different ($P<0.001$). However, chlamydospores from chicken manure and *C. ochroleuca* were not viable. The percentage viability of chlamydospores from the other organic substrates were above 50% with the exception of rice husks and filtermud (Table 5.1).

Table 5. 1. Number and percentage viability of chlamydospores from different organic substrates.

Organic amendment	[1]Number of chamydospore/g	[2]Chlamydospore viability(%)
Cabbage	0(0)	*
Cow manure	0(0)	*
Sawdust	0(0)	*
Chicken manure	1,000,000(5.9)	0(0.00)
Rice husk	1,333,333(6.06)	16(23.69)
Maize stover	2,666,667(6.42)	78.33(62.31)
Filtermud	5,833,333(6.73)	26(29.87)
Bean straw	9,500,000(6.86)	77.33(61.67)
Crotalaria ochroleuca	12,833,333(7.09)	0(0.00)
Goat manure	12,666,667(7.12)	79(62.74)
Tagetes minuta	15,000,000(7.14)	69.67(56.69)
Tithonia diversifolia	20,333,333(7.24)	72.33(58.29)
Maize cobs	43,000,000(7.56)	65(53.73)
Mucuna pruriens	42,166,667(7.62)	60(50.77)
Rice (Standard)	7,833,333(6.88)	55(47.93)
LSD	0.37	7.71

Values in brackets are [1]Log_{10} and [2] arcsine transformed means used in comparison with LSD, *OA with no chlamydospores were not included in viability analysis.

5. 3. 2 Effect of extracts from organic materials on chlamydospore production

The differences in chlamydospore counts of extracts from different OA were significant ($P<0.001$) from each other (Fig. 5.1). Most of the extracts used supported growth and production of large numbers of chalmydospores, with exception of extracts from sawdust which did not support chlamydospore production. However, cabbage which was the worst when used in form of powder was the best when used as extract.

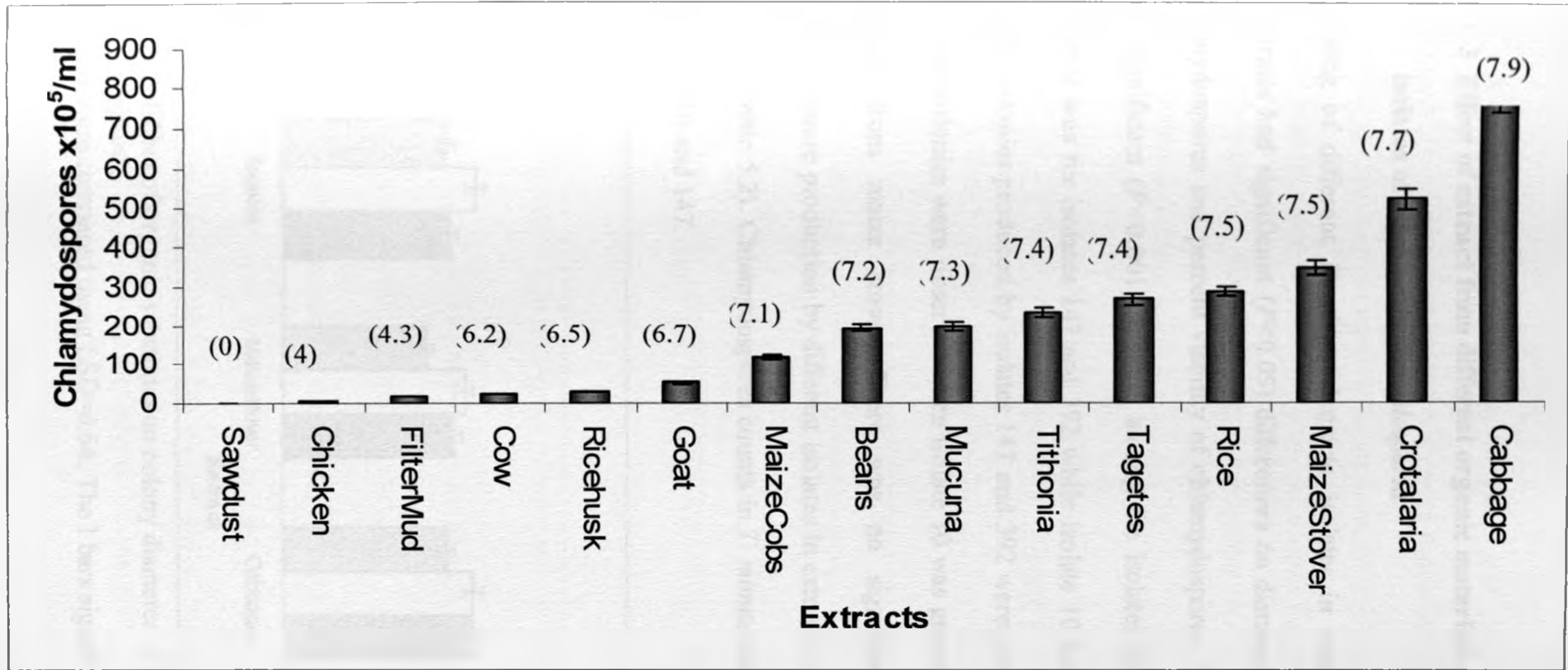


Fig. 5. 1. Numbers of chlamydospores produced from different extracts of various organic substrates.
Values in brackets are Log_{10} transformed data and were compared using LSD=2.201. The I bars signify 5% error.

5. 3. 3 Effect of extract from different organic materials on growth of different isolates of *Pochonia chlamydosporia*

Growing of different *P. chlamydosporia* isolates in extracts from different organic substrates had significant ($P<0.05$) differences on diameter of the colonies, number of chlamydospores and percent viability of chlamydospores. The diameter of the colonies was significant ($P<0.001$) different among the isolates (Fig. 5.2). The largest colony diameter was for isolates 147 and 392 while isolate 10 had the smallest diameter. The largest colonies produced by isolates 147 and 392 were grown in extract from rice. The smallest colonies were observed when isolate 10 was grown in a medium amended with extracts from maize stover. There was no significant difference ($P>0.05$) in chlamydospore production by different isolates in extracts from OA with exception of *T. minuta* (Table 5.2). Chlamydospores counts in *T. minuta* were higher in isolate 392 than in isolate 10 and 147.

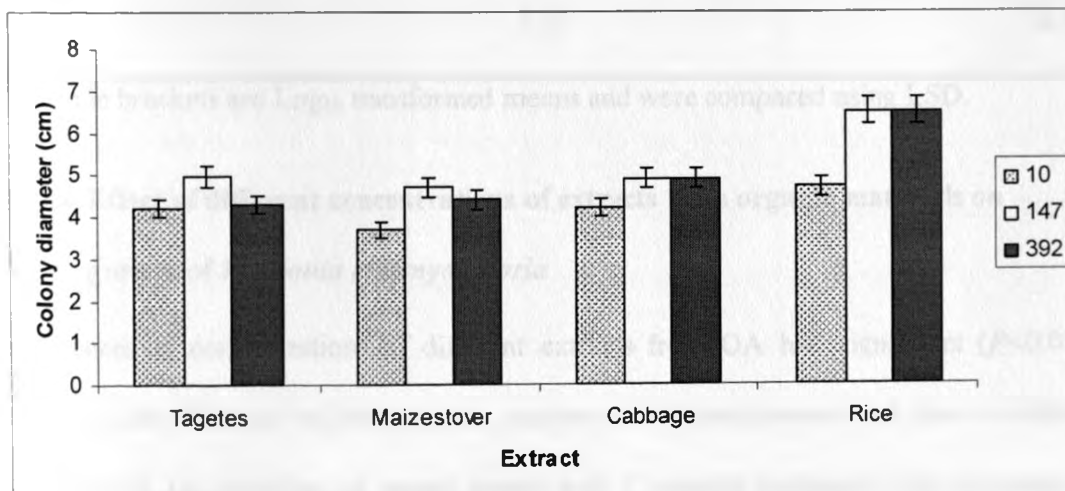


Fig. 5. 2. Effect of organic substrates on colony diameter of isolates of *Pochonia chlamydosporia*.
The data were compared using LSD=0.64. The I bars signify 5% error.

The percent viability of the chlamydospores was higher in extract from cabbage compared to that of the other three which were not different from each other (Table 5.2). In contrast to the other parameters, the percentage viability of the chlamydospores were generally significantly ($P<0.001$) higher for isolate 10 in all substrates and lower for isolate 147. However, the highest percent viability was for isolate 147 when grown on cabbage extract.

Table 5. 2. Effects of extracts from organic materials on chlamydospore production and percent viability of isolates of *Pochonia chlamydosporia.*

Substrates	Chlamydospores counts/ml			Chlamydospores viability(%)		
	10	**147**	**392**	**10**	**147**	**392**
Cabbage	53,333(4.73)	56,667(4.75)	453,333(5.66)	83.92	91.43	72.87
Maize stover	33,333(4.49)	150,000(5.17)	370,000(5.57)	81.19	46.06	71.35
Rice	170,000(5.13)	156,667(5.16)	493,333(5.69)	87.75	40.10	71.53
Tagetes minuta	6,667(1.43)	3,333(1.33)	80,000(4.89)	67.82	63.07	69.59
LSD		1.67			6.75	

Values in brackets are Log_{10} transformed means and were compared using LSD.

5. 3. 4 Effect of different concentrations of extracts from organic materials on growth of *Pochonia chlamydosporia*

Differences in concentrations of different extracts from OA had significant ($P<0.05$) effects on the diameter of the colonies, number of chlamydospores and their viability. Diameter of the colonies in maize stover and *T. minuta* increased with decreasing concentrations (Fig. 5.3). The diameters of the colonies were generally higher in rice and

lower in maize stover when compared to the others. The largest diameter was from rice and *T. minuta* both at 25% concentration.

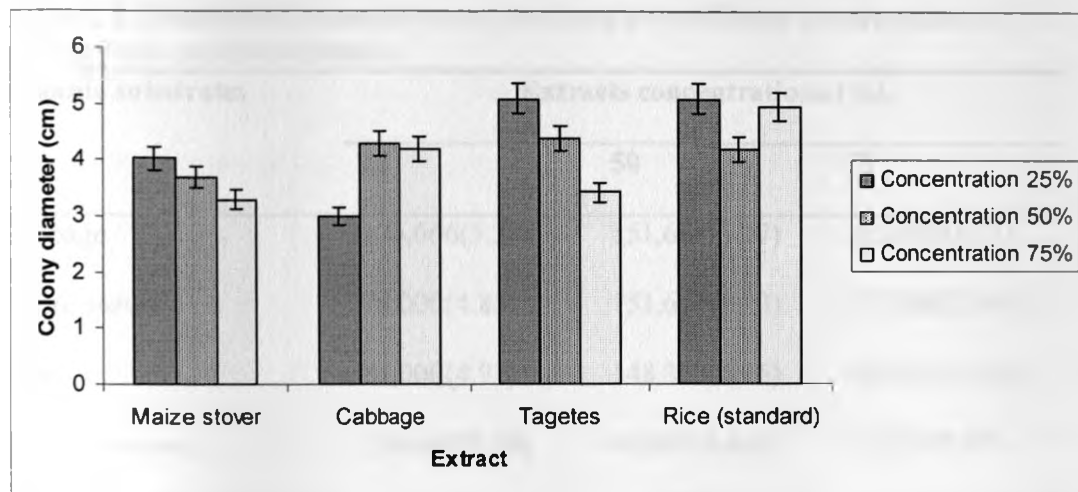


Fig. 5. 3. Colony diameter of *Pochonia chlamydosporia* on agar amended with extracts from organic materials of different concentrations.
Means were compared using LSD 0.6115. The I bars signify 5% error.

Different concentrations of extracts from different OA significantly ($P<0.05$) affected chlamydospore production (Table 5.3). Chlamydospore counts increased with increasing concentrations with all extracts with the exception of *T. minuta* which decreased with increasing concentration. *Tagetes minuta* also had the lowest counts while others had counts which were not significantly ($P>0.05$) different from each other. Also, concentrations of extracts from organic substrates led to highly significant ($P<0.001$) differences in percent viability of the produced chlamydospores (Fig. 5.4). In general the viability of chlamydospores was higher at high concentration (75%) compared to others (50% and 25%). The data for chlamydospores viability indicate that extracts from maize stover, cabbage and *T. minuta* supported significantly ($P<0.001$) higher viability which are similar between concentrations than in to rice. However, the chlamydospore viability

in rice decreased significantly ($P<0.001$) with decreasing concentration. Among all the extracts, rice had the highest and lowest viability percent.

Table 5. 3. Numbers of chlamydospores produced from different concentrations of extracts from organic substrates.

Organic substrates	**Extracts concentrations (%)**		
	25	**50**	**75**
Cabbage	176,666(5.23)	151,667(5.17)	325,000(5.51)
Maize stover	75,000(4.85)	151,667(5.23)	325,000(5.54)
Rice	85,000(4.93)	148,333(5.15)	180,000(5.22)
Tagetes minuta	186,667(5.18)	46,667(4.64)	13,333(2.87)
LSD		1.24	

Numbers in brackets are $\log_{10}$ transformed means and were compared using LSD

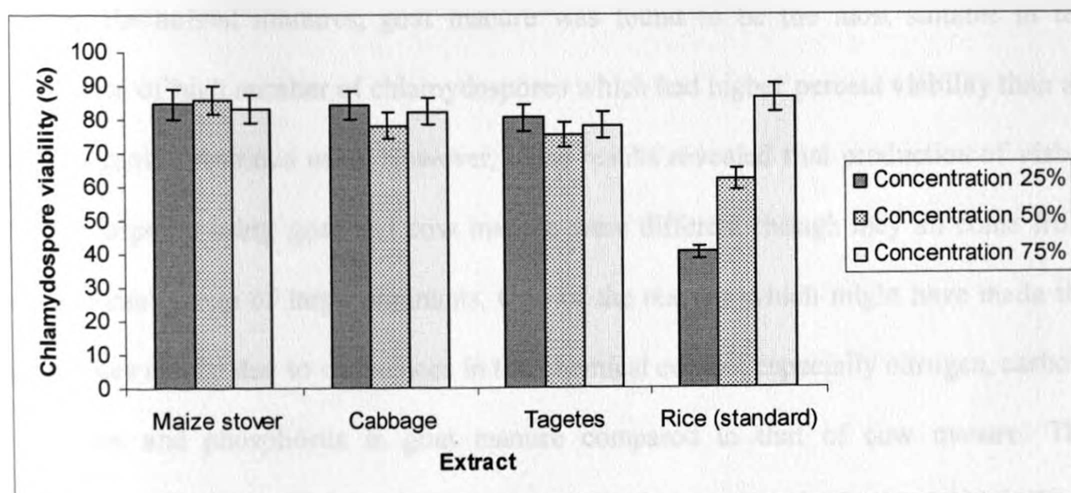


Fig. 5. 4. Percentage viability of chlamydospores of *Pochonia chlamydosporia* on xtracts from organic materials at different concentrations.
Means were compared using LSD 8.085. The I bars signify 5% error.

5. 4 DISCUSSION

This study has established that most of the locally available organic materials can be used to support the production of chlamydospores of the biocontrol agent *P. chlamydosporia*. All green manures except cabbage leaves used in this study as substrates supported chlamydospore production more or less at the same level with rice. Similarly, Lopez-Llorca and Olivares-Bernabe (1991) reported that leaf litters from different plants vary in suitability for growth and development of nematophagous and entomopathogenic fungi due to differences in their phenol content. Also, with the exception of *C. ochroleuca*, the percent viability for the chlamydospores produced was higher in many materials than that of rice. The ability of the green manure to support production of fungal chlamydospores could be due to the high content of major nutrients (nitrogen, phosphorus and potassium) in their shoots.

Among the animal manures, goat manure was found to be the most suitable in the production of high number of chlamydospores which had higher percent viability than all other organic substrates used. However, these results revealed that production of viable chlamydospores using goat and cow manure were different though they all come from same animal group of large ruminants. One of the reasons which might have made the differences can be due to differences in the chemical content especially nitrogen, carbon, potassium and phosphorus in goat manure compared to that of cow manure. The differences in chemical content in the manures of the same group of animals might have been caused by differences in the chemical content of the food taken by the animal during the period when manures were collected. It has also been argued that when fungi are grown under different nutritional conditions different compounds accumulate

intracellularly that may contribute to differences in germination of fungal spores (Ypsilos and Magan, 2004).

Yields of chlamydospore on plant residues were the same or lower than rice. This could be due to low nutrient content left in the crop residues due to senescence after the maturity of the crop. According to Liu and Chen (2003) differences in the components of the substrates such as sources of carbon and nitrogen may lead to differences in growth of *P. chlamydosporia.*

Most of the industrial by-products were weak in the production of chlamydospores and the viability of produced chlamydospores was lower than 30%. The low capacity of the industrial products to support viability of chlamydospores could be due to additives used in industries during processing which might have hindered chlamydospore production and their viability. These results suggest that there was variation in the suitability of different organic materials as substrates for chlamydospore production but, most had indicated capacity to support chlamydospore production. The variation in the capacity of substrates to support the growth of the biological agent has been reported by Larena *et al.* (2004) who found that production of the conidia for the inoculum of *Epicoccum nigrum* was higher in peat/vermiculite/lentil meal than in peat/vermiculite substrate. The production of chlamydospores using organic substrates was similar to or greater than the production on rice substrate. This is a reflection of the fact that this fungus is facultative that forms chlamydospores as a resting stage when subjected to stressful conditions, such as a decrease in food supply (Atkins *et al.*, 2003b).

Most of the effective materials used in this study had nitrogen, carbon, potassium and phosphorus content within the ranges of those for rice. The materials whose chemical

contents are different from those of rice such as sawdust did not support production of chlamydospores. This suggests that nutrients are important in supporting growth of the fungus and formation of chlamydospores. According to Ludmilla *et al.* (2002) growth of saprophytic fungi depends on the supply of nutrients, especially carbon and nitrogen. Hence, materials that are able to supply these nutrients in good proportion can be a good substrate for *P. chlamydosporia* production and deliver results similar to the standard substrate (rice). Also, the study on the effects of nutrients to fungal growth conducted by Liu and Chen (2003) found that growth of *P. chlamydosporia* is influenced by the nutrient composition of the substrate.

The viability of chlamydospores is important for fungal growth and for its persistence in the soil. In this study, more than 50% of of the chlamydospores produced were viable. *Crotalaria ochroleuca* and chicken manure produced chlamydospores but were not able to maintain viability and hence were not suitable to be used as substrates. Substrates which support production of chlamydospores and maintain viability are therefore suitable to be used for the production of *P. chlamydosporia* inoculum. Chlamydospores need to maintain viability before use and this is an important attribute especially in storage of the inoculum for future use. According to Jenkins and Grzywacz (2000), viability of a biological control inoculum is one of the criteria of a good quality inoculum.

The study on the use of the extracts revealed that when the same materials were used in the form of extracts, most substrates performed better and similar to rice than the whole materials. This might be due to the disruption of cell walls, thus allowing the release of important nutrients needed by the fungus. These results are supported by the findings by Lopez-Llorca *et al.* (1999) who found that good spore production of mycoparasitic fungi

(*Trichoderma harzianum* and *Gliocladium virens*) was from substrates which had empty cell spaces such as *Phoenix dactylifera* seeds and almond mesocarp. Production of chlamydospore using extracts from organic substrates has been found to be effective and enhanced the materials that were not able to support chlamydospore production when the materials were used in powder form. Differences in condition of the media may also affect the utilization of nutrients by fungus for example solid condition may provide more oxygen than liquid while liquid may provide more water than solid media (Liu and Chen, 2003). The difference between leaves and extract of cabbage might have been caused by vitamin B which is soluble in water and is easily lost into water when cabbage are extracted or boiled and makes easily available (Moreno *et al.*, 2003). Moreover, extraction of vitamin B, influences extraction of other substances such as mineral salts, nitrogenous substances and carbohydrates that are important for the growth of fungus (Whipple, 1920; Moreno *et al.*, 2003).

Different concentrations of extracts from organic substrates had impact on the growth of colonies and percent viability. This study has established that the growth of the colonies of *P. chlamydosporia* increased with decrease in the level of concentration of extracts from the organic substrates. This could be due to low nutrients which could be the optimum level for mycelium growth and supporting production of chlamydospores. However, the percentage viability of chlamydospores decreases with decreasing concentration of extracts and is high on high organic substrate concentration. The high viability in high concentrations could be due to the fact that the chlamydospores from high nutrient media may have high nutrient reserves provided by high concentrate extracts from organic substrates as compared to chlamydospores produced on low

concentration. The extracts from organic materials with large colony growth (diameter) are those which have a low nutrient concentration while those with high nutrient concentration had small colony growth. It has been noted that the extracts from organic amendments were able to support large colonies at the low nutrient concentration. But, most of the extracts produced high number of chlamydospores at high concentrations. This could be due to availability of nutrients in high concentration which allows the fungus to grow well and hence produce many chlamydospores. The effect of high nutrient concentration and its effect on fungal inoculum have also been observed by Ludmilla *et al.* (2002). However, it was different for *T. minuta* where chlamydospore counts increased with decreasing concentration. *Tagetes minuta* may have chemicals or nutrients which are in excess when the concentration is high and hinder *P. chlamydosporia* growth. The chemicals which are higher in *T. minuta* than in cabbage and maize stover that may not support fungal growth are phosphorus, aluminium, copper, iron and nickel. The differences of different organic substrates to support chlamydospore production has been supported by Liu and Chen (2003) who found that growth of biocontrol fungi like *P. chlamydosporia* varies depending on the components like carbon and nitrogen and vitamins of the material used. In order to obtain highly viable chlamydospores using extracts from the organic substrates, this study suggests that concentration of substrates needs to be high.

Different isolates of *P. chlamydosporia* on extracts fom cabbage and maize stover were similar to rice in producing viable chlamydospores. This study demonstrated that isolate 392 was able to grow well even on materials which have elements that can limit the growth of other isolates. The reason for the differences in growth of various isolates on

different media given by Ludmilla *et al.* (2002) was due to the difference in carbohydrate content of the medium. The ability of the locally available materials that were tested to produce viable chlamydospores shows that they have potential to replace rice in the production of fungal inoculum and thereby reduce production costs.

CHAPTER 6

EFFECT OF ORGANIC AMENDMENTS ON THE ACTIVITY OF *Pochonia chlamydosporia* AGAINST ROOT-KNOT NEMATODES

6. 1. INTRODUCTION

Root-knot nematodes, *Meloidogyne* spp., are among the major pests that are affecting tomato production by causing severe damage to the crop. Management of the nematodes is more complicated compared to other plant pests due to their soil inhabitancy and broad host range (Akhtar, 1997). Several methods have been developed for their control that include crop rotation, resistant cultivars and chemical nematicides (De Jin *et al.*, 2005). Although nematicides have been found to be the most effective in controlling the nematodes, their adoption in small scale agriculture is restricted (Sikora and Fernandez, 2005) due to environmental concerns associated with nematicide application and by the high cost and limited availability (Akhtar and Malik, 2000). As a result, non-chemical measures such as intercropping and crop rotation with non-host or antagonistic crops, fallowing, biological control and application of organic amendments (OA) are becoming widely accepted (Akhtar, 1997). Efficacy of these methods have been found to be low compared to chemical nematicides, making it necessary to integrate them with other methods (Sikora *et al.*, 2000; Anastasiadis *et al.*, 2008).

Use of nematophagous fungi that are parasitic on nematode eggs has been of interest to many researchers (Atkins *et al.*, 2003b; Mauchline *et al.*, 2004). Among them, *Pochonia chlamydosporia*, being one of the most widely studied has attracted much attention on its potential to control root-knot and cyst nematodes (Kerry and Bourne, 1996; Atkins *et al.*, 2003a; Atkins *et al.*, 2003b; Liu and Chen, 2003). Research on integration of the egg

parasitic fungus *Paecilomyces lilacinus* with other management practices has been done in many parts of the world (Mittal *et al.*, 1995; Khan and Saxena, 1997; Anastasiadis *et al.*, 2008). For example, Khan and Saxena (1997) used organic amendments together with *P. lilacinus* in the management of *Meloidogyne javanica* in tomato.

Organic amendments alone have often been used by small farmers, but often with not so positive results (Sikora *et al.*, 2000). This method can, however, be of great use in organic farming systems where growers face a big challenge due to RKN infestation in tomato (Sikora *et al.*, 2000). However, for organic amendments to be effective on their own, high amounts are needed and this has proved to be highly expensive (Akhtar and Malik, 2000). Due to this, OA have been used in integrated management as a supplement to other methods of controlling plant parasitic nematodes (PPN) (Akhtar and Malik, 2000). Integrating biological agents with organic amendments as a management practice of nematodes has been recommended in order to improve their efficacy (Kerry, 2001). Application of OA stimulates the activity of microbial antagonists to nematodes which in turn results in the reduction of harmful nematodes (Akhtar, 1997).

Many researchers have reported that addition of OA causes a decline in nematode numbers due to the stimulation of nematode antagonistic micro-organisms in the soil (Akhtar and Malik, 2000; Huang *et al.*, 2006) among other factors. This has been said to be caused by the supply of nutrients into the soil from the OA (Akhtar and Malik, 2000). Akhtar (1997) reported that addition of materials like crustacean shells rich in chitin, increased the population of *P. lilacinus* and decreased *M. arenaria* population. Different sources of organic matter differ in supporting growth of biological agents as in some cases, addition of these materials to the soil may not enhance the efficacy of the

biological agent (Akhtar and Malik, 2000). The fungus, *P. chlamydosporia* is a facultative parasite of RKN eggs therefore can survive using OA when available in the soil, thereby potentially reducing its activity in controlling RKN. The efficiency of the fungus is therefore, variable depending on the organic materials used (Akhtar and Malik, 2000).

It is recommended that with appropriate application techniques, biological agents can be integrated with OA and have positive results (Akhtar and Malik, 2000; Atkins *et al.*, 2003b). It was also important to know whether the time of incubation of the OA in soil can influence the activity of the nematophagous fungi in controlling RKN.

The main objective of this study was to determine the efficacy of *P. chlamydosporia* in the presence of organic amendments (OA) in RKN management. The specific objectives were:

- To assess the ability of organic materials to enhance growth and activity of *P. chlamydosporia* against root-knot nematodes.
- To assess the effect of different incubation periods of *P. chlamydosporia* with organic materials on growth and activity of the fungus.
- To determine the effect of extracts from different organic materials on the parasitic capacity of *P. chlamydosporia.*

6. 2 MATERIALS AND METHODS

6. 2 1 Efficacy of organic materials on growth of *P. chlamydosporia* and suppression of root-knot nematodes

Organic amendments used in this study were *Tagetes minuta* , *Mucuna pruriens, Tithonia diversiforia,* maize stover and maize cobs. One set of OA were mixed with *P. chlamydosporia* while the other was not, making a total of 10 treatments. The experiment had a total of 12 treatments that include five mentioned OA all either applied with fungus or not applied and two controls where one had fungus alone and second one with no OA and no fungus. The experiment was laid out in a glasshouse in a randomised complete block design (RCBD) and each treatment was replicated five times. Pots of 500g capacity were filled with sterile soil. The chlamydospores of *P. chlamydosporia* isolate 392 were prepared using rice substrate as described in section 3. 3. 2. The *P. chlamydosporia* inoculum (chlamydospores) was mixed with the soil at the rate of 5000 chlamydospores per gram of soil (Kerry and Bourne, 2002). Each OA was pulverized into powder as described in section 3. 5. 1 and applied to the soil in pots at the rate of 0.5% of the soil weight. One seedling from pre-germinated tomato seeds was transplanted into each pot 24 h after the fungus and OA were added into the soil. Two weeks after planting, 3000 eggs and J2 of RKN were added into the soil in each pot. All appropriate agronomic practices were done throughout the experimental time, including maintaining soil moisture, fertiliser and pesticide applications. The numbers of CFU were enumerated after 30 and 90 days of application of *P. chlamydosporia* into the soil. Damage by nematodes (galling), numbers of J2 and dry shoot weight were recorded after 90 days.

6. 2. 2 Effect of different incubation periods of *Pochonia chlamydosporia* and maize stover in the management of RKN in tomato

This experiment was conducted in a glasshouse in pots of 500g capacity with seven treatments where the soil with fungus was either amended with maize stover or without maize stover and incubated for one day, 14 and 30 days before planting. The control treatment with neither organic amendment nor fungus was included. The treatments were replicated three times each and arranged in a RCBD. Pulverized maize stover was mixed with soil at 0.5% w/w. Also, *P. chlamydosporia* isolate 10 inoculum prepared using rice substrate was added at the rate of 5000 chlamydospores per gram of soil. Soil amended with maize stover and applied with the fungus was incubated at 20±2°C for varying durations, viz., one day, 14 and 30 days after mixing prior to planting. Also another set of pots with the stated time span as above were incubated with fungus but without maize stover. Tomato seedlings were transplanted into all the pots considering the respective incubation periods. Root-knot nematode eggs/juveniles were applied into the pots two weeks after transplanting the seedlings. The first data on CFU were taken at 30 days after transplanting. Three months (90 days) after planting, data on soil and root CFU, root galling index, dry shoot weight of the plants and root fresh weight and the number of J2 in the soil and roots were collected.

6. 2. 3 Effect of time of incubation and media on the parasitic activity of *Pochonia chlamydosporia*

The experiment was set up using the extracts from the most effective OA (*Crotalaria ochroleuca*) and least effective OA (sawdust) in supporting the production of fungal

chlamydospores, in an earlier experiment to test the parasitic activity of *P. chlamydosporia*. The data were collected at two time intervals; 24 and 48 h. Each experiment had seven treatments: each of the two OA in yeast extract and in sterile distilled water, yeast extract with no fungus, yeast extract with fungus, and water with fungus. The experiment was laid in a RCBD and each treatment was replicated three times, where the replications were incubated at different intervals. Nematode eggs were obtained from the roots of six week-old tomato plants infected with *M. incognita* prepared and diluted to make a suspension of 1000 eggs/ml which was used. Isolate 10 of *P. chlamydosporia*, was grown on corn meal agar (CMA) for conidia production and the fungal inoculum was prepared at a strength of 5.5×10^4 spores/ml. Using universal bottle of 25ml capacity, 4ml of extract from OA, 4ml of yeast extract, 1ml of fungal suspension and 1ml of nematode egg suspension were mixed, closed and incubated at 26±1°C in a Gallenkamp orbital incubator (150rpm). Similarly, sterile distilled water was also used instead of yeast extract medium. After each incubation period the percentage of infected eggs was recorded.

6. 2. 4 Effect of extracts from organic materials on parasitism of root-knot nematodes eggs by *Pochonia chlamydosporia*

The OA extracts evaluated were from green manures (maize stover, cabbage leaves, *Crotalaria ochroleuca, Tagetes minuta, Mucuna pruriens, Tithonia diversifolia*), animal manures (cow, goat and chicken) and crop residues (maize cobs, sawdust, rice bran, filtermud and bean straw). The materials were prepared using methods and techniques as described in section 3. 5. 1. There were 18 treatments of which 15 were extracts from OA

with three controls which were with yeast extract alone as medium with the fungus, 50% yeast extract and 50% of water with fungus and with yeast extract but no fungus. The treatments were arranged in a RCBD and each treatment was replicated three times. The treatments were mixed with 5. 5 × 10^4conidia/ml suspension of *P. chlamydosporia* isolate 10 and 1000 eggs/ml of *M. incognita* (see section 3. 4. 2) at a ratio of 8:1:1, by volume, in sterile universal bottles. The pH of the materials was also taken using a Jenway pH meter before and after incubation. The bottles were incubated at 26±1^{0}C in Gallenkamp orbital incubator at 150rpm for 48 h. After incubation, 3ml of the suspension was drawn from each bottle and 100 eggs used to estimate infected eggs, under a dissecting microscope and expressed as percentage egg infection.

6. 2. 5 Effect of different concentrations of extract from organic materials on the parasitism of *Pochonia chlamydosporia* on root-knot nematode eggs

Different concentrations; 25, 50, 75 and 100% of extracts from maize cobs, *C. ochroleuca* and sawdust which were made from 25g of OA in a litre of water were used in this experiment to test the most effective concentration. The extracts at these dilutions were mixed with yeast extract (0.0125g/l) 50% v/v. The controls were four; with yeast extract and water alone each with and without fungus making a total of 16 treatments which were replicated three times and arranged in a RCBD. In each dilution, 5.5×10^4 conidia/ml of *P. chlamydosporia* isolate 10 and 1000 eggs /ml of *M. incognita* were added. The treatments were incubated at 26±1°C in a Gallenkamp orbital incubator at 150rpm. After 48 h of incubation the percentage of eggs parasitized was recorded.

6. 2. 6 Statistical analysis

Data collected in each experiment above were either log_{10}, arcsine or Logit transformed when required and then subjected to ANOVA using Genstat package version 11. The means were compared using least significant difference (LSD).

6. 3 RESULTS

6. 3. 1 Effect of organic materials on growth of *Pochonia chlamydosporia* and activity against effect on root-knot nematodes

Application of OA had a significant ($P<0.05$) effect on population density of *P. chlamydosporia* at 30 days, galling index, and the number of second stage juveniles at 90 days after planting of tomato (Table 6.1). At 30 days, the numbers of fungal propagules were the same in the soil amended with all OA with the exception of soil amended with *T. diversifolia* where no fungal propagules were detected. Application of *P. chlamydosporia* into the soils amended with *T. minuta* significantly reduced the root galling and the number of second stage juveniles than where only *T. minuta* was added without the fungus. The lowest numbers of J2 were recorded in soil where the fungus was applied and amended with *T. minuta* and *M. pruriens* (Table 6.1).

Application of *P. chlamydosporia* together with OA significantly ($P<0.001$) increased dry shoot weight of tomato (Fig. 6.1). Most of the treatments with fungus had higher dry shoot weight compared to those where the fungus was not added. Among the OA with significantly higher ($P<0.001$) dry shoot weight compared to the others, were *T. diversifolia* and maize stover.

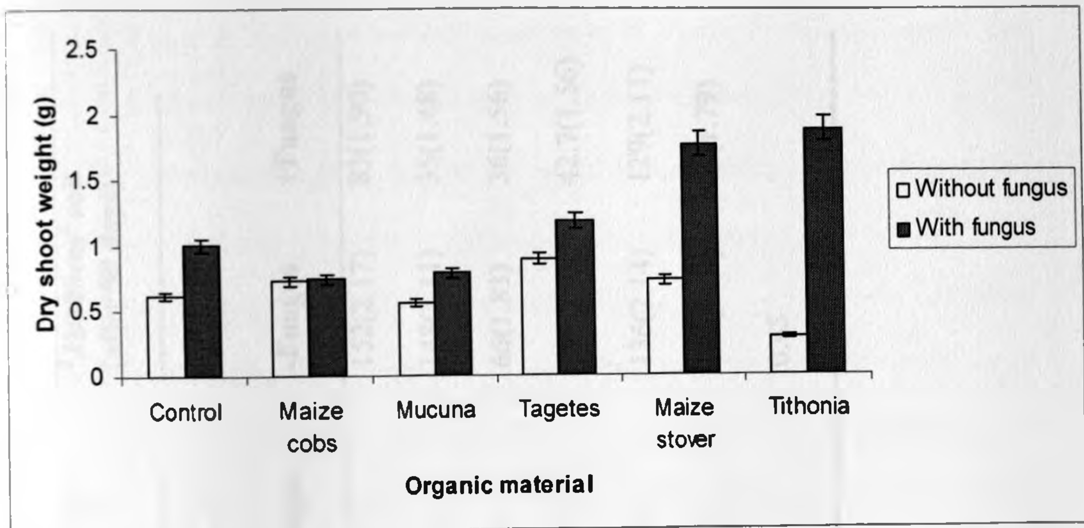


Fig. 6. 1. Effect of organic materials and *Pochonia chlamydosporia* on dry shoot weight of tomato.
The I bars signify 5% error.

Table 6. 1. Effect of organic materials on colony forming units of *Pochonia chlamydosporia* in the soil, galling index and second stage juveniles/200cm^3 soil (J2) associated with tomato.

Treatment	[1]Colony forming units/g soil			Galling index (1-5) (after 90 days)		[2]J2/200cm^3 soil (after 90 days)	
	30 days		90 days				
	-Fungus	+Fungus	+Fungus	-Fungus	+Fungus	-Fungus	+Fungus
Control	0(0.00)	3,482(3.35)	58,943(4.74)	4.00	3.67	152(2.17)	83(1.90)
Tagetes sp	0(0.00)	8,025(2.63)	70,393(4.79)	3.67	2.67	148(2.11)	35(1.48)
Mucuna sp	0(0.00)	7,952(2.66)	53,079(4.69)	3.00	3.33	66(1.83)	36(1.56)
Tithonia sp	0(0.00)	0(0.00)	33,085(4.45)	3.33	2.67	189(2.25)	42.7(1.56)
Maize stover	0(0.00)	5,944(2.54)	52,803(4.67)	3.00	3.00	136(2.14)	129(2.11)
Maize cobs	0(0.00)	7,477(3.71)	35,610(4.48)	3.00	3.33	113(2.00)	65(1.79)
LSD		..........1.91	0.34	0.86		0.45	

Values in brackets are [1]Log_{10} and [2]Arcsine transformed means used in comparison with LSD.

6. 3. 2 Effect of different incubation periods of *Pochonia chlamydosporia* and maize stover in the management of RKN in tomato

Mixing maize stover and *P. chlaymdosporia* for varied durations before planting had significant ($P<0.05$) effect on the number of fungal propagules, and the number of second stage juveniles in the roots of tomato (Table 6.2). Addition of maize stover significantly increased the number of fungal propagules in the soil where maize stover was mixed with the fungus and incubated for one day before planting. However, the numbers of fungal propagules, both in soil and roots, were significantly ($P<0.05$) higher where the fungus was incubated for 14 and 30 days compared to one day.

The number of J2 in the roots were significantly lower ($P<0.001$) in treatments where maize stover was not applied compared to where maize stover was applied. In treatments where maize stover was not added the number of J2 increased with increase time of incubation. The lowest J2 population was on treatments where only fungus was applied and only left in the soil for one day before planting.

Galling index increased significantly ($P<0.05$) with time of incubation as well as the root weight (Figs. 6.2 & 6.3). Decomposing maize stover with *P. chlamydosporia* for 14 days before planting significantly ($P<0.05$) increased galling index and root weight than when the fungus was incubated alone for the same period. The galling index and the root weight were highest on roots from the soil where fungus was incubated for 30 days prior to planting.

Table 6. 2. Effect of time of incubation of maize stover and *Pochonia chlamydosporia* before planting of tomato on the population of fungus (CFU), dry shoot weight and number of second stage juveniles (J2).

Time of incubation	CFU/g soil		CFU/g root		Shoot weight (g)		J2/200 cm^3 soil		J2/5g root	
	-OA	+OA	-OA	+OA	-OA	+OA	-OA	+OA	-OA	+OA
1 day	34,857(4.53)	123,467(4.92)	14,000(4.05)	19,000(4.26)	2.95	6.25	40	37.5	15	67.5
14 days	287,665(5.45)	337,674(5.5)	27,250(4.43)	24,750(4.38)	3.97	5.59	30	55	35	65
30 days	377,223(5.57)	406,192(5.6)	28,375(4.45)	30,125(4.47)	2.80	3.53	42.5	50	47.5	65
LSD	0.38		0.23		3.51		39.11		29.47	

Values in brackets are Log_{10} transformed means used in comparison with LSD.

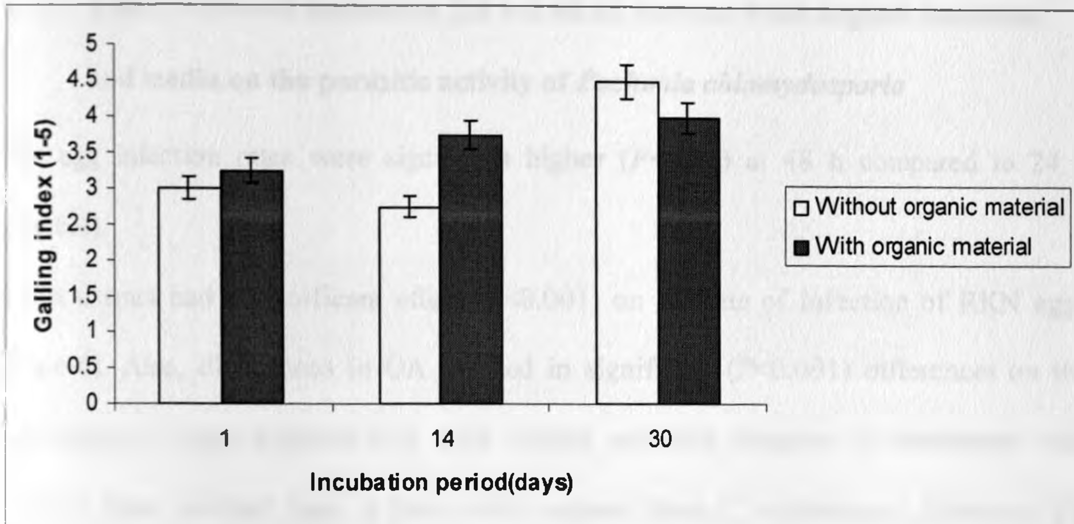


Fig. 6. 2. Effect of incubating maize stover and *Pochonia chlamydosporia* on root galling of tomato.
Means were compared using LSD=0.99. The I bars signify 5% error.

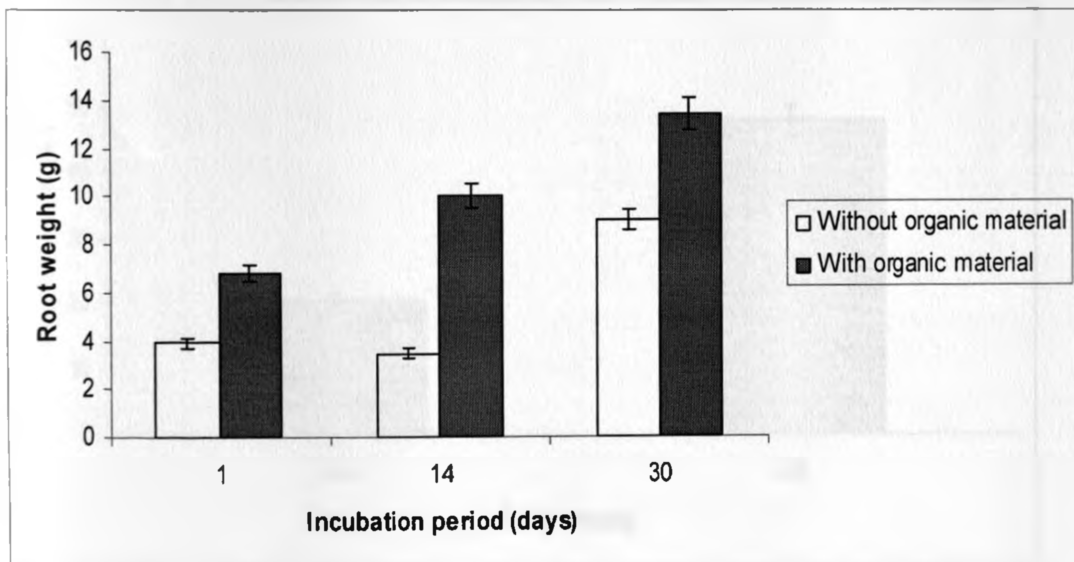


Fig. 6. 3. Effect of incubating maize stover together with *Pochonia chlamydosporia* on the root weight of tomato.
Means were compared using LSD=4.21. The I bars signify 5% error.

6. 3. 3 Effect of time of incubation (24 and 48 h), extracts from organic materials and media on the parasitic activity of *Pochonia chlamydosporia*

The egg infection rates were significant higher ($P<0.05$) at 48 h compared to 24 h (Fig.6.4).

Yeast extract had a significant effect ($P<0.001$) on the rate of infection of RKN eggs (Fig.6.5). Also, differences in OA resulted in significant ($P<0.001$) differences on the percentage of eggs infected (Fig. 6.6). Higher infection occurred in treatments with extracts from sawdust than in those with extracts from *C. ochroleuca*. However, the percent egg infections in organic amendments were lower than the yeast extract.

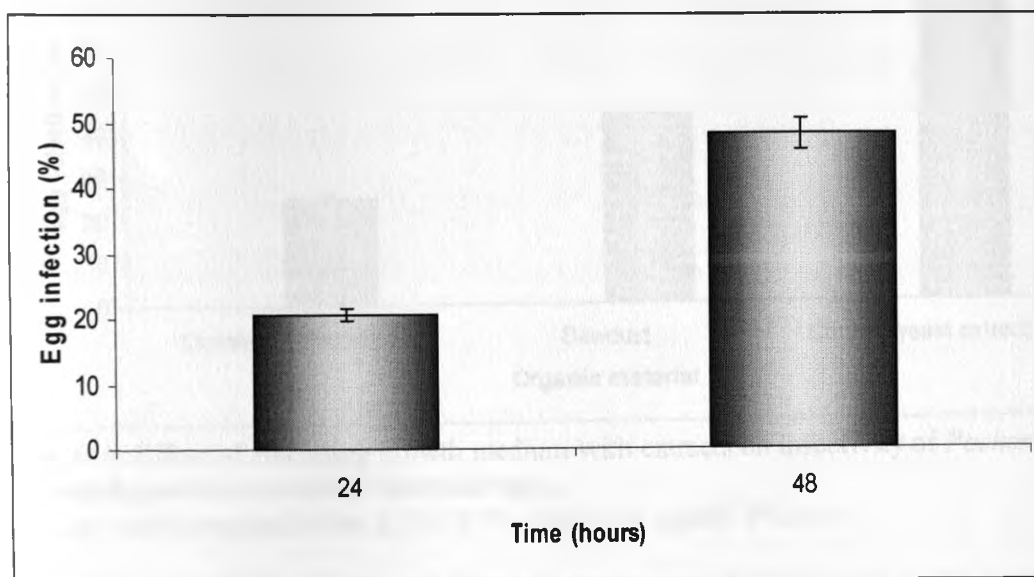


Fig. 6. 4. Effect of duration of exposing root-knot nematode eggs to *Pochonia chlamydosporia* on percent egg infection.
Means were compared using LSD of 16.68. The I bars signify 5% error.

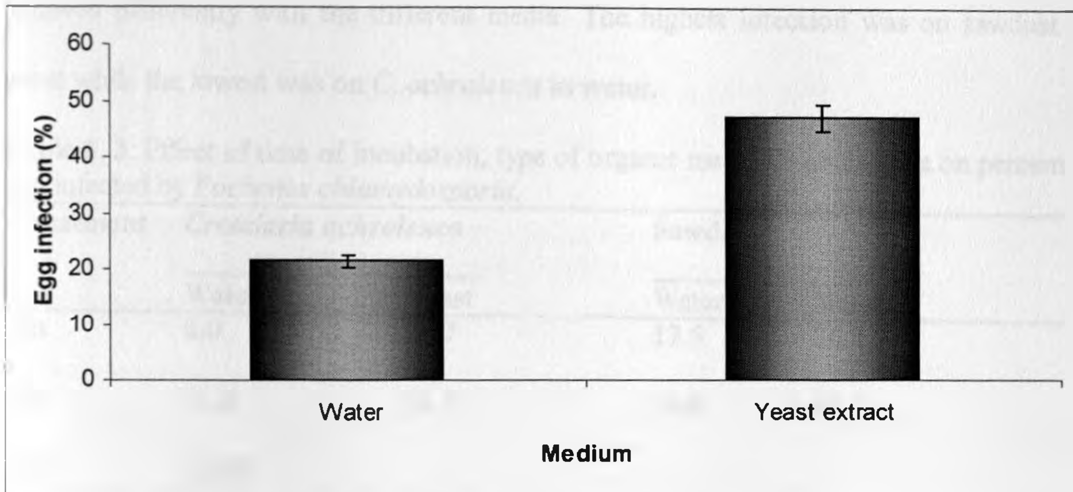


Fig. 6. 5. Influence of water and yeast extract media on the percent eggs infected by *Pochonia chlamydosporia.*
Means were compared using LSD 11.79. The I bars signify 5% error.

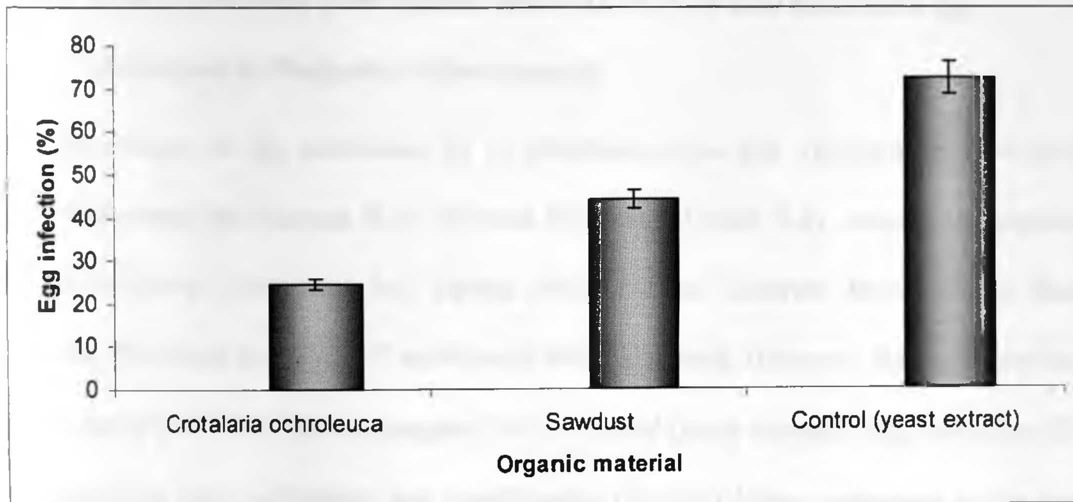


Fig. 6. 6. Effect of amending growth medium with extracts on infectivity of *Pochonia chlamydosporia* to root-knot nematode eggs.
Means were compared using LSD 11.79. The I bars signify 5% error.

There was a significant ($P<0.05$) effect on percent egg infection due to the interaction between time, OA and media (Table 6.3). The highest percent egg infection was after 48 h when extract from sawdust was used with yeast extract while there was no infection when the extract from *C. ochroleuca* was used in water at 24 h. At 48 h, organic material

behaved differently with the different media. The highest infection was on sawdust in yeast while the lowest was on *C. ochroleuca* in water.

Table 6. 3. Effect of time of incubation, type of organic materials and media on percent egg infected by *Pochonia chlamydosporia.*

Treatment	*Crotalaria ochroleuca*		Sawdust	
	Water	Yeast	Water	Yeast
24h	0.0	28.7	17.5	36.1
48h	31.4	38.3	36.8	85.7
LSD	16.68			

6. 3. 4 Effect of extracts from organic materials on root-knot nematode egg parasitism by *Pochonia chlamydosporia*

The percentage of egg parasitism by *P. chlamydosporia* was significantly ($P<0.001$) different among the extracts from different OA tested (Table 6.4). Among the extracts tested the extract from rice had highest effect but not different from extracts from sawdust, filtermud, rice husk, *C. ochroleuca* and maize cobs. However, their efficacy was significantly ($P<0.001$) lower compared to the control (yeast extract). Egg parasitism for the remaining nine substrates was significantly ($P<0.05$) lower compared to the egg infection in rice extract (Table 6.4).

The pH of the extracts were also highly significantly ($P<0.001$) different among each other. Out of the materials tested, five extracts from OA which were as good as rice in infection of RKN eggs, the pH of filtermud and sawdust among the five were not significantly ($P<0.001$) different from rice and were also similar to the standard, yeast extract medium (YEM). However, *M. pruriens* and *T. diversifolia*, were also not

significantly ($P<0.001$) different in their pH from the standard and rice extracts but the percent egg infection were significantly ($P<0.001$) lower (Table 6.4).

Table 6. 4. Effect of extracts from organic materials and their pH on root-knot nematodes eggs infected by *Pochonia chlamydosporia.*

Organic material extract	pH	Egg infection (%)
Beans	6.86	0 (-5.30)
Cabbage	6.67	0 (-5.30)
Maizestover	4.87	0 (-5.30)
Chicken manure	7.1	0.6 (-4.79)
Mucuna pruriens	6.27	1.25 (-4.31)
Tithonia deversifolia	6.32	6.32 (-3.34)
Tagetes minuta	4.76	7.81 (-2.41)
Goat	7.00	10.81 (-3.78)
Cow manure	7.07	11.64 (-2.16)
Maize cobs	5.35	22.04 (-1.27)
Crotalaria ochroleuca	5.24	25.52 (-1.25)
Rice husk	6.94	36.62 (-0.59)
Filtermud	6.48	37.66 (-0.56)
Sawdust	6.11	41.87 (-0.40)
Rice	6.18	53.33 (0.14)
YEM (standard)	6.49	91.88 (2.42)
LSD	0.64	1.58

Data in brackets are Logit transformed means used in comparison with LSD. YEM=yeast extract medium.

6. 3. 5 Effect of different concentrations of extracts from organic materials on parasitic activity of *Pochonia chlamydosporia* on root-knot nematode eggs

The percentage egg infection by the fungus was significantly ($P<0.001$) influenced by extracts from OA used and their concentrations (Table 6.5). Sawdust supported the highest percent egg infection while *C. ochroleuca* was not suitable. Yeast extract medium as a standard, showed a significantly higher ($P<0.001$) infection of RKN eggs than the extracts from the other OA. However, water as a medium used for negative control was more effective in supporting egg infection than *C. ochroleuca.* The concentration of OA

played a significant ($P<0.001$) role in the infection of RKN eggs by the fungus. The lower concentration of the extracts from OA had a higher percent infection compared to the higher concentrations (Table 6.5). The four levels of concentration gave significantly lower ($P<0.001$) egg infection than yeast (+ve control). However in comparison with water as a negative control, the 25 and 50 % concentrations had significantly higher ($P<0.001$) egg infection than water (Table 6.5).

Sawdust had the highest infection at both high and low concentrations while *C. ochroleuca* had the lowest of all the OA. The percentage of egg infection decreases with increasing OA concentration for all the materials tested. In general, percent egg infection in all extracts from OA significantly ($P<0.05$) decreased with increase in the concentration of the extracts. The egg infection in sawdust for all concentrations was higher than in maize cobs and *C. ochroleuca*. The extract from sawdust was more effective at 25% concentration than all other extracts and concentrations.

Table 6. 5. Effect of varying concentrations of extracts from organic materials on root-knot nematode egg infected by *Pochonia chlamydosporia*.

Treatment	**Concentration of the extracts from organic materials (%)**			
	25	50	75	100
Crotalaria	16.29(-1.63)	10.63(-2.15)	3.87(-3.11)	1.70(-3.83)
Maize cobs	69.14(1.05)	60.57(0.44)	36.37(-0.63)	40.09(-0.42)
Sawdust	95.38(2.97)	89.17(2.08)	87.23(2.03)	83.79(1.62)
Water				45.04(-0.20)
YEM		98.64(4.02)		
LSD	0.92			

Values in brackets are Logit transformed means used in comparison with LSD.

6.4 DISCUSSION

This study has revealed that addition of OA with fungus into the soil caused an increase in the fungal population and dry shoot weight, decrease in galling and the population of nematodes in the soil. This finding concurs with the results reported by Jaffee (2004) who observed an increase in the number of biological control agents due to application of OA. The increase in fungal population could be attributed to the release of nutrients needed by the fungus such as N and C from the OA (Chavarria-Carvajal *et al.*, 2001). The study also recorded that there was low contribution of OA alone in reducing galling and RKN J2. Similarly, Jaffee *et al.* (1994) noted that application of compost from chicken and cow manure had no effect on the population of the parasitic nematode, *Criconemoides xenoplax,* in peach orchards. It is possible that materials did not have enough nematicidal compounds such as alkaloids, glucosinolates and phenols for the control of nematodes on its own (Perez *et al.*, 2003).

However, application of *T. minuta* and *T. diversifolia* in combination with *P. chlamydosporia* led to a decrease in root galling and numbers of nematodes in the soil compared to OA alone. Increase in the ability of egg parasitic fungi to control nematodes after integrating with OA has also been reported by Khan and Saxena (1997), who found increase in the ability *Paecilomyces lilacinus* in reducing nematode population and root galling after integrating with groundnut cakes. Integration of *P. chlamydosporia* and OA not only resulted in reducing the root galling and lowering the population of nematodes but also increased shoot weight. The increase in dry shoot weight could be due to increase in the fungal population density which reduced the number of J2 and hence reduced plant infection by RKN. Chen *et al.* (1998) observed an increase in yield of

peanut by 58 to 94% due to application of a biological agent, *Pasteuria penetrans*, in the control of *M. arenaria*. Also, increase in dry shoot weight can be attributed to the provision of nutrients released by OA after decomposition (Akhtar and Malik, 2000). This suggests that *P. chlamydosporia* could be more effective as a biocontrol agent in the management of RKN when used in integration with selected OA.

Mixing of OA and fungus in the soil and allow them to stay for the duration of time before planting resulted in increased population of fungus in the plant rhizosphere, galling index, root weight and number of nematodes in roots. The increase of fungal population with increase in time is similar to what has been found by Jaffee *et al.* (1994) who noted an increase in the fungus population between 27 and 55 days when cow manure and straw were added into the soil. The increase of fungal propagules with time could be due to the time given the fungus and allow them to sporulate and establish (Anastasiadis *et al.*, 2008). The high fungal colonisation with increased incubation time and maize stover has not increased the possibility of the fungus to infect many RKN eggs in the roots. This could be due to the incubation of OA with time which may have released nutrients required by this facultative saprophyte and make them less dependent on RKN eggs. The increase in RKN J2 may have caused formation of large galls and probably increased the weight of the roots. According to Akhtar and Maliki (2000), addition of OA increased the population of the fungus *Hirsutella rhossiliensis* in the soil and decreased its nematicidal activities which also occurred in the present study.

The *in vitro* assay study established that longer time was better for higher egg infection. This could be because prolonged incubation could stabilise the fungus, germinate, produce infection pegs, infect and erode the defence mechanism of eggs and hence

colonize a high number of eggs. Normally, eggs are protected themselves from chemicals and attack from microbial antagonists (Olivares-Bernabeu and Lopez-Llorca, 2002). The *in vitro* parasitism of RKN eggs has been found to be effective at 48 h after incubation with up to 80% being infected depending on the isolate used (Esteves, 2007). *Crotalaria ochroleuca* was more suitable than sawdust in chlamydospore production but, its efficacy is lower based on the number of eggs infected. The growth of fungal mycelium on *C. ochroleuca* was faster and higher compared to sawdust but did not cause a higher infection of the eggs. Abundant availability of nutrients in *C. ochroleuca* could have promoted high fungal growth at the expense of infection activity. Similarly, application of alfalfa in the soil enhanced the population of *Arthrobotrys oligospora* but few or no nematodes were trapped (Jaffee, 2004).

The efficacy of fungus in sawdust could be attributed to lower nitrogen, potassium and phosphorus and higher carbon compared to *C. ochroleuca.* Availability of these nutrients in *C. ochroleuca* might have played a major role in reducing the dependency of the fungus on eggs of RKN. The nutrient content of the OA can determine the dependence of this fungus on nematodes and may make them less active in its parasitism (Morton *et al.*, 2004). Studies on the effects of this fungus as a saprophyte have been reported by Kerry (Kerry, 2000) who concluded that efficacy of egg-parasitic fungi including *P. chlamydosporia* depends on the available nutrients in the soil which may interfere with parasitic habits of the fungus. In general, this study also suggests that for testing high infection levels *in vitro*, yeast is a preferred medium. The higher egg infection when yeast is used as medium compared to sterile distilled water has been reported by Esteves

(2007). Moreover, this study also suggests the importance of knowledge of the nutrient content of the OA which plays a major role in facilitating parasitism.

Among the extracts from fifteen OA used in this study, it has been noted that rice supported fungal parasitism with high percent egg infection. Extracts from five OA that were comparable to rice in their egg parasitism suggest that there is a wide range of choice of different locally available OA which can facilitate egg infection. The wide range of available OA that can favour fungal infection will allow the farmer to base a choice on a material that will be both economical and compatible to their farming practices, as advised by Akhtar and Maliki (2000). One of factors involved in selecting OA for the control of nematodes for the resource-poor farmers, is the availability and reduced costs. However, this study noted the influence of pH of the extracts from the OA in the support of fungal activity. The increase in pH from pH 6.5 and decrease to 4.5 lowers the percentage eggs infected. This shows that the fungus was more effective when the condition is slightly acidic. It is also known that the pH ranges for this fungus to grow well is between 4 and 8 (Kerry *et al.*, 1986). In this case, it is important to know the pH of the materials used as well as that of the soil.

Variation in the concentrations of OA extracts showed a marked variation on the fungal infection of RKN eggs. Sawdust, continued to promote high egg parasitism making it the most suitable OA in RKN management. Other interesting effect is that of concentrations of extracts which showed that the lower the concentration of the extract the higher the infection rate. Similarly, the effects of concentrations of different OA extracts have been studied by Owino *et al.* (1993) who found that high concentrations of extracts from some materials such as mustard hinder activity of *P. chlamydosporia* due to the release of

compounds which have fungicidal effects. The high infection for a low concentration could be due to a low nutrient content that stimulated the fungus to switch to alternative nutrient source which could be the RKN eggs. This implies that some amendments can be good at favouring infection when used in low concentrations.

CHAPTER 7

EFFECT OF SOIL TEMPERATURE, PH AND CARBON:NITROGEN RATIO ON GROWTH AND INFECTIVITY OF *Pochonia chlamydosporia* ON ROOT-KNOT NEMATODE EGGS

7.1 INTRODUCTION

Most of the biocontrol agents require certain environmental conditions for optimum growth, infection or predacious activity (Sayre and Walter, 1991). According to Duponnois *et al.* (2002), the environmental conditions of the soil are of vital importance when introducing any biocontrol agent into a particular environment. In addition to this, knowledge of the environmental conditions that affect the growth of the biocontrol agent is essential when determining their effect on their ability to control plant pathogens like root-knot nematodes (Sayre and Walter, 1991). *Pochonia chlamydosporia,* a parasite of root-knot and cyst nematode eggs, its establishment in the soil has been difficult due to its weak saprophytic nature leading to suppression by other soil micro-organisms (Kerry, 2000).

Fungi need optimum temperature levels that differ from one isolate to the other for their growth and infectivity (Viaene *et al.*, 2006). The optimum temperature for growth has been stated as 25°C but this varies and not necessarily to be optimum for infection, depending on the isolate (Kerry *et al.*, 1986). For example, the optimum temperature for hyphal growth of strain II of *P. chlamydosporia* is 25°C and for parasitism is 12°C (Irving and Kerry, 1986). In general, the survival of the fungus in the soil is limited by both high and low temperatures (Van Damme *et al.*, 2005). Temperatures below 5°C hinder growth of the fungus while little growth occurs at temperatures above 30°C (Kerry, 2000). In

most regions, temperature can vary depending on the geographic location, altitude and season.

In order to facilitate growth, the fungus requires nutrients from different sources. Addition of OA into the soil is known to increase the population of *P. chlamydosporia* in the soil but lowers the infection ability due to availability of nutrients to the fungus (Jaffee, 2002). Addition of the fungus on already decomposed OA might enhance fungal growth and its ability to infect more eggs. For instance, decomposing broccoli in temperatures of 25 and 30-35 for 20 and 10 days respectively released the biofumigants and effectively controlled nematodes (Ploeg and Stapleton, 2001). Organic materials are comprised mainly of carbon (C) and nitrogen (N), both of which are required by micro-organism and higher plants. The supply of C and N from OA is one of the major factors responsible for fungal activity and growth (Segers, 1996). It is known that the ability of *P. chlamydosporia* to infect nematode eggs depends on the level of C and N released into the soil after decomposition of the materials (Chen and Dickson, 2004).

Parasitic activity of *P. chlamydosporia* in the soil is challenged by many factors including chemical and physical factors. pH being one of them can be a limiting factor on the growth and infectivity of parasitic fungi on RKN eggs. As with other soil micro-organisms, decrease in pH has been said to be one of the factors accounting for growth inhibition of antagonists such as bacteria (Verschuere *et al.*, 2000). The growth of *P. chlamydosporia* has been found to be optimum at pH 5 (Kerry *et al.*, 1986). Also, variation in soil pH from acidic to alkaline decreased the infectivity of *P. chlamydosporia* against RKN eggs (Jaffee and Zasoski, 2001). Little knowledge is available on the factors affecting the parasitic activity of the fungus, particularly in soils rich in organic

substrates. Therefore, the objective of the study was to determine the effect of soil temperature and pH and levels of C and N of the organic materials on the parasitic activity of *P. chlamydosporia.*

7. 2 MATERIALS AND METHODS

7. 2. 1 Effect of exposing organic materials in various temperatures on growth and parasitic activity of *Pochonia chlamydosporia*

This experiment was conducted to assess decomposition of different OA under different temperatures that affected the growth and ability of *P. chlamydosporia* to infect RKN eggs. These OA were selected from the results of an earlier study among the best, medium and poor in chlamydospore production. The treatments for the experiment were sawdust, *Crotalaria ochroleuca* and maize cobs each decomposed at 15, 20 and 25°C for 30 days. Non-decomposed (fresh materials) OA were included as controls. In addition, treatments without organic amendments but with and without fungus were included making a total of 14 treatments. The treatments were replicated three times and arranged in a RCBD.

Using 177g of sterile soil in 200g capacity pots, the soil was mixed with OA powders prepared as described in section 3. 5. 1 at the rate of 0.5% of the weight of soil and incubated (Gallenkamp cooled incubator) for 30 days. After incubation, the pots that had received the nine treatments were transferred to an incubator which had optimum temperature (25 ± 2°C) for *P. chlamydosporia*. Another four sets of treatments with three organic amendments (*Crotalaria ochroleuca,* sawdust and maize cobs) and one without OA were added and incubated at 25 ± 2°C. Chlamydospores of isolate 10 of *P.*

chlamydosporia were added to all pots at the rate of 5000/g soil before incubation at 25°C for 30 days. After incubation, ten egg masses of *M. incognita* were buried into the soil of each pot (all treatments) using the baiting technique (Lumsden, 1981) where plastic slide mounts (24 × 36mm), with glasses removed, were used to hold ten egg masses wrapped in nylon fabric and the slides were buried into the soil. The slides were left in the soil for seven days.

After incubation, the egg masses were recovered from the soil by pulling out the slides, placed into excavated glass blocks and cleaned using three millilitre of sterile distilled water. The eggs were then released from egg masses using a cyst crusher. From the ensuing egg suspension, 200µl was pipetted onto sorbose agar with mixture of antibiotics (Apendix 1), in three replicates, and incubated at 25°C for 72 h. After incubation, a total of 100 eggs were used to estimate the percent egg infection as described in section 3. 6. 1. After retrieving the egg masses, the soil in the pots was assessed for the number of fungal propagules. One gram of moist soil from each pot was diluted in 9ml sterile water agar (0.05%) up to 10^{-3} dilution. The last two dilutions were plated onto a semi-selective medium (Appendix 1) in Petri dishes and incubated at 25°C for 14 days. After incubation, the colonies emerged were counted and the number of CFU was calculated using the formula illustrated by Kerry and Bourne (2002).

7. 2. 2 Effect of pH on the infectivity of *Pochonia chlamydosporia* on eggs of root-knot nematodes

The experiment was set up in the laboratory to test the effect of pH *in vitro* egg parasitism using yeast extract and water media each at five different pH levels. The pH

levels were 3.42, 4.47, 5.73, 7.06 and 8.77, and were made using a buffer solution made from sodium phosphate (0.2M) and citric acid (0.1M).as shown in Appendix 4. The experiment had four controls, two for yeast extract (normal pH=6.63) and two for water (normal pH=5.81) each with and without fungus and made a total of 14 treatments which were replicated three times and arranged in a RCBD.

Eight millilitre of either water or yeast extract media made from the prepared buffer was placed in universal bottles. To this one millilitre each of 1000 of *M. incognita* eggs (prepared as in section 3. 4. 2) and 5.5×10^4 fungus conidia from *P. chlamydosporia* isolate 10 (prepared as in section 3. 3. 1) were mixed and incubated at 25°C in a Gallenkamp orbital incubator (150 rpm) for 48 h. After incubation, the bottles were taken out and whirl mixed before removing a 3ml aliquot and placing it onto a counting slide. Data on parasitized and non-parasitized eggs per 100 eggs were collected and used to estimate the percentage of eggs parasitized.

7. 2. 3 Effect of carbon and nitrogen ratios on the efficacy of *Pochonia chlamydosporia* to parasitize root-knot nematode eggs *in vitro*

The experiment had a total of 12 treatments that included 10mM C with different rates of N (1, 5, 10, 50 and 100mM), and different rates of C (1, 5, 10, 50 and 100mM) with 100mM N, and two controls (yeast extract medium with and without fungus). They were replicated three times and arranged in a RCBD. Glucose (30g/l) was used as a source of carbon while ammonium nitrate (40g/l) was used as a source of nitrogen. Glucose was added into water, stirred and sterilised using a Minisart® single filter unit (0.20µm) while ammonium nitrate was dissolved in water and autoclaved at 120°C for 15 minutes. The

glucose was not autoclaved to avoid chemical changes due to heat. All treatments were buffered using 0.1M potassium buffer (pH 6.80±0.01).

Eight millilitre of the C:N containing media was mixed with one millilitre suspension containing 5.5×10^4 conidia of *P. chlamydosporia* /ml and 1000 *M. incognita* eggs /ml in universal bottle. There were two controls where yeast extract medium (0.0125g/l=0.4mM C, 0.1mM N) was used to test the eggs in the presence and in the absence of the fungus. The treatments were incubated in an INNOVA® 40 incubator shaker (150rpm) at 25.5°C for 48 h. After incubation the data on parasitized and non parasitized eggs were counted using 100 eggs and the percentage of parasitized eggs was calculated.

7. 2. 4 Statistical analysis

Data on number of fungal propagules (CFU) and percent egg infection were square root and Logit ((%infection+0.5)/(100.5-%infection)) transform respectively. The transformed data were subjected to analysis of variance (ANOVA) using Genstat package version 11. Means from ANOVA output were compared using least significance differences (LSD).

7. 3 RESULTS

7. 3. 1 Effect of exposing organic materials in various temperatures on growth and parasitic activity of *Pochonia chlamydosporia*

Decomposition of OA at various temperatures significantly (P<0.05) affected the number of fungal propagules (7.1). Sawdust had a significantly (P<0.001) higher number of fungal propagules compared to the other OA (Table 7.1). Also, the comparison of the

number of fungal propagules in each decomposed OA was significantly ($P<0.05$) higher compared to undecomposed materials (Table 7.2). Interaction of OA and temperature significantly ($P<0.001$) influenced the numbers of fungal propagules (Table 7.2). The number of fungal propagules was highest in sawdust at 15 and 20°C. In general, the fungal population density increased with increase in temperature for *C. ochroleuca* and maize cobs, with exception of sawdust.

Percent egg infection was significantly ($P<0.05$) affected by OA tested and temperature (Table 7.2). Sawdust which had highest number of fungal propagules had the lowest egg infection. The percentage egg infection in *C. ochroleuca* and maize cobs were not significantly different ($P<0.001$) from one another but were both significantly ($P<0.001$) higher compared to sawdust. The 20°C treatment had higher percent infection and was significantly different ($P<0.05$) from that at 25°C.

Table 7. 1. Effect of decomposing organic materials at various temperatures on the population density of *Pochonia chlamydosporia*.

Organic amendment		Decomposition temperature (°C)		
	Undecomposed	15	20	25
Crotalaria sp	613(14.3)	32,976(177.9)	66,354(256.7)	77,171(273.9)
Maizecobs	0(0)	3,442(45.2)	20,797(117.7)	40,405(196)
Sawdust	645(14.7)	141,072(373.5)	116,368(329.4)	72,964(268.4)
Control	12,666(107.9)			
LSD		83.82		

Values in brackets are square root transformed means used in comparison with LSD.

Table 7. 2. Effect of decomposing organic materials at different temperatures on the percent egg infected by *Pochonia chlamydosporia*.

Organic amendment	Decomposition temperature (°C)			
	Undecomposed	15	20	25
Crotalaria sp	7.14(-2.58)	13.81(-1.92)	16.19(-1.76)	15.71(-1.75)
Maizecobs	7.14(-2.58)	9.52(-2.21)	15.71(-1.79)	4.76(-2.91)
Sawdust	3.33(-3.25)	2.38(-3.99)	1.91(-3.75)	0.95(-4.66)
Control	1.43(-4.2)			
LSD	1.15			

Values in brackets are Logit transformed means used in comparison with LSD.

7. 3. 2 Effect of pH on the parasitic activity of *Pochonia chlamydosporia* to root-knot nematode eggs

The results for percent egg infection were significantly ($P<0.05$) affected by the media used and also by the pH (Fig. 7.1). The percentage of egg infection in water was significantly ($P<0.001$) lower compared to infection in yeast extract. The results for the different pH levels indicates that among the pH treatment tested, the highest infection was at pH 4.47 and that the lowest was at pH 5.73. However, an increase or decrease in pH did not correlate ($P>0.05$) with percent egg infection. Below pH 4.47, the infection was low and the infection varied as pH increased. The infection on yeast extract medium (control) was high compared to all pH treatments, even those that had a pH closer to the pH of yeast extract medium. The pH values for water and yeast extract media (controls) were between 5.73 and 7.06 but the percent egg infections at different pH levels was significantly ($P<0.001$) different from yeast extract or water media.

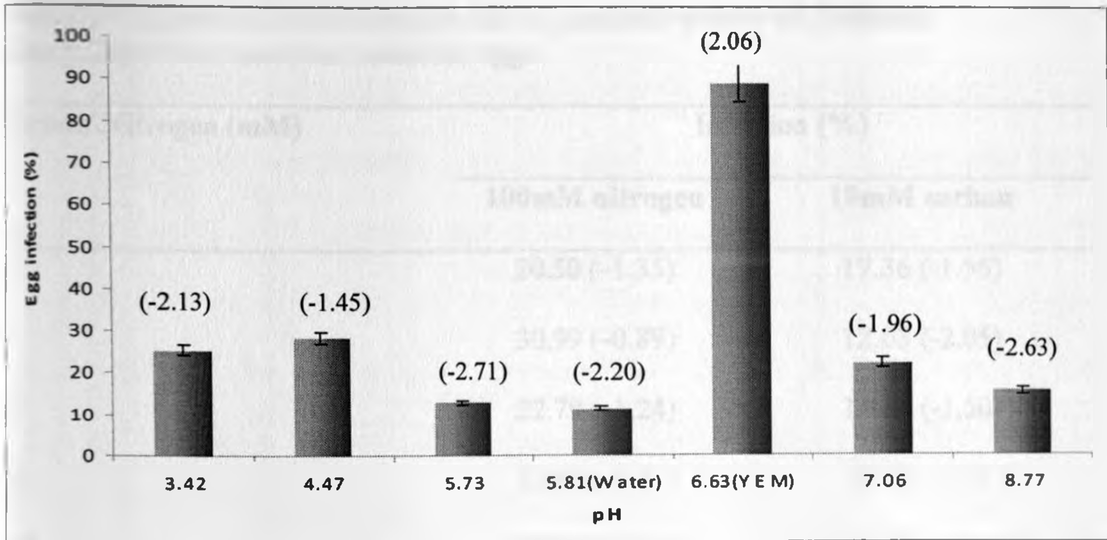


Fig. 7. 1. Effect of different pH on parasitic activity of *Pochonia chlamydosporia* to root-knot nematode eggs.
Values in brackets are Logit transformed means used in comparison with LSD=1.020.

7. 3. 3 Effect of carbon and nitrogen ratios on the parasitic activity of *Pochonia chlamydosporia* to root-knot nematode eggs *in vitro*

Differences in C and N levels significantly ($P<0.05$) reduced the percentage of eggs infected by *P. chlamydosporia* (Table 7.3). The highest infection was recorded in yeast medium (control). Among the CN: ratios tested, the highest and lowest egg infections were recorded in media containing C:N ratios of 5:100 and 5:10 respectively (Table 7.3). Also, the percent egg infections significantly ($P<0.05$) increased with increase nitrogen from level 5 to 100mM when carbon was at level 10mM. (Table 7.3).

Table 7. 3. Effects of carbon:nitrogen ratio on parasitic activity of *Pochonia chlamydosporia* to root-knot nematode eggs.

Carbon\Nitrogen (mM)	**Infection (%)**	
	100mM nitrogen	**10mM carbon**
1	20.50 (-1.35)	17.36 (-1.56)
5	30.99 (-0.89)	12.03 (-2.05)
10	22.79 (-1.24)	18.00 (-1.50)
50	23.63 (-1.31)	20.08 (-1.43)
100	22.99 (-1.24)	22.79 (-1.24)
Control (yeast extract) C:N (4:1))	49.73 (-0.02)	
LSD	0.78	

Values in brackets are Logit transformed means used in comparison with LSD.

7. 4 DISCUSSION

This study has demonstrated that fungal population increased with increase in the temperature at which organic substrates were decomposed. Decomposing OA at 25°C before application of fungus led to increased population of the fungus. This temperature has also been reported Kerry *et al.*(1986), as optimum for *P. chlamydosporia* growth. The numbers of fungal propagules when using *C. ochroleuca* and maize cobs as OA increased with increase in temperature but the reverse was recorded in sawdust. The increase in the numbers of fungal propagules by *C. ochroleuca* and maize cobs at higher temperature could be a result of enhanced decomposition and the release of available nutrients such as N and P to the fungus. The decrease in the numbers of fungal propagules in soil amended with sawdust with increase in temperature may have facilitated the release of elements

that are not suitable for fungal growth. According to Oka *et al.* (2007), decomposition of organic amendments with high C:N ratio can result in the release of toxic substances such as phenols that may have nematicidal and/or fungicidal activity. The sawdust used had a high C:N ratio and it is possible that at temperatures above 15°C toxic compounds released may have suppressed growth of *P. chlamydosporia.* In general, the results indicate that presence of OA and temperature are important factors influencing the efficacy of the fungus. This was observed when OA were mixed with *P. chlamydosporia* without allowing time to decompose.

This study has also demonstrated that decomposing *C. ochroleuca* and maize cobs in the soil for 30 days before application of *P. chlamydosporia* resulted in increased egg parasitism. This implies that the time given for the OA to decompose was enough for the released nutrients to deplete and this might have increased the ability of fungus to parasitize RKN eggs and reduced its saprophytic tendency. As a facultative parasite, the fungus has been reported to be good in utilizing nutrients saprophytically but the saprophytic ability differs depending on isolate, colonized crop and how rich is the soil (Liu and Chen, 2003; Mauchline *et al.*, 2004). The nutritional status of the rhizosphere determines the switching on and off from saprophytic stage (Esteves, 2007). The inability of sawdust to support high infection, as in previous study, implies that not all OA can have beneficial effects after one month of decomposition. It has been argued that sawdust, as one of the cellulosic materials, has wide C:N ratio and poor nematode management (Akhtar and Malik, 2000). Due to the wide C:N ratio, decomposition process is slow and takes longer to release nutrients required by the fungus (McSorley and Frederick, 1999). This suggests that the time given was short for sawdust and may

have contributed to low fungal population to infect large numbers of nematode eggs. In this study, *Crotalaria ochroleuca* and maize cobs have led to promising results for the fungal infection and could be suitable when decomposed at the temperature range of 15 and 20°C. The effectiveness of the decomposed OA in this study has also been reported to be effective in broccoli decomposed for 20 days and hence reduced galling index from 9.5 to 4.8 in melon (Ploeg and Stapleton, 2001). The population of the fungus in sawdust was not low as its infection rate when you compare to others. This indicates that increase in fungal population may not always translate into higher parasitism (Jaffee, 2004).

The study on testing influence of different pH levels found that the difference in pH did not indicate strong or direct effect on egg infection rate. Similarly, the indirect influence of pH on the activity of nematophagous fungi on RKN eggs had been noted by Jaffee and Zasoski (2001). There was a slight difference in pH between yeast and water but the difference in percentage eggs infected was significant. When comparing egg infection in yeast extract medium, the pH 5.73 or 7.06 which were closer to the pH of yeast extract (pH 6.63) had lower percentage eggs infected. The ability of yeast to enhance egg infection could be due to the additional nutrient components, including C and N, when compared to sterile distilled water. Also, the differences in infection rates among pH treatments and media containing yeast extract, even those closer to the pH of the yeast extract medium, indicates that the reagents used to make the buffer (potassium phosphates) could be having a negative effect on infectivity of the fungus on RKN eggs. According to Eayre *et al.* (1990), application of potassium chloride into the soil did not enhance parasitism of nematodes by the nematophagous fungus, *Hirsutella rhossiliensis*. The highest egg infection at pH 4.47 among pH levels tested, can be compared to the

results reported by Jaffee and Zasoski (2001) who achieved a higher parasitic activity of *H. rhossiliensis* to nematodes at pH 4.5 and a lower egg parasitism at lower or higher pH. Increase in the levels of C and N in the liquid medium did not increase the proportion of eggs infested by the fungus. This finding confirms the results reported by Irving and Kerry (1986) where differences in nutrient concentration levels of the agar did not cause significant differences in fungal parasitism to *Heterodera avenae* eggs. The highest level of carbon at which highest infection attained is at the lowest level of N. This implies that C and N act differently from one another. Also, in this study, percentage egg infection was reduced at increasing carbon and increased at decreased C levels. This result is similar to that of Segers (1996) who found a low egg infection by the fungus when C was equal to or greater than ten mill Molar, and independent of N levels. This finding suggests that high amounts of C do not support parasitism of nematode eggs. Generally, the percent egg infection was low in the C:N ratio media used in this study compared to that found in yeast extract medium (standard). Compared to the C and N content in the standard yeast extract medium, this study used higher C and N levels and this could be one of the reasons of the low percent egg infection.

CHAPTER 8

INTEGRATED MANAGEMENT OF ROOT-KNOT NEMATODE USING BIOLOGICAL AGENTS, ORGANIC MATERIALS AND CROP ROTATION

8. 1 INTRODUCTION

Tomato is one of the most economically important crops to small-scale farmers in Kenya. It is, however, one among the many crops that are highly susceptible to root-knot nematodes (RKN) and cause heavy yield losses (Nono-Womdim *et al.*, 2002). Dependence on the use of available control measures has proved to be unreliable, even with those methods which initially seemed to be effective, such as nematicides and resistant cultivars (Akhtar, 1997). Continuous use of resistant cultivars leads to breakdown of resistance while repeated use of nematicides has led to concerns about the environment (Whitehead, 1998). Integrated management has been gaining popularity in recent years due to the absence of new nematicides and the banning of methyl bromide (Sikora *et al.*, 2005; Anastasiadis *et al.*, 2008). Also, in developing countries where there are often many resource-poor farmers, nematicides are expensive and not always readily available (Akhtar, 1997).

Demand for sustainable management of nematodes has increased the interest on different production systems which includes crop rotation, application of OA such as chicken manure and biological control. Rotation with non-host crops has been recommended and used in many countries in order to reduce the build up of RKN (Widmer *et al.*, 2002). However, due to the wide host range of RKN, rotation crops have to be selected with care (Kiewnick and Sikora, 2006). Some of the vegetable crops used in rotation to control

RKN are cabbage, cauliflower and onions as they are considered as poor host crops while other high value crops like tomato, okra and lettuce are among the very susceptible ones (Sikora and Fernandez, 2005). Some of the weed plants, such as *Tagetes minuta*, can be used to control nematodes when planted in rotation and used as an amendment (Akhtar and Malik, 2000). One of the successfully tested integrated management options used in vegetable production systems for the control of RKN was application of the nematophagous fungus on a RKN tolerant crop, followed by growing of a susceptible crop (Sikora *et al.*, 2005). Maize has also been found to be less susceptible to *M. incognita* and significantly reduces nematode populations when used in rotation with tomato and chilli (Sikora and Fernandez, 2005).

Addition of OA, has been suggested to be beneficial especially when integrated with other methods such as crop rotation and biological control (Akhtar, 1997). The modes of action include release of toxic compounds like phenols and fatty acids and enhancement of nematode antagonists (Arim *et al.*, 2006). It has been reported that application of OA increases populations of biocontrol agents in the soil especially in the rhizosphere and reduces nematode populations when applied together with biocontrol agents (Jaffee, 2004). For example, application of a mixture of cattle manure and *P. chlamydosporia* in less susceptible crops (beans and cabbage) prior to growing of tomato in the following season had greatly increased fungal population (Atkins *et al.*, 2003b). Besides the direct and indirect effect to nematodes, OA also improve the soil structure and fertility thus encouraging stronger plants (Sikora and Fernandez, 2005).

Pochonia chlamydosporia is more effective when incorporated to crops like cabbage and maize that favour its growth in their rhizosphere (Bourne and Kerry, 1999). For instance,

addition of *P. chlamydosporia* on cabbage, bean and kale reduced RKN in the soil before the susceptible tomato crop was planted (Sikora and Fernandez, 2005). Crops that favour fungal growth in the rhizosphere also increase the chances of the fungus surviving in the soil even with different cropping systems (Leij de and Kerry, 1991). In Cuba, chlamydospores of *P. chlamydosporia* are applied to a poor host crop used in rotation with tomato in a vegetable production system to enhance growth and survival of *P. chlamydosporia* (Sikora and Fernandez, 2005).

Integrated management where *P. chlamydosporia* has been incorporated, have been successfully done with a poor host followed by a susceptible crop, either in the following year or after two seasons (Kerry and Bourne, 1996; Atkins *et al.*, 2003b). However, the plant species that are poor hosts to RKN differ in their ability to allow fungal growth on their root surface (Bordallo *et al.*, 2002). This brings the need for identification of poor hosts of the target nematode that are suitable for fungal colonisation.

This study was undertaken with the aim of determining the potential for integrating *P. chlamyodosporia*, crop rotation and organic amendments in the management of root-knot nematodes. Therefore, the specific objectives of this study were,

- To evaluate different locally grown and commonly found plants that support growth and multiplication of *P. chlamydosporia* in their rhizosphere.
- To determine the potential of combining crop rotation, *P. chlamydosporia* and organic materials in root-knot nematode management.

.2 MATERIALS AND METHODS

.2. 1 Selection of commonly found plant species that support fungal abundance

This experiment was carried out in a glasshouse using five different plants that are known to be poor hosts of RKN and which can be rotated with tomato. The plants tested were cabbage (*Brassica oleracea),* maize (*Zea mays), Tagetes minuta* (African marigold), *Crotalaria ochroleuca* (sunn hemp) and *Mucuna pruriens* (velvet beans). The experiment had six treatments (five poor host crops and tomato as a control) arranged in a randomised complete block design (RCBD) and was replicated five times. Seeds of the plants were pre-germinated in Petri dishes and transferred into 500g capacity pots containing loam soil mixed with sand at 3:1 w/w. The potted soil was also inoculated with *P. chlamydosporia* at a rate of 5000 chlamydospores/g of soil making a total of 2,500,000 chlamydospores per pot.

Other agronomic practices such as watering and fertilisation were carried out regularly. Soil and root samples were collected at 30 and 90 days after application of the fungus into the soil and used to estimate the fungal propagules (CFU) as described by Kerry and Bourne (2002). One gram of soil was suspended in nine millilitre water which had 0.05% agar and diluted to 10^{-2} and 10^{-3}. From the dilutions, 0.2ml was cultured on semi-selective media (Appendix 1) in Petri dishes and incubated at 25°C for 14 days. After incubation, the colonies of *P. chlamydosporia* were counted and were estimated as number of fungal propagules per gram of soil. Numbers of fungal propagules in the roots were also estimated per gram of ground roots using the same procedure as above.

8.2.2 Integrated management of root-knot nematode

The experiment was conducted in a glasshouse and in a field located at the College of Agriculture and Veterinary Sciences of the University of Nairobi, for two seasons. The treatments for glasshouse and field experiments for the two seasons were as shown in Table 8.1. In the first season, chlamydospores of *P. chlamydosporia* (isolate 10) were incorporated into the soil (Appendix 2) in the pots and in the field. In the second season, maize stover was incorporated into the soil for the appropriate treatments (Table 8.1).

Table 8. 1. Treatments for integrated management of root-knot nematode in two seasons.

Treatment	First season	Second season
1 (Control)	Tomato	Tomato
2	Tomato	Tomato + maize stover
3	Tomato + *P. chlamydosporia* isolate 10	Tomato
4	Tomato + *P. chlamydosporia* isolate 10	Tomato + maize stover
5	Maize	Tomato
6	Maize	Tomato + maize stover
7	Maize + *P. chlamydosporia* isolate 10	Tomato
8	Maize + *P. chlamydosporia* isolate 10	Tomato + maize stover

8. 2. 2. 1 Glasshouse experiment

Pots were filled with one kilogram soil (Appendix 2) taken from the site where the field experiment was conducted. Chlamydospore inoculum of *P. chlamydosporia* isolate 10 was mixed with soil at the rate of 5000 chlamydospores/g soil. The eight treatments

Table 8.1) were arranged in a RCBD with five replications. A control, where neither fungus nor amendment was maintained, but tested for the natural occurrence of *P. chlamydosporia*. Pre-germinated tomato and maize seeds were transferred into the appropriate pots. Three months after planting, the experiment was terminated and data on number of fungal propagules (CFU) of the fungus and second stage juveniles (J2) of RKN from soil and tomato roots were taken. Root galling index and dry shoot weight of tomato plants were also recorded. The numbers of CFU was collected as described in section 8.2.1. Second stage juveniles were extracted from the soil and roots using the modified Baermann funnel technique as described by Hooper *et al.* (2005). The root galling indices were determined using a scale of 1-5 (Appendix 5) (Coyne *et al.*, 2007) while dry shoot weight was obtained after drying fresh materials in an oven at 70°C until constant mass was achieved in about 72 h.

8. 2. 2. 2 Field experiments

The experiments were laid out in a RCBD and each treatment (Table 8.1) was replicated four times. The field experiments were done for two seasons; between April to October 2008 and October to March 2009. A plot size of 4m × 4m with a spacing of 60cm × 75cm were used. The planting holes were made and about three kilogram soil from the hole was taken and mixed with *P. chlamydosporia* isolate 10, at the rate of 5000 chlamydospores/g before being placed back into the hole. The plot was irrigated and maize seeds sown while six weeks-old tomato seedlings were transplanted into the appropriate plots.

The plants were watered regularly depending on the weather conditions. Ridomil 25 W.P (a.i metalaxyl+mancozeb) Syngenta (2kg/ha, mixed as 2gm/liter water) and Polytrin (a.i

rofenofos + cypermethrin) (400ml/ha, mixed as 2.5ml/10 liter water) were sprayed when ecessary to control fungal diseases (early and late blight) and insect pests (mites), espectively, on a weekly basis. A compound fertilizer (17:17:17) was applied after one month at 100 kg/ha. Yield data was collected by harvesting mature fruits once a week until the termination of the experiment. The number and yield (weight) of fruits was recorded at every harvest. On termination of the experiment, data on the number of fungal propagules, second stage juveniles and galling index were recorded using the techniques and procedures described in section 8. 2. 2. 1. The experiment was repeated once following the same practices as in the first one.

8. 2. 3 Statistical analysis

All data were checked for normal distribution and, when needed, Log_{10} or square root were used to transform the data. The data from all experiments were subjected to two way analysis of variance (ANOVA) using Genstat package version 11. The means were compared using least significance differences (LSD).

8. 3 RESULTS

8. 3. 1 Growth and survival of *Pochonia chlamydosporia* in the soil and rhizosphere of different plants

At 30 days after fungal application, the root and soil fungal populations were significantly ($P<0.05$) different between different plants (Table 8.2). The fungal propagules in the soil and roots were increased in all plants with the exception of *M.*

uriens which had low numbers of CFU in the roots and soil. After 90 days, differences plant tested resulted into significant ($P \leq 0.001$) difference on the numbers of *P. chlamydosporia* propagules in the soil and root tissues. The root CFU counts after three months indicated that cabbage, *M. pruriens* and maize had higher fungal propagules than others while tomato and *C. ochroleuca* had no fungal propagules (Table 8.2). In the soil, the CFU counts were higher in maize rhizosphere compared to the other plants.

Table 8. 2. Colonisation of *Pochonia chlamydosporia* in the soil and in roots of six different crops at 30 and 90 days after application.

Crop	CFU /g at 30 days		CFU/g at 90 days	
	Root	Soil	Root	Soil
Cabbage	67,500(4.83)	392,086(5.58)	13,250(4.11)	284(1.38)
Crotalaria sp	85,000(4.92)	500,133(5.70)	0(0.00)	0(0.00)
Maize	110,000(5.04)	369,507(5.57)	12,250(4.04)	2,245(3.34)
Mucuna sp	10,000(2.15)	10,478(2.16)	12,500(4.09)	307(1.39)
Tagetes sp	20,000(4.29)	201,323(5.27)	4,250(3.62)	283(1.38)
Tomato	182,500(5.26)	164,889(5.21)	0(0.00)	0(0.00)
LSD	1.55	1.52	0.19	1.36

Values in brackets are Log_{10} transformed means used in comparison with LSD.

8. 3. 2 Integrated management of root-knot nematodes

8. 3. 2. 1 Glasshouse experiment

Application of *P. chlamydosporia* had a significant ($P \leq 0.001$) effect on the number of J2 and CFU in the roots of the tomato plants in the first season (Table 8.3). The fungus

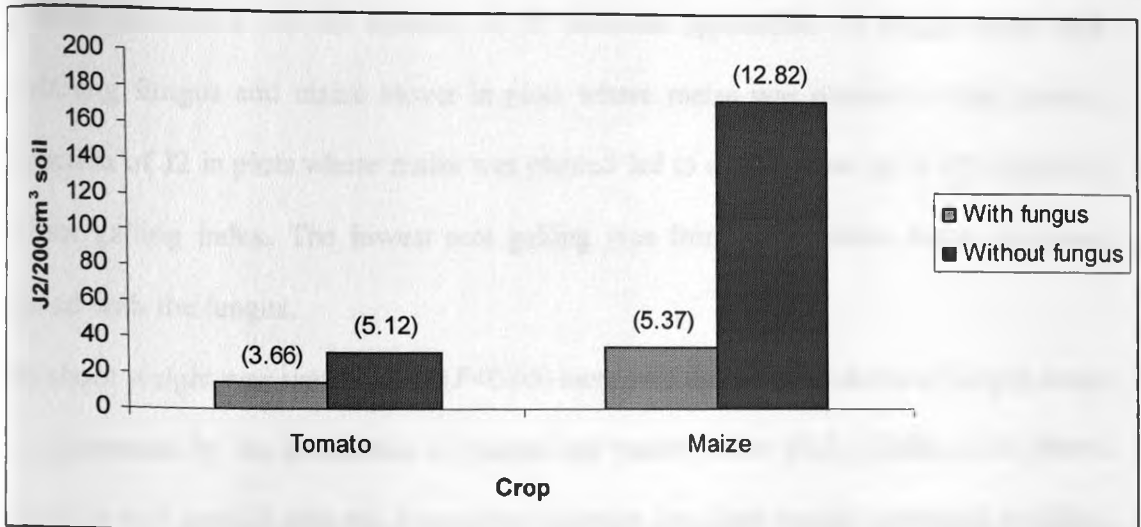
caused a decrease in the number of J2 from the roots of tomato where fungus was added. There was increase in the number of fungal propagules from the amount applied in the soil with no fungus detected in soil where fungus was not added. However, the root galling index and dry shoot weight in tomato grown in soil applied with the fungus were not significantly ($P>0.05$) different from tomato grown in soil not applied with the fungus.

Table 8. 3. Survival of applied *Pochonia chlamydosporia* and its effects on second stage juveniles (J2), galling index and dry shoot weight of tomato.

Parameters	With fungus	Without fungus	LSD
[1]CFU/g roots	29,000(10.14)	0(0)	0.42
[2]J2/5g roots	1(0.1)	43(1.78)	0.88
[2]Galling index (1-5)	3.9(2.01)	4.1(1.95)	0.23
[2]Dry shoot weight (g)	4(1.97)	4.37(2.05)	0.39

Values in brackets are [1]Log_{10} and [2]square root transformed means used in comparison with LSD.

The numbers of J2 in the soil were significantly ($P<0.001$) affected by the type of crop, presence of fungus and their interaction (Fig. 8.1). The number of J2 was lower in soil where tomato was grown in presence of the fungus than where fungus was not applied. However, the level of nematode suppression due to the fungus was higher in maize than in tomato (Fig. 8.1 upper panel). *Pochonia chlamydosporia* isolate 10 reduced the numbers of J2 by 58 and 28% in maize and tomato plots, respectively. The fungal propagules in the soil were significantly ($P<0.001$) different as a result of application of the fungus, with soil in pots applied with the fungus having higher counts compared to soil where fungus was not applied (Fig.8.1 lower panel).



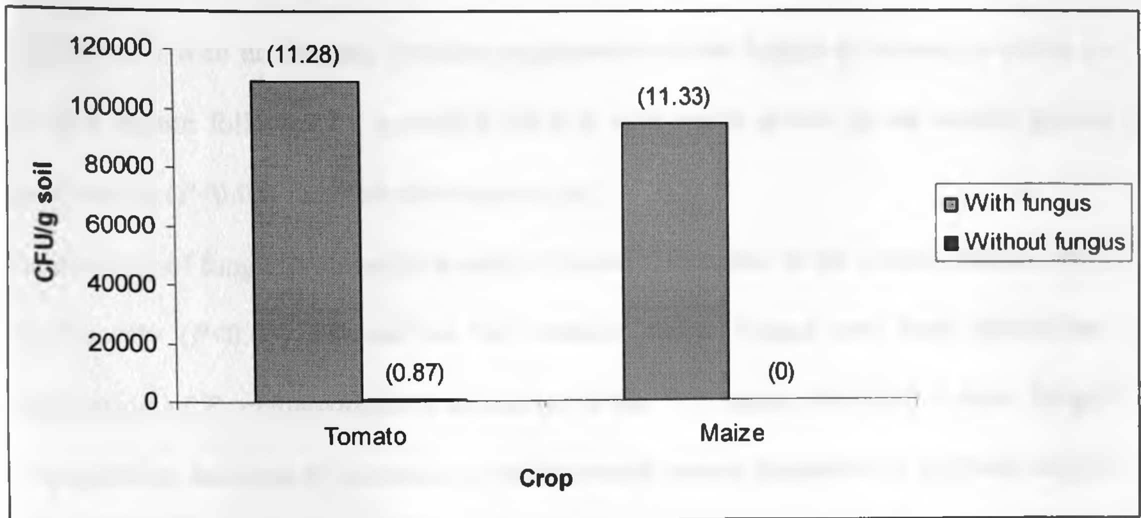


Fig. 8. 1. Effect of maize and tomato on numbers of root-knot nematodes (J2) and fungal propagules (CFU) in the soil.
Values in brackets in upper panel are square root transformed means were compared using LSD 2.16 and lower panel are Log_{10} transformed means were compared using LSD 1.31

In the second season, the number of second stage juveniles in the roots and soil were significantly (P<0.05) reduced in plots where maize was planted in the first season (Table 8.4). However, application of the fungus in tomato in the first season followed by maize stover in the second season significantly decreased (P<0.05) the number of J2 in tomato roots than application of fungus alone (Table 8.4). There was no significant

($P<0.05$) difference on the number of J2 between application of fungus alone and combining fungus and maize stover in plots where maize was planted in first season. Reduction of J2 in plots where maize was planted led to a significant ($P<0.05$) reduction of root galling index. The lowest root galling was from plots where maize crop was planted with the fungus.

Dry shoot weight was significantly ($P<0.05$) increased due to application of fungus alone and decreased by the interaction of fungus and maize stover (OA) (Table. 8.4). Plants grown in soil applied with the fungus had a higher dry shoot weight compared to plants from the soil with no fungus. However, application of the fungus in tomato or maize in the first season followed by amending the soil with maize stover in the second season significantly ($P<0.05$) reduced dry shoot weight.

The number of fungal propagules in roots of tomato, recorded in the second season, were significantly ($P<0.05$) affected by the rotation crops, fungus and their interaction. Application of *P. chlamydosporia* to tomato in the first season resulted in more fungal propagules in the roots of tomato crop in the second season compared to applying fungus in maize in the first season followed by tomato in the second season. But applying maize stover in plots where maize was grown in presence of fungus significantly ($P<0.05$) increased the fungal propagules in the roots more than applying fungus alone.

Table 8. 4. Effect of crop rotation, *Pochonia chlamydosporia* and maize stover on root-knot nematode population, galling [illegible] shoot weight and fungal propagules in the second season for the glasshouse experiment.

Season I	Season II	[1]Root J2/5g	[1]Soil J2/200cm^3	[1]Galling index (1-5)	[1]Dry shoot weight(g)	[2]Root CFU/g	[2]Soil CFU/g
Tomato	Tomato	460.8(6.08)	330(5.05)	3.8(1.94)	1.79(1.57)	0(0.00)	0(0.00)
Tomato	Tomato+Mstover	180.6(4.09)	348(5.81)	3.2(1.77)	4.87(2.16)	0(0.00)	0(0.00)
Tomato+F	Tomato	450.4(6.47)	288(4.97)	3.2(1.77)	10.08(2.90)	1,400(5.91)	15,924(7.76)
Tomato+F	Tomato+Mstover	100(3.14)	280(5.04)	3.4(1.84)	2.53(1.47)	4,300(7.99)	15,000(5.93)
Maize	Tomato	56(1.84)	100(2.94)	2(1.39)	3.40(1.62)	0(0.00)	0(0.00)
Maize	Tomato+Mstover	72(2.12)	170(4.04)	2.2(1.46)	2.57(1.55)	0(0.00)	0(0.00)
Maize +F	Tomato	90(2.7)	102(3.06)	1.4(1.66)	9.58(2.76)	800(2.98)	61,388(4.71)
Maize +F	Tomato+Mstover	96(3.0)	276(4.31)	1.6(1.25)	3.81(1.93)	700(4.23)	61,583(6.89)
LSD		2.18	2.77	0.311	1.06	3.01	5.51

Values in brackets are [1]square root and [2]Log_{10} transformed means used in comparison with LSD. Mstover= maize stover, F= Fungus,

8.3.2.2 Field experiments

8.3.2.2.1 Field trial one

The results of the field experiment for the first season taken from tomato crop alone indicated that application of fungus had a significant ($P<0.05$) effect on the survival of fungal propagules in the roots and tomato yield (Table 8.5). The roots taken from the soil applied with the fungus had higher fungal population density than those from soil not applied with fungus (Table 8.5). Also, application of the fungus increased the yield of tomato and reduced root galling index (disease severity) significantly ($P<0.05$) (Fig. 8.2).

Table 8. 5. Effect of application of *Pochonia chlamydosporia* on fungal propagules (CFU) and second stage juveniles (J2) in the roots, number and yield of tomato.

Parameters	With fungus	Without fungus	LSD
[1]CFU/g roots	1,625(7.23)	187.5(1.64)	1.91
[2]J2/5g roots	22.5(1.38)	130(3.04)	1.80
[2]Number of fruits/ha	22,950(9.96)	17,475(9.59)	0.59
[2]Tomato yield t/ha	20.8(2.99)	15.4(2.71)	0.21

Values in brackets are [1]Log_{10} and [2]square root transformed means used in comparison with LSD.

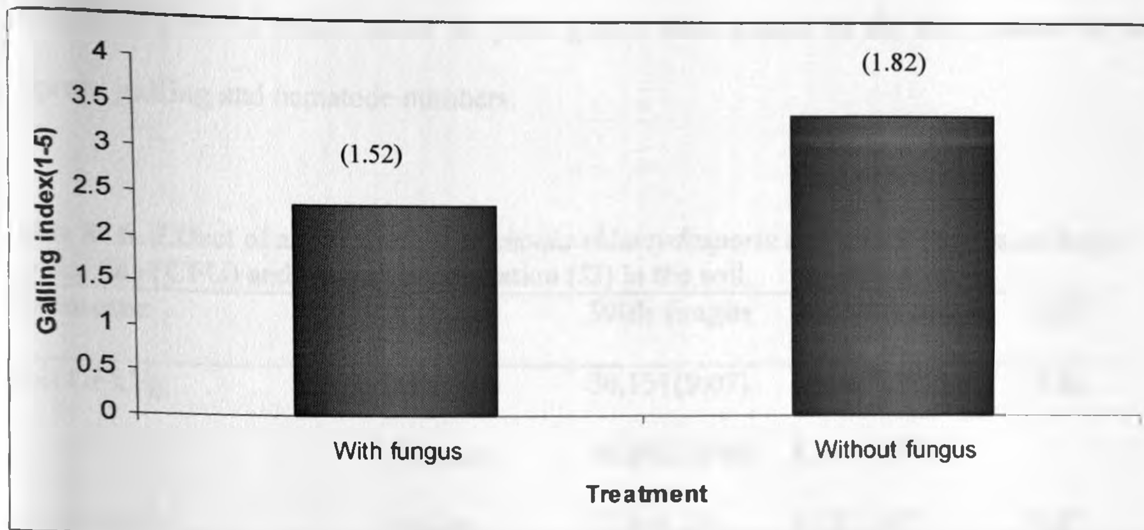


Fig. 8. 2. Effect of fungus on root galling of tomato.
Values in brackets are square roots transformed means compared using LSD = 0.24

The analysis using the two crops and fungus as factors in the first season showed that the fungal propagules in the soil increased significantly (*P*<0.001) due to addition of the fungus (Table 8.6) and not (*P*>0.05) by type of crop or their combination. The soil nematodes population (J2) was significantly (*P*<0.001) decreased by differences in crop grown and application of fungus. The number of J2 was lower in soil where maize was grown than in tomato plots. Also, the number of J2 decreased with application of fungus. The lowest number of J2 was on soil where maize was grown and applied with the fungus.

The results for the second season indicate that the numbers of J2 and galling index were significantly (*P*<0.05) reduced by the rotation and the interaction between rotation crop and fungus (Table 8.7). The number of J2 and galling index were lower in plots where tomato was planted in the second season after maize in the first season than where tomato was followed by tomato. Application of fungus in tomato in the first season resulted into lower number of J2 in the roots of tomato grown in the second season than where fungus was not

added. Addition of maize stover in plots grown with tomato in the first season did not suppress galling and nematode numbers.

Table 8. 6. Effect of application of *Pochonia chlamydosporia* and rotation crops on fungal propagules (CFU) and nematode population (J2) in the soil.

Parameter	Crop	With fungus	Without fungus	LSD
[1]Soil CFU/g	Maize	30,151(9.07)	1,656(2.2)	3.86
	Tomato	44,692(10.64)	4,930(4.57)	
[2]J2/200cm^3	Maize	22.5(1.23)	31.2(1.57)	0.87
	Tomato	57.5(2.37)	92.5(2.83)	

Values in brackets are [1]Log$_{10}$ and [2]square root transformed means used in comparison with LSD

Continuous growing of tomato significantly ($P<0.05$) reduced the number and yield of tomato compared to rotating maize and tomato. The highest yield was obtained in the treatments where maize was planted in the first season followed by tomato, and also where fungus was applied in the first season. Addition of *P. chlamydosporia* and maize stover in plots where tomato was planted in the first season did not cause a difference in yield compared to only where the fungus was applied. The fungal propagules in the soil were significantly ($P<0.001$) increased by addition of the fungus in the first season. The fungal propagules were higher in soil applied with fungus compared to soil not applied.

Table 8. 7. Effect of rotation crop, *Pochonia chlamydosporia* and maize stover on [illegible] propagules, number and yield of tomato in the second season in trial one of the field.

Season I	Season II	[1]Root J2/5g	[1]Soil J2/200cm^3	[1]Galling index (1-5)	[2]Soil CFU/g	[2]Fruit counts	[2]Tomato yield t/ha
Tomato	Tomato	150(3.04)	67.5(2.35)	3(1.68)	0(0.00)	302,500(12.54)	14.44(2.66)
Tomato	Tomato+Mstover	65(2.45)	72.5(2.64)	3.75(1.62)	0(0.0)	306,250(12.61)	14.38(2.64)
Tomato+F	Tomato	22(1.47)	40(1.92)	2.25(1.49)	823.2(3.21)	293,750(12.54)	17.9(2.83)
Tomato+F	Tomato+Mstover	28(1.64)	45(1.99)	3.75(1.84)	375.7(5.85)	281,875(12.47)	18.51(2.88)
Maize	Tomato	5(0.5)	30(1.49)	1.75(1.29)	176.3(1.64)	450,000(13.00)	23.14(3.12)
Maize	Tomato+Mstover	18(1.14)	15(1.18)	1.5(1.21)	0(0.00)	465,000(13.05)	23.09(3.10)
Maize +F	Tomato	22(1.29)	25(1.26)	1.25(1.10)	231.6(4.19)	548,125(13.21)	39.09(3.63)
Maize +F	Tomato+Mstover	10(0.85)	10(0.85)	1.5(1.21)	144.2(2.72)	503,750(13.06)	30.85(3.40)
LSD		1.64	1.26	0.54	3.22	0.50	0.43

Values in blackest are [1]square root and [2]Log_{10} transformed means used in comparison with LSD. Mstover= maize stover, F= Fungus

8.3.2.2.2 Field trial two

In the first season, application of the fungus significantly ($P<0.001$) increased fungal propagules in the roots of tomato grown in the first season (Table 8.8). The CFU were in roots from plants grown in presence of the fungus while the fungus was not detected in roots of the plants grown in pots where the fungus was not applied. Application of fungus had no significant ($P>0.05$) effect on the number of J2, number and yield of tomato. Application of *P. chlamydosporia* and crop differences significantly ($P<0.05$) increased the fungal propagules and reduced the number of J2 in the soil in the first season (Table 8.9). Fungal propagules were significantly ($P<0.05$) higher in soil applied with fungus than where fungus was not applied (Table 8.9). The numbers of J2 were significantly ($P<0.05$) higher in soil where tomato was planted and where fungus was not applied than where maize was grown and applied with fungus.

Table 8. 8. Effect of application of *Pochonia chlamydosporia* on colony forming units (CFU) and nematode population (J2) in the roots, number and yield of tomato.

Parameters	With fungus	Without fungus	LSD
[1]CFU/g roots	5,000(6.56)	0(0)	2.53
[2]J2/5g roots	32.5(1.45)	91.25(2.42)	1.69
[2]Number of fruits/ha	200,100(12.03)	174,300(11.96)	0.67
[2]Tomato yield t/ha	14.39(2.59)	15.51(2.6)	0.4

Values in brackets are [1]Log_{10} and [2]square root transformed means used in comparison with LSD.

Table 8. 9. Effect of combining *Pochonia chlamydosporia* and rotation crops on the fungal propagules (CFU) and nematode populations (J2) in the soil.

Parameter	Crop	With fungus	Without fungus	LSD
[1]Soil CFU/g	Maize	9,858(5.98)	835(1.1)	3.89
	Tomato	14,022(8.25)	824(1.1)	
[2]J2/200cm^3 soil	Maize	11.2(0.97)	26.2(1.41)	0.78
	Tomato	32.5(1.74)	67.5(2.38)	

Values in brackets are [1]Log_{10} and [2]square root transformed means used in comparison with LSD.

In the second season, planting tomato after maize caused significant (P<0.05) decrease in population of J2 and galling of the tomato roots while an increase was found when tomato was planted in the two seasons (Table 8.10). The numbers of J2 were lowest in plots where maize was planted in soil applied with the fungus and maize stover in the first and second seasons respectively. The type of crop planted in the first season also had a significant (P<0.05) effect on number and yield of tomato recorded in the second season (Table 8.10). The number and weight of tomato fruits in the second season was higher in plots where maize was followed by tomato than where tomato was followed by tomato (Table 8.10).

Table 8. 10. Effect of crop rotation, *Pochonia chlamydosporia* and maize stover on root-knot nematode population (J2), galling index, fungal propagules (CFU), yield (number and weight) of tomato in the second season in trial two of the field.

Season I	Season II	[1]Root J2/5g	[1]Soil J2/200cm^3	[1]Galling index(1-5)	[2]CFU/g of soil	[2]Total number of fruits	[2]Tomato yield t/ha
Tomato	Tomato	158(3.9)	145(3.73)	3.25(1.46)	45,543(8.16)	615,562(13.31)	5.61(1.64)
Tomato	Tomato+Mstover	160(3.78)	107.5(3.25)	3(1.72)	13,985(4.89)	815,062(13.55)	5.69(1.56)
Tomato+F	Tomato	242(4.63)	145(3.74)	2.75(1.64)	16,856(7.49)	773,062(13.50)	5.21(1.55)
Tomato+F	Tomato+Mstover	152(3.66)	162.5(3.93)	2.75(1.64)	25,562(7.79)	576,188(13.18)	3.63(1.03)
Maize	Tomato	60(2.39)	107.5(3.27)	2(1.39)	3,108(2.36)	1,008,000(13.76)	13.58(2.39)
Maize	Tomato+Mstover	65(2.45)	115(3.29)	2.25(1.49)	50,315(10.57)	771,750(13.53)	6.72(1.86)
Maize +F	Tomato	48(2.08)	72.5(2.67)	1.5(1.21)	38,615(5.32)	1,013,250(13.80)	9.22(2.09)
Maize +F	Tomato+Mstover	42(2.04)	62.5(2.45)	1.5(1.21)	10,842(4.81)	1,006,688(13.78)	9.59(2.21)
LSD		1.71	1.08	0.39	7.67	0.47	0.83

Values in brackets are [1]square root and [2]Log_{10} transformed means used in comparison with LSD. Mstover= maize stover, F= Fungus,

8. 4 DISCUSSION

This study has demonstrated that different plants differ in their capacity to support growth of *P. chlamydosporia* on their roots and in their rhizosphere. All the crops tested had some ability to support fungal growth on their root surfaces with the exception of *C. ochroleuca* and tomato. The decline in fungal populations associated with the two plant species which were nematode free, can be attributed to the root exudates they release were not attracting the fungus. According to Kerry (2000) root exudates from tomato infected by RKN contain more water soluble and several metal ions which support more colonisation of *P. chlamydosporia* than healthy roots. Also, it can be said that the decline may have been occasioned by the degradation of nutrients in the rhizosphere and their subsequent uptake by the growing plants. This study has shown that *C. ochroleuca* and tomato do not support external root colonization of the fungus for an extended period of time. Inability of these plants to support *P. chlamydosporia* for extended periods implies that a one time application of the fungus may not sustain high enough fungal propagules to control root-knot nematodes. Addition of *P. chlamydosporia* to crops that are poor in supporting fungal growth in their rhizosphere may not be useful (Bordallo *et al.*, 2002).

A similar trend was observed for the fungal populations in soil in the crops tested which increased after 30 days of application and subsequently decreased three months after fungal application with the exception of *M. pruriens*. These results suggest that, different plant species have different capacities of supporting the fungus in their rhizosphere. Variability in the ability of different plants to support the fungus in the rhizosphere has been reported by several authors (Bourne and Kerry, 1999; Kerry, 2000; Vargas-Ayala *et al.*, 2000). In this experiment maize and cabbage exhibited good support of fungus

establishment and growth in the rhizosphere. Compared to the other crops tested, maize was able to maintain high fungal population density in the soil and roots, throughout the growing period.

The differences in capacity to support fungal growth could be due to differences in availability of chemical substances such as glucose and nitrogen emanating from the host plant, that might either promote or suppress fungal growth (Kerry, 2000). It has been noted that maize supports a higher degree of fungal growth, even in the presence of high nematode populations compared to tomato (Bourne and Kerry, 1999). It can be suggested from this study that maize is a promising crop in cropping systems where *P. chlamydosporia* is used as a biocontrol agent. The use of maize as a rotational crop may provide double effect on RKN management; as a poor host to RKN and a better host to this fungus hence suppressing the nematode populations (Bourne *et al.*, 1996). A report by Desaeger and Rao (2000) shows that rotating maize with a RKN susceptible crop (sesbania) significantly reduced RKN infestation. In addition, maize is a readily acceptable crop to smallholder farmers since it improves food security.

The study on integrating *P. chlamydosporia,* crop rotation and maize stover established that, addition of fungus resulted in higher colonization of the rhizophere, reduced the population of nematodes and galling in roots and increased growth of tomato. The population of *P. chlamydosporia* recovered from the soils at the end of the experiments was higher than the number applied. This signifies that this fungus was able to adapt, compete with other soil micro-organisms, survive and then colonise the soil. Van Damme *et al.* (2005) evaluated the long term effect of *P. chlamydosporia* and reported that the fungus persisted in soil for at least 5-7 months. Establishment of this fungus in the soil

after introduction may lead to soil suppressiveness for long periods without repeated applications (Anastasiadis *et al.*, 2008).

Maize was effective in lowering the nematode population in the soil and roots in presence of *P. chlamydosporia.* This study established that percentage reduction of J2 in the soil applied with fungus was higher in maize (58%) than in tomato (28%). The differences in the ability of different crops to affect the activity of the fungus on RKN conforms with the results by Bourne and Kerry (1999) who found that the reduction of J2 population in the soil due to *P. chlamydosporia* was higher in maize, kale and *Phaseolus* bean than in tomato. Kerry and Bourne (2002) also found that egg parasitic fungi are less effective in crops that support large gall formation on their roots that retain and protect some of the eggs, making the fungus unable to penetrate and infect them. Moreover, the reduction of J2 might have been due to the increase in soil fungal population which could have contributed to high RKN egg infection and hence reduced number of J2 in the soil. A similar study by De Leij *et al.* (1993) recorded a 90% reduction of *M. incognita* in tomato fields where *P. chlamydosporia* was applied.

The ability of the fungus to colonise the roots of the tomato might have contributed to high infection of RKN eggs by the fungus followed by reduction of the number of second stage juveniles and hence reduced galling in tomato roots. These findings relating to the ability of the fungus to reduce nematode population and root galling are consistent with previous reports by Atkins *et al.* (2003b) who noted a reduction in numbers of second stage juveniles from 35 to 6 and root galling from 4 to 2.5 due to application of *P. chlamydosporia.*

The population of the fungus and its efficacy on RKN in the glasshouse was not much different from that in the field. The small inconsistency can be attributed to differences in environmental conditions which could have influenced the populations of soil micro-organisms some of which are antagonistic to *P. chlamydosporia* (Kerry, 2000). Similarly, Kok *et al.* (2001) found that, infectivity of *P. chlamydosporia* to nematode eggs was hindered by antagonistic micro-organisms such as bacteria from the pseudomonad group.

Growing tomato in the second season after maize reduced of RKN and root galling and increased the number and yield of tomato fruits compared to plots where tomato was planted throughout the two seasons. The reduction of galling and increase in tomato yield as a result of rotation confirmed that maize is a poor host to RKN. Crop rotation with non-host crops is often adequate in maintaining nematode populations below economically damaging levels. Crop rotation with non host crops to nematodes has been widely promoted as an appropriate, effective and practical management option against RKN, among other plant pathogens. In vegetable production, the rotation of beans with grain crops such as maize, wheat, barley, rye, or oat has been reported to decrease severity and damage of several root pathogens in beans (Abawi and Widmer, 2000).

Crop rotation has been intensively studied with several crops. For instance, crop rotation was associated with reduction of plant parasitic nematode population and increased soybean yield after velvetbean (Vargas-Ayala and Rodríguez-Kábana, 2001). Although crop rotation has been proved to reduce soil-borne pathogens, many of the beneficial effects of crop rotations have also been attributed to their direct and indirect effects on the physico-chemical characteristics of soil as well as to promoting the activities of beneficial microorganisms (Sauerborn *et al.*, 2000).

Contrary to widespread reports, this study established that incorporation of maize stover alone has not much influence in managing RKN. Application of OA in the soil has been known to have variable effects on different nematodes. A similar study by Widmer and Abawi (2000) found that the incorporation of Sudan-grass as an amendment increased population of soil micro organisms such as bacteria, fungi and nematodes. Also, Kimpinski *et al.* (2003) noted increase in RKN populations in the soil after amending the soil with compost and manure. These can be due to the reason that, effects of organic materials to micro-organisms depends on soil type, source of the materials and the decomposition status (Widmer *et al.*, 2002).

However, adding maize stover in soil where fungus was applied in tomato during first season suppressed the numbers of J2 in the following tomato. Amending the soil with maize stover and integration of *P. chlamydosporia* may have a synergistic effect causing a decline in the numbers of the J2. Certain OA have been found to have nematicidal properties once incorporated into the soil. The mode of action of organic amendments leading to stimulation of free living nematodes is complex and dependent on the nature of the amendments (Kimpinski *et al.*, 2003). Degradation of organic amendments release NH4+, formaldehyde, phenols, and volatile fatty acids which individually or collectively stimulate build up of beneficial microbes, some of which are antagonistic to nematodes (Perez *et al.*, 2003). According to Jaffee (2002) addition of OA resulted in increased numbers of nematode trapping fungi (*Dactlellina hyptotyla*) that lead to decreased plant parasitic nematodes. The resulting nematicidal compounds together with the increased root fungal propagules may account for the suppression of RKN in the soil.

The study found no difference in yield when maize stover was applied together with *P. chlamydosporia* to when *P. chlamydosporia* or maize stover is applied alone. This finding is consistent with the report by Kimpinski *et al.* (2003) who reported increase in yield of potato and barley due to application of compost and manure as organic amendments into the soil. Increase in yield may be attributed to the release of important plant nutrients after mineralization of maize stover. According to Widmer (2002) decomposition of materials with C:N ratio less than 30 release available nutrients such as organic carbon, nitrogen, potassium, calcium and magnesium to plants and hence increase yield.

This study has demonstrated that maize, a poor host of RKN as a rotational crop has great potential in suppression of RKN in tomato production. In addition, there was increased suppression of the RKN when the crop rotation was used together with biological control agent *P. chlamydosporia.* Further studies need to be done to ascertain the most appropriate OA that promote population of *P. chlamydosporia* whilst suppressing RKN more than when used alone.

CHAPTER 9

CONCLUSIONS AND RECOMMENDATIONS

9.1 Introduction

The aim of this work was to determine the potential of the nematophagous fungus; *P. chlamydosporia*, as a biocontrol agent of root-knot nematodes under varying conditions. The thrust of the work was to develop a practical strategy of applying the fungus in small-scale vegetable production systems. The alternative substrates for use in cost-effective production of the fungus were identified. In addition, the environmental conditions that are likely to influence growth and infectivity of the fungus were elucidated. The potential of an integrated strategy incorporating *P. chlamydosporia*, organic amendments and crop rotation was ultimately evaluated under field conditions.

9. 2 Conclusions

The use of different species of *P. chlamydosporia* had established that isolates 10 and 392 were more effective and can be used as biocontrol agents. Screening these isolates in the laboratory and under glasshouse conditions showed that the two isolates are effective in the management of RKN under controlled conditions. These isolates and others were tested and compared to *P. lilacinus*, a commercial product, which is already on the market and proved to be effective.

This study identified that among the 14 different materials used, *M. pruriens*, maize cobs, *T. diversifolia*, *T. minuta*, goat manure, bean straw and maize stover can be used in the production of *P. chlamydosporia* inoculum. These materials produced chlamydospores

with viability percent of >50%. However, some materials such as *C. ochlroleuca* and chicken manure were able to produce chlamydospores but their viability was poor which makes them desire unacceptable for inoculum production.

Application of OA together with the fungus increased the population of the fungus and reduced the population of nematodes and galling index under glasshouse conditions. More time given for incubation of fungus and OA before planting resulted in increased in population of fungus. Also the longer RKN eggs were exposed to a suspension containing OA extracts and the fungus the greater was the percent egg infection. Percentage egg infection in extract from materials with low C:N ratio such as *C. ochloleuca* is lower than in extract from OA with high C:N ratio like sawdust.

Differences in factors such as soil temperature and pH and C:N ratio of soil and OA differed in support of fungal population that reduced nematode population. A temperature of 25°C for 30 days was optimum for decomposition of the OA with low C:N ratio before application of *P. chlamydosporia.* Moreover, it has been found that the effect of pH is minor in the ability of the fungus to parasitize RKN eggs. Also the process of egg infection by the fungus is lowered by increase in C content and raised when the level of C is decreased. High level of C/N ratio is associated with low egg infection while low C/N ratio is associated with high egg infection.

Integrating *P. chlamydosporia* with crop rotation and OA demonstrated that maize, a poor host of RKN, can be used as a rotational crop and has great potential in suppression of RKN in tomato production. In addition, the suppression of RKN population was more when the crop rotation was used together with biocontrol agent *P. chlamydosporia.* Other

advantage of adding the fungus to maize which is a poor host crop to RKN has increased the yield of the tomato crop up to 39t/ha from normal production which is <20t/ha.

9. 3 Recommendation for future work

1. To develop methods of storing chlamydospores and modes of application of the fungus should be undertaken.
2. The efficacy of the fungus in different fields and areas with varying soil conditions should be determined.
3. The potential of applying *P. chlamydosporia* together with other biocontrol agents like *P. lilacinus* should be explored with the aim of improving their efficacy.
4. Monitoring the residue effects of the integrated management with time.
5. More isolates of *P. chlamydosporia* should be screened in a continuous search for more effective isolates in managing nematodes.

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APPENDICES

Appendix 1. The content of different media

Media	Content
Corn meal agar (CMA)	17g CMA/litre distilled water
Potato dextrose agar (PDA)	39g PDA/litre distilled water
Sorbose agar plus antibiotics	12g technical agar/litre distilled water, 2g sorbose, 50mg streptomycin sulphate, 50mg chloramphenical, 50mg chlortetracycline
Semi-selective medium	17g CMA, 17.5 sodium chloride, 75mg rose bengal 50mg streptomycin sulphate, 50mg chloramphenical, 50mg chlortetracycline, 37.5mg thiabendazole, 37.5mg carbendazim, 3ml Triton X-100
Rice broth	40g rice/litre tap water
Water agar plus antibiotics	8g technical agar/litre distilled water, 50mg streptomycin sulphate, 50mg chloramphenical, 50mg chlortetracycline
Dilution medium (0.05% agar)	0.5g/litre distilled water
Media for *Paecilomyces lilacinus*	39g PDA/ litre distilled water, 10g sodium chloride, 28mg of PCNB, 50mg chlortetracycline 100mg streptomycin sulphate, 1ml tergitol
Yeast extract medium	0.125g/l litre distilled water

Appendix 2. Physical and chemical properties of the soil collected from Kabete Field Station.

Property	Content
Texture	Clay
pH in water	6.29
C (%)	2.32
N (%)	0.24
P (ppm)	31.67
K (Cmol/kg)	1.80
Na (Cmol/kg)	Trace
Ca (Cmol/kg)	8.25
Mg (Cmol/kg)	2.67
CEC (Cmol/kg)	21.40

Analyzed by Soil Science Department, Kabete.

Appendix 3. Nutrient analysis for 15 organic materials used in the study

SAMPLE	%N	%C	C:N	K 766.490PPM	P 213.617PPM
Bean straw	1.97	41.73	21.18	25,175.39	1,634.29
Cabbage	2.10	39.31	18.75	40,657.14	1,221.60
Chicken	2.52	31.05	12.30	11,809.56	18,398.04
Cowmanure	1.65	35.97	21.77	20,483.94	2,455.54
Crotalaria	4.54	45.98	10.13	19,505.99	2,943.80
FM	1.68	28.43	16.97	8,646.96	1,132.31
Goat	2.76	37.95	13.77	21,356.81	5,426.93
Maizecobs	0.59	47.93	81.22	9,821.42	843.44
Maizestover	1.54	43.85	28.53	17,630.81	1,420.85
Mucuna	2.99	44.56	14.93	19,073.38	2,435.33
Rice	1.61	44.90	27.91	621.65	991.35
Ricehusk	0.47	33.82	71.67	3,529.90	909.70
Sawdust	0.07	51.53	724.04	412.40	35.97
Tagetes	2.06	46.76	22.71	16,069.80	2,575.85
Tithonia	4.38	42.54	9.71	55,396.20	4,082.22

Analyzed by Department of Soil Science, Rothamsted.

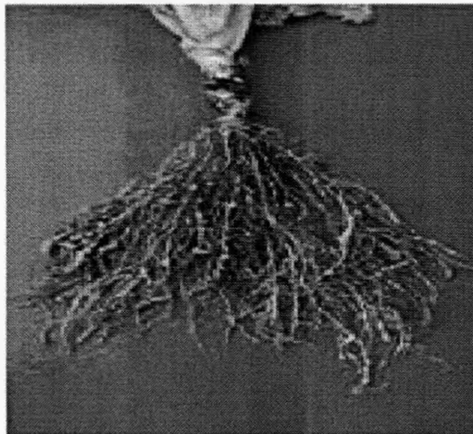
Appendix 4. Component and amount used to make different buffer mixtures used for pH.

0.2MNa2HPO4/ml	0.1MCitric acid/ml	pH measured
20.55	79.45	3.42
38.55	61.45	4.47
51.50	48.50	5.73
63.15	36.85	7.06
82.35	17.65	8.77

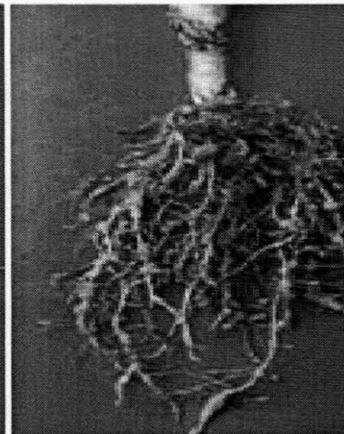
Appendix 5. Galling index key (From Coyne *et al*., 2007)

1. No galling damage.

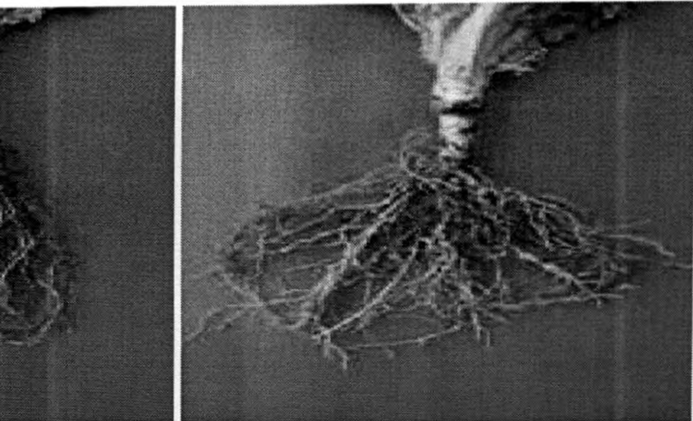
2. Slight galling.



4. Moderate galling.



5. Severe galling



3. Mild galling