OCCURRENCE OF PLANT PARASITIC NEMATODES IN COMMERCIAL PINEAPPLE FIELDS AND EFFECT OF BIOCONTROL AGENTS ON *Meloidogyne* SPECIES IN KENYA

BY

AGNES WARINGA KIRIGA (B.Sc)

A145/26321/2013

A thesis submitted in partial fulfilment of the requirements for the degree of Master of Science in Crop Protection (Plant Pathology) in the School of Agriculture and Enterprise Development, Kenyatta University

May, 2018

DECLARATION

I (Agnes Kiriga) declare that this thesis is my original work and has not been presented for the award of a degree in any other university or any other award.

Signature _____Date_____

Declaration by the supervisors

We confirm that the work reported in this thesis was carried out by the candidate under our supervision and has been submitted for examination with our approval as the supervisors.

Dr. George M. Kariuki

Department of Agricultural Science and Technology

Kenyatta University

Signature_____Date_____

Dr. Solveig Haukeland

Plant Health

ICIPE, International Centre for Insect Physiology and Ecology

Signature_____Date_____

Dr. Danny L. Coyne

International Institute of Tropical Agriculture (IITA)

ICIPE campus, Nairobi

Signature_____Date_____

DEDICATION

This thesis is dedicated to my dear husband Joseph K. Chege and our son Salmon Chege for their support, understanding, prayers and encouragement during the pursuit of this degree. To my parents for laying down the foundation of success and being a source of inspiration, to my brothers and sisters for their prayers and support and to my friends who stood by me and encouraged me during my study period at Kenyatta University. May the Lord bless you all.

ACKNOWLEDGEMENT

My sincere gratitude goes to my supervisors Dr. George M. Kariuki, Department of Agricultural Science and Technology, Dr. Solveig Haukeland, Department of Plant Health, ICIPE and Dr. Danny Coyne, IITA. Their mentorship, advice, suggestions, comments and general support are invaluable. My special credit goes to Dr. N. Van Beek for offering me a sponsorship for this project which was a big step to actualizing my dreams and career development. To Dr. Christiaan Kooyman, your unwavering support and guidance was key to the success of this work.

Special thanks to all the staff at the Department of Agricultural Science and Technology and the School of Agriculture and Enterprise Development for giving me the opportunity to pursue the Masters program. I appreciate the technical assistance granted me at ICIPE. I also highly appreciate the support accorded to me by my colleagues and friends especially at the Department of Nematology, ICIPE. My appreciation also goes to APU Laboratory colleagues and friends for their technical input in the management of fungi. Thank you so much Jane Kimemia and Oliver Chitambo, you will remain an integral part of this research.

I am forever indebted to my family particularly my husband for his moral and financial support. My heartfelt gratitude also goes to my son Salmon Chege for the understanding and cooperation during my research. I am also grateful to my entire family for the encouragement during the research journey.I am grateful to God without whose blessings, protection and providence this work would never have seen the light of day. Thank you to all who supported me in one way or another. God bless you all.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF PLATES

ABBREVIATIONS AND ACRONYMS

ANOVA	Analysis of Variance
BCA	Biological Control Agents
FAO	Food and Agricultural Organization
HCDA	Horticultural Crops Development Authority
IJ2s	Infective Juveniles
J2s	Second Stage Juveniles
KBL	Kenya Biologics Limited
LSD	List Significant Difference
MWP	Mealybug Wilt of Pineapple
µm	Micrometer
NaOCl	Sodium Hypochlorite
PCPB	Pest Control Products Board
PPN	Plant Parasitic Nematodes
RKN	Root Knot Nematodes
USDA	United States Department of Agriculture

ABSTRACT

Plant-parasitic nematodes, in particular *Meloidogyne* spp., cause significant yield reduction in commercial pineapple (*Ananas comosus)* worldwide. In Kenya limited studies have been conducted on nematodes in pineapple although the main commercial producer in Kenya has sole mandate to use Telone II (1, 3-Dichloropropene) indicating the seriousness of the problem. The current study was conducted to provide an update on the occurrence of plant-parasitic nematodes in commercial pineapple farms and to evaluate the effect of selected biocontrol agents on *Meloidogyne* species. Sampling was done in Delmonte and Kakuzi farms in Kiambu and Murang'a Counties respectively. Soil and root samples were collected from different stages of the crop. The top 5 cm of the soil was removed and 25 root and soil sub-samples were collected from randomly selected locations in each pineapple field. After extraction using modified Baermann technique, the recovered vermiform nematodes were counted and identified to genus level using morphological features including molecular techniques for the *Meloidogyne* species. The remaining soils were baited for *Meloidogyne* with pineapple plants cv. Smooth cayene to obtain nematode inoculum for the biocontrol experiments. Greenhouse experiments on rooted pineapple crowns were conducted to evaluate the efficacy of three isolates of *Trichoderma* spp. *Trichoderma asperellum* M_2RT_4, *T. atroviride* F_5S_{21}, *Trichoderma* sp. MK_4 and two isolates of *Purpureocillium lilacinum* KLF_2 and MR_2 against *Meloidogyne* spp. in pot experiment. Data on the occurrence of PPNs was analyzed using two-way analysis of variance (ANOVA) while one on effect of biocontrol agents against *Meloidogyne* spp. was analyzed using one-way ANOVA. All the means were separated using Tukey-HSD at $P \leq 0.05$. There was widespread distribution of plant parasitic nematodes and the most frequently occurring were *Meloidogyne* spp., *Helicotylenchus* spp. and *Tylenchus* spp. both in soil and root samples. More nematodes were recovered from older fields, 24 and 36 months after planting, 4380 and 3260 nematodes per 100mls soil respectively than in younger fields, fallow and 3 months old, (2919 and 1687 nematodes per 100 mls soil respectively). All the three *Trichoderma* isolates successfully colonized pineapple roots endophytically. The application of two isolates of *Trichoderma* (*T. asperellum* M_2RT_4 and *Trichoderma* sp. MK_4) individually and the two isolates of *P. lilacinum* significantly reduced nematode egg and egg mass production reducing root galling damage by up to 60.8% and increased the plant root mass growth compared to the untreated control. *T. asperellum* M2RT4 most effectively reduced galls, egg mass and eggs, by 81.8, 78.5 and 88.4%, respectively. Treatment with *T. asperellum* M_2RT_4 increased root fresh weight by 91.5%, *Trichoderma* sp. MK_4 by 63.8%, *T. atroviride* F_5S_{21} by 50.0%, *P. lilacinum* KLF_2 by 43.8% and MR_2 by 32.3%. *Trichoderma atroviride* F_5S_{21} application, however, had no significant effect on nematode multiplication or root damage compared to the control. Results indicate that both *Trichoderma* spp. and *P. lilacinum* isolates directly and indirectly affected nematode reproduction (eggs counts and egg masses) and host response (host growth and root galling), demonstrating their control potential against *M. javanica* on pineapple. The results provide alternative options for managing *Meloidogyne* spp. that are more environmentally sensitive and can be combined with other management methods towards more sustainable pineapple production systems.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background information

Pineapple (*Ananas comosus*) is an important fruit crop ranking third, after banana and citrus, and contributes over 23% of the global production of tropical fruits (Kormelinck and Janssen, 2012; FAOSTAT, 2015). Pineapple production is concentrated in the tropical regions of the world in over 82 countries (Ndungu, 2014).

The main pineapple producers in the world are Brazil, Costa Rica, Philippines and Thailand commanding nearly 50 % of the total output (UNCTAD, 2016). Other important producers are India, China, Nigeria, Kenya, Mexico and Indonesia (Kormelinck and Janssen, 2012; FAOSTAT, 2015). According to FAO, over 2.1 million acres are put under the crop worldwide (Pay, 2009) with an annual production of over 23% (24.8 million tons) of the global tropical fruit production (FAOSTAT, 2015; Kormelinck and Janssen, 2012; UNCTAD 2016). In Kenya, pineapple is mostly cultivated on large scale commercial plantations using cv. Smooth Cayenne by farms like Delmonte in Kiambu (Thika), Kakuzi in Murangá and Ndemo farm based in Kilgoris, Narok Counties. However, smallholder farmers are increasingly turning to pineapple production for both home consumption and commercial purposes (USAID 2005; HCDA 2008; Koech *et al*., 2014).

Plant-parasitic nematodes have been described as important pests of pineapple causing significant reduction in yield worldwide. The most important are root-knot nematodes (*Meloidogyne javanica* and *M. incognita*) (Rohrbach and Apt, 1986) alongside

Rotylenchulus reniformis (reniform nematode) and *Pratylenchus brachyurus* (lesion nematode) (Stirling, 1993; Gianessi, *et al*., 2002; Sipes *et a*l., 2005; Daramola and Afolami, 2014). *Meloidogyne* spp. are highly adapted obligate plant parasites with a life cycle of 21-25 days in the tropics. The second-stage juveniles (J2s) penetrate and develop in the roots modifying the cells of the plant roots and make feeding sites causing formation of root-knots or galls (Jones *et al*., 2013), which interfere with nutrient and water uptake in the plant.

In Kenya, the root-knot disease is more pronounced because the country is located in the tropical region, where the environment favors nematode reproduction and survival throughout the year (De Waele and Elsen, 2007; Kariuki *et al*., 2010). They are also difficult to control due to their polyphagous nature, ability to reproduce fast and form multiple generations within a short time, and their endoparasitic nature (Trudgill and Blok, 2001; Manzanilla-Lopez *et al*., 2004). The availability of susceptible pineapple crops cultivated continuously in the same field often aggravates the problem. Under commercial production systems synthetic chemical pesticides (e.g. 1, 3-Dichloropropene-Telone II) are widely used to manage these pests (Stirling and Pattison, 2008; Daramola and Afolami, 2014). However, environmental and human health concerns regarding the use of nematicides have led to increased interest in alternative strategies that are more environmental friendly (Singh *et al*., 2012).

1.2 Biological control of *Meloidogyne* spp.

Biological control is broadly defined as the use of living organisms or their metabolites to reduce the population density or disease impact of a specific pest organism (Sikora *et al*., 2003; Lamovšek *et al*., 2013). Biological control agents (BCAs) have shown promise as

both an economical and ecological approach to reducing pest damage (Singh *et al*., 2012). Well-known antagonists of *Meloidogyne* spp. are soil borne fungi from the genera *Trichoderma* (Sharon *et al*., 2007), *Paecilomyces* (Kumar *et al*., 2009), *Pochonia* and *Arthrobotrys* (Lamovšek *et al*., 2013). *Arthrobotrys* spp. are nematophagous fungi that form hyphae with adhesive structures to capture and feed on nematodes (Lamovšek *et al*., 2013). *Arthrobotrys dactyloides* reduced the number of *M. incognita* when applied before planting tomato seedlings (Kumar and Singh, 2006). *Pochonia chlamydosporia* is one of the extensively studied biological control agents (BCA) against plant parasitic nematodes (Van Damme *et al*., 2005; Siddiqui *et al*., 2009; Manzanilla-Lopez *et al*., 2013) and is known to parasitize on the eggs and/or females of *Meloidogyne* spp. (Hallmann *et al*., 2009; Lamovšek *et al*., 2013).

Biocontrol activity of *Trichoderma* spp. against plant pathogens occurs through various mechanisms: induced resistance in the host plant, antibiosis, competition, direct parasitism and enzymatic hydrolysis (Elad and Freeman, 2002; Howell, 2003; Harman *et al*., 2004). Moreover, these fungi may also promote plant growth (Yedidia *et al*., 1999; Sharon *et al.,* 2001). *Trichoderma* spp. have been used to successfully suppress juvenile (J2) densities and egg production of *Meloidogyne* spp. in tomato roots by parasitism (Sharon *et al*., 2007). The fungus *Purpureocillium lilacinum*, previously called *Paecilomyces lilacinus* (Lopez-Lima *et al*., 2014) is a saprophyte that parasitizes stationary stages of nematodes, particularly their eggs that are usually deposited in a gelatinous matrix, and female nematodes (Mukhtar *et al*., 2013). It has attracted more attention because of its high potential for the biological control of nematodes and has been successfully used against *Meloidogyne javanica* and *M. incognita* on tomato (Van

Damme *et. al*., 2005). This fungus can saprophytically survive well in the rhizosphere, is relatively easy to mass-culture and more effective in infecting because their host is sessile (Bishop, *et al.*, 2007). To date, neither of these BCAs has been tested or used in pineapple production fields.

If soil fungi are to be used successfully as BCAs, they must be able to establish and survive in the soil, and to enable this, the fungus must be compatible with the host cultivar (Al-Hazmi and TariqJaveed, 2016). Once a BCA has established it should be able to persist in the rhizosphere and readily colonize the formed roots, and hence compete for space and nutrients (Howell, 2003; Sariah *et al*., 2005). A BCA cannot compete for food and space if it cannot establish in the rhizosphere and grow readily along with the formed root system of the treated plant (Harman 2000, Howell *et al.*, 2000). *Trichoderma* spp. has demonstrated ability to colonize the root surface of plants. This has been linked to its successful suppression of root knot disease, (Yedidia *et. al*., 1999; Sharon *et al*., 2001; Siddiqui and Shaukat 2003) and competition with nematodes (Harman *et al*., 2004). To enhance maximum root colonization once the seedling is transplanted, Van Damme *et al*. (2005) and Dababat and Sikora (2007) recommended that *Trichoderma* spp. be applied before planting the crop. For pineapple, crowns, suckers or slips are inserted into the soil to act as the "seedling" material (Rohrbach and Apt, 1986). Roots are produced after three to four weeks, but to our knowledge, no studies have been done on the ability of *Trichoderma* spp. to colonize these developing roots.

The egg-pathogenic fungus *P. lilacinum* has been widely tested for the biological control of plant-parasitic nematodes (Atkins *et al*., 2005) and has shown significant success against *Meloidogyne* spp. (Siddiqui *et al*., 2000; Khan *et al*., 2006a; Oclarit and

Cumagun, 2009; Mukhtar *et al*., 2013). Diverse mechanisms have been suggested for the biological activity of *P. lilacinum* against parasitic nematodes, with direct parasitism of the egg stage (Kiewnick and Sikora, 2006) and females (Holland *et al*., 1999) after the formation of appressoria being the main mechanisms of action. The production of proteases and chitinases by the fungus is associated with the infection process (Khan *et al*., 2004; Kiewnick and Sikora, 2006). The enzymes dissolve the vitelline layer of eggshell; hence the fungal hyphae are able to penetrate, grow inside the eggs and destroy the embryonic developmental stages at an early stage. Once the egg contents are depleted, the hyphae penetrate the eggshell and proliferate to parasitize other eggs within the egg mass (Khan *et al*., 2006b; Mukhtar *et al*., 2013).

This study investigated the occurrence of PPNs in commercial pineapple farms of Kenya, and the potential use of two BCAs, *Trichoderma* isolates (*T. asperellum* M2RT4, *T. atroviride* F5S21, *Trichoderma* sp. MK4) and *Purpureocillium lilacinum* isolates KLF2 and MR2, on *Meloidogyne* sp. affecting pineapple in Kenya.

1.3 Statement of the problem and justification

Plant parasitic nematodes are devastating to pineapple, reducing total yields and altering fruit size. They have been described as important pests of horticultural crops (Stirling and Pattison, 2008) with *Meloidogyne* spp. causing significant reduction in pineapple yield (Gianessi *et al*., 2002; Sipes *et al*., 2005). Damage to pineapple roots by *Meloidogyne* spp. is amplified by the non-regenerative nature of the roots once infected and damaged (Rohrbach and Apt, 1986).

In commercial production of pineapple, nematicides have been widely used to manage *Meloidogyne* spp. However, nematicides pose serious threat to the ecosystem (Sharma and Pandey, 2009), leading to most countries banning their use due to these adverse effects (Li *et al*., 2011; Singh *et al*., 2012). Environmental and human health concerns regarding nematicide use against *Meloidogyne* spp. has led to an increased interest to explore alternative strategies which are environmental friendly. Biological control has shown promise as an economically and ecologically friendly approach to reduce pest damages (Davies *et al*., 1991; Hussain *et al*., 2011; Singh *et al*., 2012). Several organisms are known to be antagonistic to plant parasitic nematodes. It has been demonstrated in several soils that nematophagous fungi increase under some perennial crops and under those grown in monocultures, and so may control some nematode pests, including *Meloidogyne* (Stirling, 2011). Although biological control is a potential component of nematode management program in pineapple, currently, this component is lacking in commercial pineapple plantations. This study therefore explores the occurrence of plant parasitic nematodes in commercial pineapple farms in Kenya and the effect of *Trichoderma* spp. and *Purpureocillium lilacinum* on the *Meloidogyne* spp. present. This is important since there are no previous reports on use of BCAs on management of *Meloidogyne* spp. in Delmonte and Kakuzi commercial pineapple plantations.

1.4 Objectives

1.4.1 Broad objective

To determine the occurrence of plant parasitic nematodes in commercial pineapple productions fields and evaluate the effect of selected biocontrol agents and antagonists on *Meloidogyne* spp.

1.4.2 Specific objectives

i. To determine the occurrence of plant parasitic nematodes in commercial pineapple fields at Delmonte and Kakuzi.

ii. To identify and characterize the *Meloidogyne* species present in Delmonte and Kakuzi pineapple fields.

iii. To evaluate the colonization potential of *Trichoderma* spp. on roots of pineapple.

iv. To evaluate the efficacy of isolates of *Trichoderma* spp. and *Purpureocillium lilacinum* on the *Meloidogyne* sp. from pineapple fields.

1.5 Research hypotheses

1. There is significant high occurrence of PPNs in Delmonte and Kakuzi pineapple commercial fields.
2. *Meloidogyne* species are present in Delmonte and Kakuzi pineapple commercial fields.
3. *Trichoderma* spp. effectively colonizes the roots of pineapple plants.
4. *Meloidogyne* spp. from Delmonte and Kakuzi pineapple commercial fields are suppressed by isolates of *Trichoderma* spp. and *Purpureocillium lilacinum*.

1.6 Significance of the study

This study aims at surveying Delmonte and Kakuzi pineapple commercial farms to establish the occurrence and distribution of PPNs and evaluate the biocontrol potential of isolates of *Trichoderma* spp. and *Purpureocillium lilacinum* against *Meloidogyne* spp. on pineapple. This is important since no study has been done on plant parasitic nematodes occurring in commercial pineapple production farms in Kenya. The study is relevant

since it helps to reduce the use of toxic nematicides (e.g. 1, 3-Dichloropropene-Telone II), which are linked to health and environmental problems. This study therefore describes the effectiveness of two microbial antagonists, *Trichoderma* and *P. lilacinum* isolates which are specific BCAs on *Meloidogyne* spp. affecting pineapple in Kenya as an alternative to chemical nematicides.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Pineapple production

Pineapple (*Ananas comosus*) is a perennial crop, vegetatively propagated using crowns (the fruit top), suckers (formed at the base of the trunk or obtained from plantlets that develop between the leaves of the declining "mother" plant) or slips (formed underneath the fruits) (Kormelinck and Janssen, 2012) (Fig. 2.1). The type of the vegetative material determines the initial development of the root system and the duration of the first crop cycle, which usually varies between 12 and 24 months, depending on cultivars and temperature (Coppens d'Eeckenbrugge *et al*., 2011). After first harvest (plant crop), crowns and slips can be replanted or suckers may be left on the plant, providing new growth axes, ratoon crop, for a further production cycle (Coppens d'Eeckenbrugge *et al*., 2011). The latter takes shorter time, as the plant is already established; however, fruit size is reduced and less uniform, so production is limited to two or three crop cycles after which the root system may no longer be functional making the production uneconomical (Rohrbach and Apt, 1986; Bartholomew *et al.* 2003; Coppens d'Eeckenbrugge *et al*., 2011). The declining plant material is therefore uprooted and incorporated back to the soil to decompose and provide organic matter (Rohrbach and Apt, 1986).

The pineapple is generally planted on low ridges or beds favourable to drainage and planting operations. The average spacing between plants is 25 to 30 cm, and the row separation approximately 80 cm. Spacing however, can vary according to planting density, which ranges from 50 000 to 70 000 plants/hectare (UNCTAD, 2016).

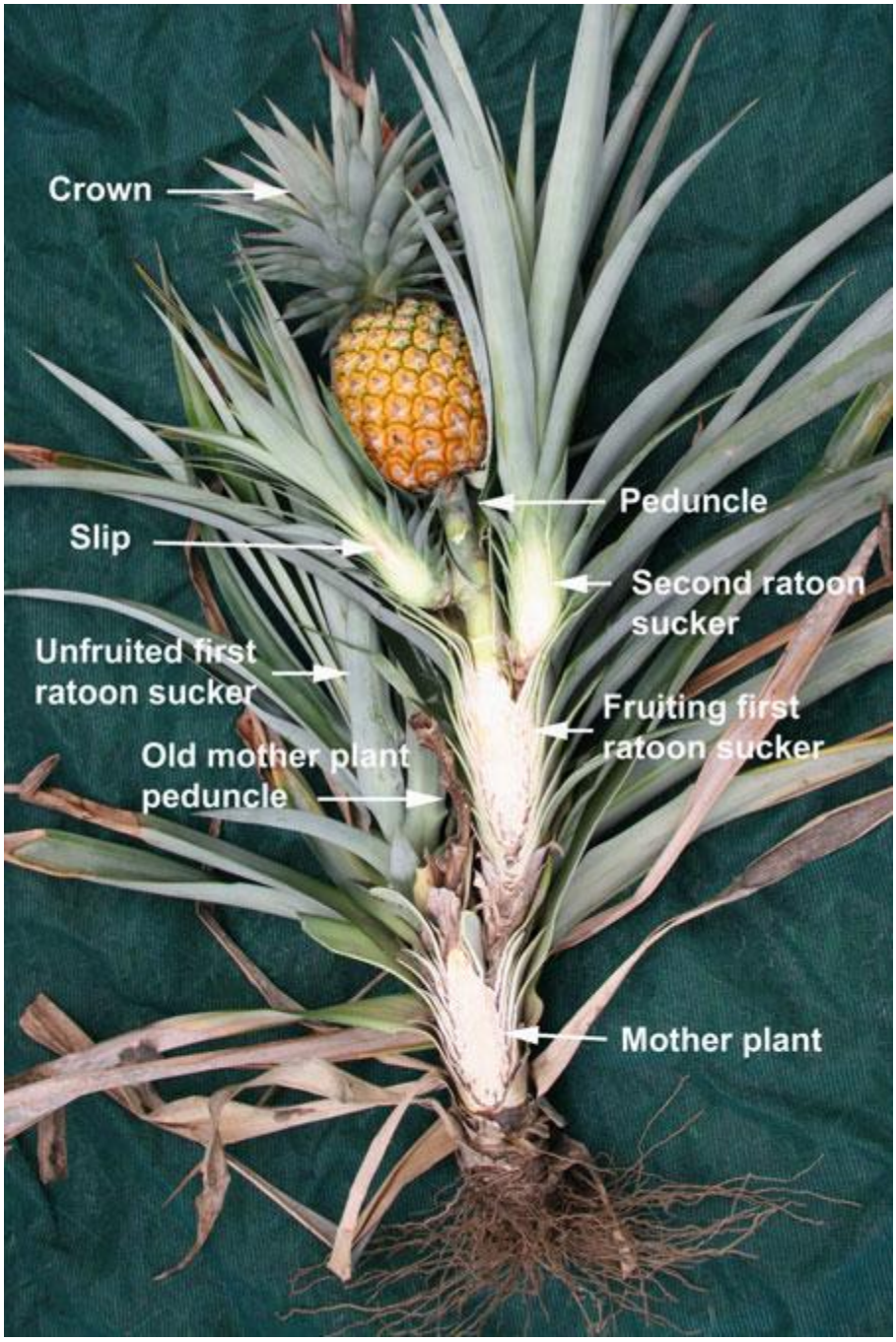

Figure 2.1:Structure of a pineapple plant (A. comosus var. comosus); showing the different types of planting materials (sucker, slip, and crown) (photograph courtesy Garth Sanewski, 2009)

2.1.1 Pineapple varieties/cultivars

Several varieties have been adopted by different countries including Smooth Cayenne, Queen, Red spanish and MD2 (Ndungu, 2014). The life cycle of pineapple takes up to 22 months. The flowering happens after attaining vegetative growth of 11-12 months after planting (Bartholomew *et al*., 2003). The first harvest (plant crop) is done around 18th month and the second harvest from ratoon crop at the 22nd month (Bartholomew *et al*. 2003). In commercial plantations, pineapple is generally produced as a monoculture with its roots originating adventitiously (Rohrbach and Apt, 1986). The plant is a xerophyte and survives well throughout the year including during drought periods (Sipes *et al*., 2005). However, a well distributed annual rainfall of at least 1000 mm and medium altitudes of 1350-1750 m above sea level are essential for it to produce well. At high altitudes growth is slow and the fruit contains much acid. Deep sandy loams with high organic matter are ideal. Other soil types can be used as long as they are not water logged and organic matter is added (Sipes *et al.*, 2005; Kormelinck and Janssen, 2012). The above and other characteristics contribute heavily to disease and nematode problems (Sipes *et al.*, 2005).

2.1.2 Economic importance of pineapple

Pineapple is cultivated predominantly for its fruit that is consumed fresh or as canned fruit and juice. Pineapple is the only source of bromelain, a complex proteolytic enzyme used in the pharmaceutical market and as a meat-tenderising agent (Bartholomew, *et al*., 2003). The stems and leaves of pineapple plant are also a source of fibre that is white, creamy and lustrous as silk. Pineapple fibre has been processed into paper with remarkable qualities of thinness, smoothness and pliability (Montinola, 1991). Parts of

the plant are used for silage and hay for cattle feed. Processing wastes in the form of shell, core materials and centrifuged solids from juice production are also used as animal feed. Alcoholic beverages can also be made from juice.

Most of the world production (about 70%), and most of the canned pineapple (about 95%), comes from the cultivar 'Smooth Cayenne'. Since 'Smooth Cayenne' does not provide the best quality fresh fruit all year round, there is pressure on distributors/growers to switch to cultivars with superior quality fresh fruit than 'Smooth Cayenne' (Sanewski and Scott, 2000).

According to Sipes *et al.* (2005), Kenya is one of the main exporters of the 10 % of pineapples that Africa produces in the world. Some reports indicate that pineapple plants infected by *M. javanica* can present symptoms of nutritional deficiency, low uptake to fertilizers, yellowish leaves, wilted leaf tips and stunted fruits (Sanches, 2005; Sipes *et al*. 2005; Lacerda *et al*., 2009).

2.2 Production constraints of pineapple

2.2.1 Pests and diseases

2.2.1.1 Pests of pineapple

The pineapple plant is affected by a wide variety of pests and diseases, with plant parasitic nematodes (PPNs) and mealybug wilt of pineapple (MWP) being the main problems (Sipes *et al.* 2005; Lacerda *et al*. 2009). Common pests infesting vegetative propagules are mealybugs, scale and pineapple red mites. Symphylans are small pseudo-millipedes which feed on the pineapple root tips, disrupting nutrient absorption by the

plant. Scale insects are primarily found on the foliar and their multiplication causes reduced plant photosynthesis, thereby slowing down its growth. They can also be found on fruits, which degrade their commercial quality. Mites are found on the above ground parts. They suck juice from the foliar thus reducing the rate of the plant growth. They also act as vectors of some diseases.

2.2.1.2 Diseases of pineapple

The pineapple plant is affected by a wide variety of diseases, with mealybug wilt of pineapple (MWP) being the main problem (Lacerda *et al*. 2009). Other diseases are fungal borne and among them are heart rot, root rot, fruit rot and butt rot which are major problems when handling, storing or planting fresh materials. Phytophthora is a fungal disease attacking both the plant and fruit. It damages the roots and spreads to the leaves which change colour from green to yellow, and then pink-red. *Penicillium* sp. and *Fusarium* sp. are fungal disease mainly affecting the fruit. Dark yellow or brown to black spots develop inside the fruit. The disease is mainly transmitted from plant to plant or from field to field by vectors like mites and scale insects. Yellow spot; pineapple yellow spot is caused by a strain of spotted wilt virus transmitted from host weeds by onion thrips, *Thips tabaci*. Infection occurs most frequently on young crowns still on the fruit or during the first few months after planting.

2.2.2 Plant parasitic nematodes

2.2.2.1 Diversity of PPN

More than 100 species of plant parasitic nematodes have been associated with pineapple root system (Sipes *et al*., 2005), among them being *M. javanica*, *Pratylenchus*

brachyurus, *Rotylenchulus reniformis*, *Helicotylenchus dihystera* and *Paratylenchus minutes* (Stirling, 1993). *Pratylenchus brachyurus* and *R. reniformis* have been reported to cause major losses while *H. dihystera* and *Criconemella ornata*, are also common on pineapple fields, although their pathogenity is unknown (Raski and Krusberg, 1984). In Nigeria, attacks by populations of *M. incognita*, *Pratylenchus* spp., *H. multicinctus and Scutellonema clathricaudatum* were identified as major causes of poor yield and reduced sucker production in pineapple (Babatola, 1985).

Surveys in the main production areas of Brazil have indicated that *M. javanica* and *Rotylenchulus reniformis* are among the most frequent and damaging PPNs (Cavalcante *et al*., 1984; Manso *et al*. 1994; Costa *et al*. 1998). Some reports demonstrated that *R. reniformis* caused yield losses which varied from 60 to 74 % in the first harvest and around 40% in the second ("ratoon") harvest (Costa and Matos, 2000; Sipes *et al*., 2005).

2.2.2.2 Root-knot nematodes

Meloidogyne spp. are polyphagous, sedentary root endoparasites. Species such as *M. javanica* and *M. incognita* are among the major limiting factors in the production of field and plantation pineapple crop (Daramola and Afolami, 2014). *Meloidogyne* second-stage juveniles (J2s), which penetrate and develop in the pineapple roots, induce changes in the host plant that lead to the formation of giant cells and galls. *Meloidogyne* spp. are difficult to control because of their wide host range, short generation times, high reproductive rates and endoparasitic nature (Manzanilla-Lopez *et al*., 2004).

Moyle and Botella (2014) found that *M. javanica* infection life cycle in pineapple takes 10 weeks with the knots appearing on the roots at approximately 4-5 weeks and the

production and release of egg outside the roots surface occur at week 10 after inoculation. Rohrbach and Apt (1986) cited that damage by *Meloidogyne* spp. pose a major limitation to pineapple production resulting in significant losses. The Hawaii pineapple industry suffered a great decline in the world as a result of damage by *Meloidogyne* spp. and the number of pineapple growers also declined reducing acreage from 61000 to 19000 (USDA 2003).

Caveness (1965) found *Meloidogyne* spp. in association with the roots of pineapple plants in parts of Nigeria, while Babatola (1985) identified populations of *M. incognita, Pratylenchus* spp., *H. multicinctus* and *S. clathricaudatum* attacking pineapple and causing poor yield and reduced sucker production. Stirling (1993) reported that in Queensland pineapple fields of Australia, *M. javanica* is the most damaging of all nematodes. Reports have also shown that *M. javanica* can cause a reduction of 10 % in the development of pineapple plants (Costa and Matos, 2000; Sipes *et al*., 2005).

2.2.2.2.1 Life cycle of RKN

Eggs of *Meloidogyne* spp. are enclosed in gelatinous egg sacs usually deposited on the surface of the galled roots and sometimes within the galls. The infective second stage juveniles (J2) hatch from the eggs (hatching is temperature dependent) and moves into the soil to search for the host root. When a suitable host root is reached the juveniles invade the root tissues towards the vascular cylinder by forcing their stylet through the root surface and producing enzymes for cell wall degradation (Abad *et al*., 2003; Curtis *et al*., 2009). After three further molts females swell, become globose and sedentary at maturity and induce the formation of five to seven giant, multinucleate cells (Gheysen and Fenoll, 2002), upon which the developing nematode feed (Fig. 2.2). Males remain

vermiform and leave the root. After maturation females lay eggs within a gelatinous matrix which are then deposited on the surface of the root gall (Gheysen and Fenoll, 2002; Karssen *et al*., 2013).

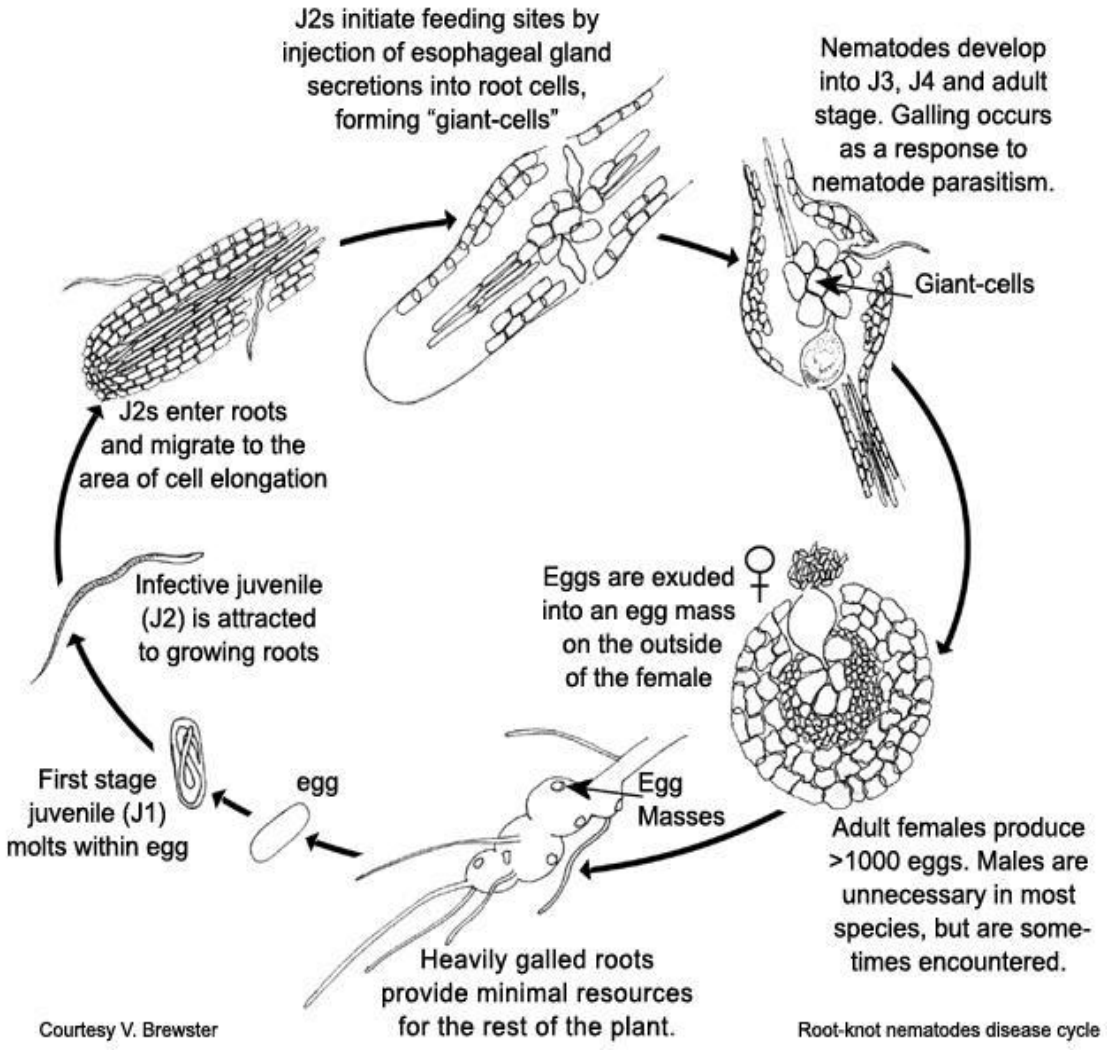

Figure 2.2: The life cycle of the *Meloidogyne* spp. Courtesy V. Brewster (Mitowski and Abawi, 2011)

2.2.3 Management of RKN

2.2.3.1 Cultural practices

Identification of *Meloidogyne* spp. is the first step in deciding the most suitable control measure (Adam *et al*., 2007). Different methods have been developed for managing the effect of *Meloidogyne* spp. on crop yield (Lichtfouse *et al*., 2009) with a number of these techniques being approved for their efficacy (Karssen *et al*., 2013). According to Coyne *et al*., (2009) use of crop rotation with resistant crops and integration of fallow periods were proven effective in managing nematode infection and populations. However, rotation alone is not effective due to the wide host range of *Meloidogyne* spp. (Aubertot *et al*., 2006). According to Thakur (2007), use of resistant plant cultivars has remained a challenge despite the fact that it is environmentally safe, due to emergence of resistance-breaking *Meloidogyne* spp. (Aubertot *et al*., 2006).

Soil flooding has also been used to reduce the density of nematodes in rice cultivation (Rhoades, 1982; Duncan, 1991). However, soil flooding method is not applicable in pineapple and other vegetable production due to the nature of the soil and the agronomic changes caused in soil e.g. lack of oxygen, soil structure degradation that might alter the overall production (Collange *et al*., 2011).

Many previous studies have focused on the use of organic amendments such as animal and green manure, neem (*Azadirachta indica*), *Tithonia diversifoliar* e.t.c as a control measure to *Meloidogyne* spp. showing suppressive effects (Akhtar and Malik, 2000; Waceke, 2002; Oka, 2010; Thoden *et al*., 2011). Although the efficacy of these products under controlled conditions is commonly recognized, results in field conditions are rather inconsistent (Abawi and Widmer, 2000). Some studies have reported no significant effect

of compost on nematode control (Szczech *et al*., 1993; McSorley *et al*., 1997). Thoden *et al*. (2011) reviewed several studies in which *Meloidogyne* populations increased after the application of organic amendments. Moreover, nematode control requires a large amount of organic amendment and therefore, it is quite expensive (Noling and Becker, 1994) and relatively difficult to implement especially in commercial pineapple production.

2.2.3.2 Chemical nematicides

From the beginning chemical nematicides have been widely used in managing *Meloidogyne* spp. (Luc *et al*., 2005; Tariq, 2008). To date, chemical nematicides remain the primary means of managing PPN in pineapple. Although chemical nematicides are effective (Tariq, 2008), they are usually expensive, of limited availability, difficult to store, pollute the environment and also lose their efficacy after prolonged use (Abawi and Widmer, 2000; Luc *et al*., 2005). Moreover, many effective nematicides are highly restricted in many countries due to their adverse effects on health and environment.

Many pineapple farm rely on highly hazardous nematicides with active ingredients ethoprophos or oxamyl, which are extremely toxic to humans and known to harm non-target soil organisms (Diepens *et al*., 2014). Ethoprophos is one of the commonest causes of acute poisoning in Costa Rica and found to contaminate surface water and drinking supplies. Mocap nematicide has been used in the control of PPN in perennial crops. However, they have adverse effects to the environment such as depleting the ozone layer (UNEP, 1995). Nematicides have a negative effect on the populations of beneficial antagonistic micro-organisms in the soil (Hasabo and Noweer, 2005). Furthermore, they are costly and unaffordable to the small scale rural resource-poor farmers (Renco and Kovacik 2012).

Delmonte farm heavily depends on use of fumigants, 1, 3-Dichloropropene- Telone II (Dow AgroSciences) in the fallow fields before planting to manage soil and plant parasitic nematodes (Salim personal communication, 2017). Telone II is a highly restricted chemical used in Delmonte commercial pineapple farm under regulation by Pest Control Products Board (PCPB) in Kenya (Ndungu, 2014). At planting the pineapple crowns are treated with fungicides and insecticides for control of mealybugs. Di-Ammonium Phosphate (DAP) is used in the nursery to enhance rooting and later NPK fertilizers applied to enhance crop growth. After the first harvest, the crop is drip irrigated with Oxamyl (Vydate) (DuPont de Nemours South Africa (Pty) Ltd) to reduce nematodes. After the second harvest, the fields are left fallow for 5-6 months. Unlike Del monte, Kakuzi farm depends on use of amore environmental friendly phytoprotect nematicide- Unrefined crude sesame Oil (Sineria Holland) during fallow periods to control nematodes and other soil pests. After the second harvest the fields are left fallow for 6 months.

Thus the development of alternative control strategies and long-term integrative approaches is urgently needed to replace chemical nematicides (Martin, 2003). There is therefore need to develop sustainable nematode management strategies to increase crop yield and crop quality while reducing reliance on nematicides (Sikora and Fernandez, 2005). This has led to an increased interest in use of biological control in order to obtain more environmentally safe methods of reducing nematode damage.

2.2.3.3 Biological control

Different antagonistic organisms such as soil fungi from the genera *Trichoderma* (Hypocreaceae), *Verticillium, Pochonia* and *Paecilomyces* (now *Purpureocillium*) have

been tested against *Meloidogyne* spp. (Sharon *et al*., 2001; Meyer and Roberts, 2002; Kerry and Hidalgo-diaz, 2004; Regaieg *et al*., 2011; Samuels *et al*., 2012), with only a few of them being developed into commercial products for use in the field (Backman *et al*., 1997). Stirling (1991) found that some fungal biocontrol agents' mode of action to plant parasitic nematodes was direct pathogenicity, some produce substances that inhibit nematode egg hatch or kill nematode juveniles (Khan and Saxena, 1997; Nitao *et al*., 1999), some degrade signalling compounds to which nematodes are attracted to, some induce plant resistance and some produce antagonistic microbes to nematodes (Ashraf and Khan, 2010).

There are a few challenges that limit the commercial use of biocontrol agents including: their inconsistent performance in the field, some affect narrow range of soil pests; some act slower to the pests than pesticides; and they are more expensive to produce than existing chemical products (Meyer and Roberts, 2002; Siddiqui and Shaukat, 2004). Attempts have been made to control *Meloidogyne* spp. using antagonistic bacteria and fungi (Khan *et al*., 2008).

2.2.3.3.1 *Trichoderma* spp.

According to Meyer *et al*. (2001) and Harman *et al*. (2004), *Trichoderma* spp. cause increased plant growth and have the ability to colonize root system, preventing the nematodes from accessing the plant and inducing systemic resistance to nematodes. Spiegel and Sharon (2005) reported that there is little known about the fungal mechanisms of actions against nematodes and widening the understanding of these mechanisms could lead to the development of improved biocontrol application methods and selection of effective isolates. Sharon *et al*. (2001) reported that tomato plants treated

with the biocontrol agent (T-203) and grown in nematode infested soil exhibited a drastic reduction in root galling when compared to the control.

The conidia of *Trichoderma* spp. attach to nematode cuticle or to egg shell and parasitize them (Sharon *et al*., 2007). Protease production by *T. harzianum* has also been associated with biocontrol of the root-knot nematode *M. javanica* on tomato plants. Dababat *et al*. (2006) recommended that the *Trichoderma* spp. should be applied to the soil before crop planting to completely colonize the root.

Several reports showed successful suppression of root knot disease by *Trichoderma* spp. (Rao *et al*., 1996; Spiegel and Chet, 1998; Sharon *et al*., 2001). *Trichoderma viride* reduced egg-hatching of *M. incognita* in the laboratory environment and nematode population in roots and soil in glasshouse experiments (Goswami and Mittal, 2004). Commercial products have also proven to be efficacious in tropical greenhouse conditions (Cuadra *et al*., 2008). Culture filtrate of *Trichoderma* spp. was highly effective in controlling reniform nematode (*R. reniformis*) and root knot nematode (*M. javanica*) on eggplant. Sharon *et al*. (2001) and Dababat and Sikora (2007) demonstrated that strains of *T. harzianum* reduced galling of the *M. javanica* and *M. incognita* on tomato plants. Windham *et al*. (1989) reported reduced egg production in the root-knot nematode, *M. arenaria*, following soil treatment with *T. harzianum* and *T. koningii* preparations. Reduction of *M. javanica* infection has been reported following treatment with several isolates of *T. lingnorum* and *T. harzianum* (Spiegel and Chet, 1998). Le *et al*. (2009) worked on the biological control of *M. graminicola* on rice using endophytic and rhizosphere fungi and reported positive results. Reddy *et al*. (1996) proved that *T. harazianum* incorporated with neem (*Azadirachta indica*) oil cakes was effective for

increasing yield and reducing the PPN numbers in soil and roots and Akhtar (1998) associated the reduction with an increase of predator and free-living nematodes.

2.2.3.3.2 Purpureocillium lilacinum

Purpureocillium lilacinum (previously *Paecilomyces lilacinus*) is a saprophyte generally specialized in parasitizing stationary stages of nematodes, particularly their eggs and females (Mukhtar *et al.*, 2013). It is well adaptable to a wide range of soil pH and establishes in the soil within a short time hence a successful biocontrol agent for nematodes (Siddiqui and Mahmood, 1996). The fungus produces serine protease and chitinase and enzymes that help it penetrate the eggshell of the nematode (Bonants *et al*., 1995; Khan *et al*., 2003). Jatala (1986) found that the fungus was able to engulf the gelatinous matrix and infect the eggs of *M. incognita* within 5 days. Siddiqui and Mahmood (1996) reported that the fungus was able to parasitize all the eggs within a very short time once it came into contact with the egg mass. It has also been reported that *Purpureocillium lilacinum* was able to control the mobile nematode *Radopholus similis* on banana (Davide and Zorilla, 1985).

Purpureocillium lilacinum has been proven to successfully control *M. javanica* and *M. incognita* on tomato and other vegetable crops (Verdejo-Lucas *et al*., 2003; Goswami and Mittal, 2004; Van Damme *et al*., 2005; Goswami *et al*., 2006; Haseeb and Kumar, 2006; Kumar *et al*., 2009), and in potato on field conditions (Jatala *et al*., 1980) and on banana (Jonathan and Rajendran, 2000) in greenhouse conditions. On the contrary, *P. lilacinum* was not effective against *M. javanica* on tobacco in microplots when it was applied individually (Hewlett *et al*., 1988. *Purpureocillium lilacinum* strain 251 that is available as a commercial formulation has been used successfully as a control agent against various

plant parasitic nematodes (Kiewnick *et al*., 2002; Brand *et al*., 2004). Direct interactions between *P*. *lilacinum* strain 251 and eggs of *M*. *javanica* were demonstrated *in vitro* by Holland *et al*. (1999).

In another study *Bacillus subtilis* and *P*. *lilacinum* were tested alone or combined against *M. incognita* on tomato in pots containing steamed soil (Gautam *et al*., 1995). In both cases plant height and weight increased and numbers of root galls, females, eggs, and J2 were suppressed. However, the synergistic effect of the two biocontrol agents on nematode populations was higher than individual effect. Kerry and Evans (1996) recorded inconsistent reports in biocontrol efficacy of *P*. *lilacinus* under glasshouse and field conditions.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Description of sampling sites

The study was carried out in the commercial pineapple plantations of Delmonte and Kakuzi. The two commercial farms have different management systems of nematodes. Delmonte (K) limited is located in Thika locality, Kiambu County in Central Kenya and partly in Murang'a County (Fig. 3.1). The plantation is located between latitude 01°03'S and longitude 37°05'E. It experiences a subtropical highland climate with sunshine throughout the year and receives an average rainfall of 1200 mm and sometimes receiving as low as 600 mm (Government of Kenya, 2015). The average annual temperature is at 19.8 °C and can go up to 27.15°C, with the hottest period in March and the coldest in July. Soils in Delmonte are well-drained, sandy loam with a high content of organic matter (www.Delmonte.co.ke). Delmonte has over 18,000 ha under pineapple production (Ndungu, 2014). Soil sampling and assessing for root knot nematodes is routinely done in Delmonte.

Kakuzi plantation has over 100 ha under pineapple production (Ndungu, 2014). The farm is located in Murang'a County, 72 km from Nairobi, off the Nairobi-Thika-Sagana highway (Fig. 3.1). The plantation lies between latitude 0°, 58' South and longitude 37° 16' East. The climatic condition of the area is sub-tropical type and experiences a maximum average annual rainfall of between 1400 mm and 1600 mm and sometimes receiving less than 900 mm per annum. The area receives minimum annual temperatures range between 16°C and a maximum temperatures of 26°C. The area is characterized by

arid and semi-arid conditions. Soil sampling for nematode diagnosis is done on a need basis i.e. if the pineapple crop is affected on a certain area.

Both plantations experience long rains in the months of March, April and May. The highest amount of rainfall is recorded in the month of April, and reliability of rainfall during this month is very high. The short rains are received during the months of October and November (Government of Kenya, 2014). Both plantations grow smooth cayenne pineapple variety. They uproot the declining plant material after the second harvest of the crop and incorporate back to the soil to decompose and provide organic matter.

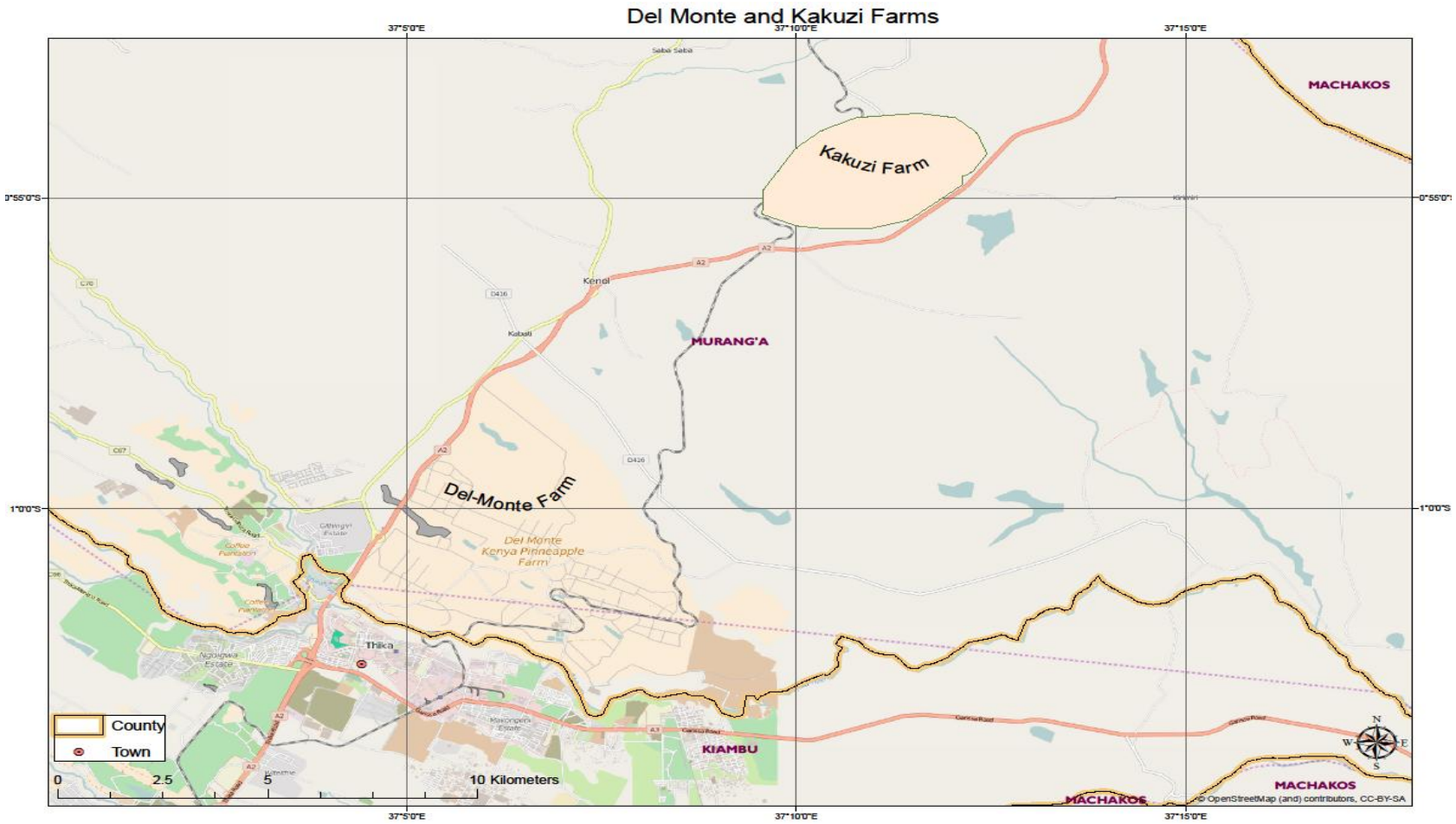

Figure 3.1: Map for the Kakuzi and Delmonte commercial pineapple farms

3.2 Sampling and experimental procedures

3.2.1 Survey and nematode identification

A survey was conducted in Delmonte and Kakuzi commercial pineapple farms. Composite soils and root samples were collected from the rhizosphere of pineapple plants (*Ananas comosus*) from both farms. Samples were collected from 7 different fields depending on age of the pineapple crop (Appendix 1). From the two farms, in each sampled field (of different age crop), the top 3 to 5 cm of the soil was removed and 25 root and soil sub-samples were collected up to a depth of 30 cm at randomly selected locations (Santhosh *et al*., 2005) using a zigzag method. They were then pooled together, mixed thoroughly and 1 kg drawn from each field. The samples were placed in polythene bags, sealed and transported to ICIPE nematology laboratory for isolation and nematode extraction. They were stored at approximately 4°C before extraction of nematodes.

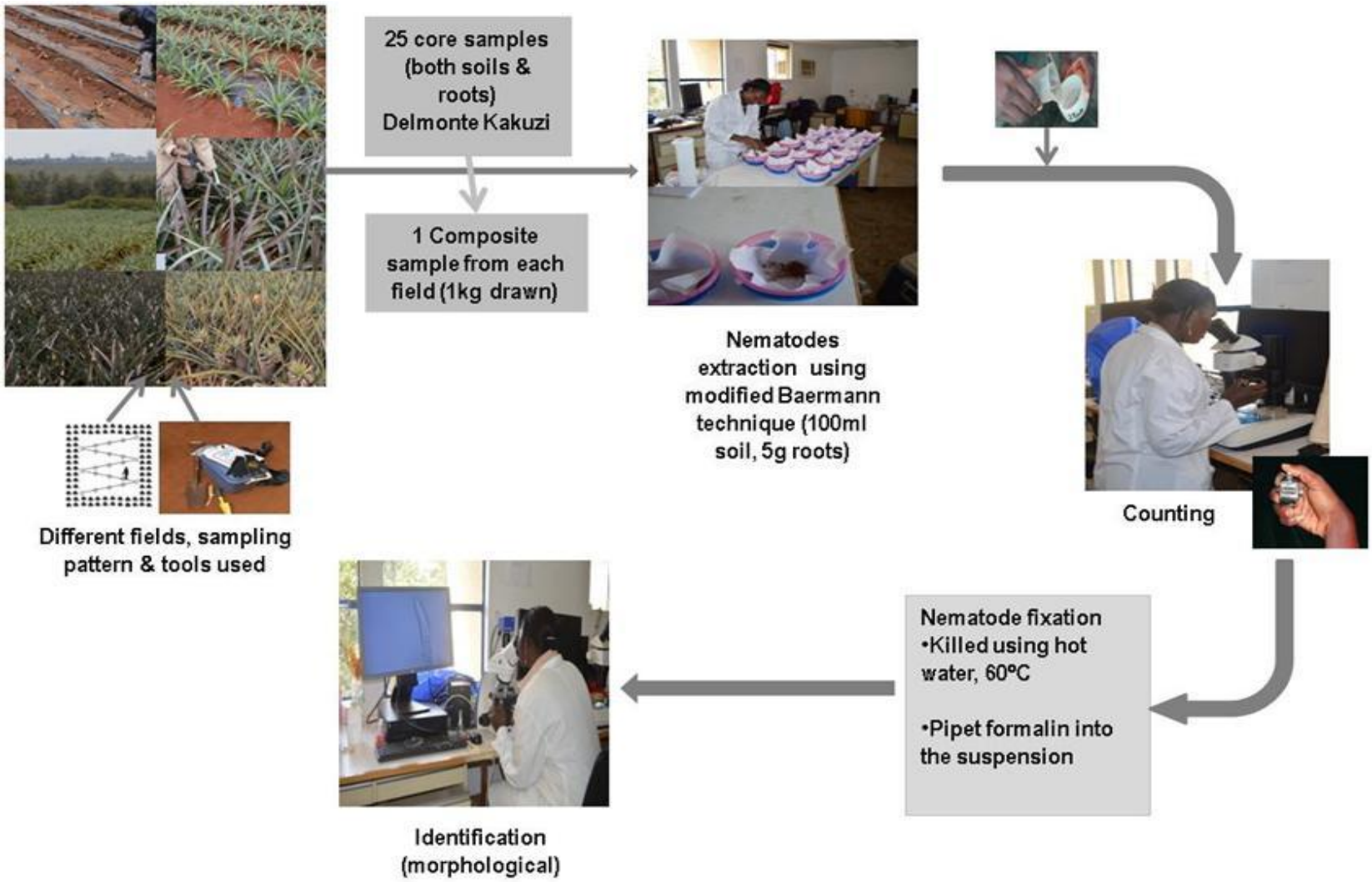

Figure 3.2: Sampling and laboratory experimental procedures (photographs courtesy Kiriga, 2017).

The modified Baermann technique (Hooper *et al*., 2005) was used to extract vermiform nematodes from 100 ml soil and 5 g root materials (Fig. 3.2). Nematodes were then counted using a single tally counter under a stereomicroscope (×100 Mg) (Fig. 3.2) then killed using hot water and fixed using three drops of formaldehyde (Coyne *et al*., 2014). They were then identified to genus level using morphological features such as J2 head, stylet and tail region morphologies under the compound microscope as described by Eisenback and Hirschmann (1980) and Hunt *et al*. (2005) (Fig. 3.2).

3.2.2 *Meloidogyne spp*. identification

Purposive sampling was conducted in Delmonte and Kakuzi farms and from 7 fields of each farm; roots with galls were randomly sampled. Twenty (20) females were obtained from each field of 1 ha where ten were used for morphological characterization using perineal pattern.

3.2.2.1 Morphological identification

Morphological features such as tail size and configuration, hyaline terminus, head region, stylet knobs and basal bulb were used to differentiate the second stage juvenile for *Meloidogyne* spp. The perineal patterns of mature live egg-laying females were obtained by cutting the vulva region according to Taylon *et al.* (1956) and Seinhorst (1966).

3.2.2.2 Molecular identification

Molecular identification was also performed using female *Meloidogyne* spp. from each field to confirm the results of morphological identification. The females were stored in 98% ethanol for 24 hours. DNA of crushed single female was then extracted using worm lysis buffer with proteinase K (Bert *et al*., 2008). It was then precipitated in iso-propanol at room temperature.

The DNA pellet was then washed twice with 70% ice-cold ethanol, re-suspended in H_2O and stored at -80°C. For molecular identification species specific primers for tropical root knot nematodes (Table 3.1) were used (Zijlstra *et al*., 2000, Tigano *et al*., 2010, Correa *et al*., 2014). The specific SCAR primers Fjav/Rjav (*M. javanica*) (Zijlstra *et al*., 2000) gave consistent results and the products were readily amplified from DNA of individual females. PCR amplification reactions were performed in 15 µl volumes of the master mix containing: template DNA, primer, PCR buffer, Magnesium Dichloride ($MgCl_2$), dNTP, nonacetylated BSA and Taq polymerase. The PCR amplification conditions used for each primer set are described in Table 3.2. All amplification tests included a no-template control.

Table 3.1: Primers used for molecular identification of root knot nematodes, *Meloidogyne* spp

Primer code	Species	Predicted fragment size (bp)	Primer sequence (5'-3')	Reference
Far Rar	*M. arenaria*	420	TCGAGGGCATCTAATAAAGG GGGCTGAATATTCAAAGGAA	(Meng *et al*., 2004)
Mi-F Mi-R	*M. incognita*	1000	GTGAG GATTCAGCTCCCCAG ACGAGGAACA TACTTCTCCGTCC	(Zijlstra *et al*., 2000)
Fjav Rjav	*M. javanica*	720	CCTTAATGTCAACACTAGAGCC GGCCTTAACCGACAATTAGA	(Zijlstra *et al*., 2000)
MK7-F MK7-R	*M. enterolobii*	600	GATCAGAGGCGGGCGCATTGCGA CGAACTCGCTCGAACTCGAC	(Tigano *et al*., 2010)
Meth-F Meth-R	*M. ethiopica*	350	ATGCAGCCGCAGGGAACGTAGTTG TGTTGTTTCATGTGCTTCGGCATC	(Correa *et al*., 2014)
JMV1 JMV	*M. hapla*	440	TTTCCCCTTATGATGTTTACCC AAAAATCCCCTCGAAAAATCCACC	(Wishart *et al*., 2002)

Table 3.2: PCR amplification profiles used with primers for identification of *Meloidogyne* spp.

Primer code	Amplification conditions	Number of cycles
Far/Rar	95 ^{o}C 5 min	
Mi-F/Mi-R	95 ^{o}C 3 min	
Rjav/Fjav	55 ^{o}C 2 min	40
	72 ^{o}C 1 min	
	72 ^{o}C 10 min	
MK7-F/MK7-R	95 ^{o}C 5 min	
JMV1/JMV	95 ^{o}C 3 min	
Meth-F/Meth-R	62 ^{o}C 3 min	35
	72 ^{o}C 1 min	
	72 ^{o}C 10 min	

3.2.3 Preparation of *Meloidogyne* spp. inocula for inoculation

Meloidogyne spp. were isolated from naturally infected pineapple plants collected from Delmonte and Kakuzi pineapple commercial farms. Single egg masses were removed from infected pineapple roots under a dissecting microscope and individually inoculated onto pineapple seedlings (cv. Smooth Cayenne) planted in pots containing autoclaved soil in the greenhouse at ICIPE.

The pineapple plants were uprooted about three months after inoculation, and the galled roots gently washed free of soil using clean tap water and then sterilized using 0.5% sodium hypochlorite (NaOCl) to ensure the absence of nematodes on the surface. The roots were blended for 30 seconds in 10% NaOCl solution (Hussey and Barker, 1973), rinsed with distilled water and eggs collected in a 25 µm sieve. The eggs were incubated at 25°C to obtain one- to five-day-old second-stage infective juveniles (IJ2s) to be used for inoculation.

3.3 Biocontrol agents used in the study

3.3.1 Sources of biocontrol agents

Four isolates of *Trichoderma* spp., three from ICIPE's Arthropod Germplasm Centre, Kenya (*Trichoderma asperellum* M_2RT_4; *T. atroviride* F_5S_{21} and *T. harzianum* F_2L_4) and one from Kenya Biologics Limited (KBL) *Trichoderma* sp. MK_4, were used in the study. KBL also provided two isolates of *Purpureocillium lilacinum* (KLF_2 and MR_2) (Table 3.3).

Table 3.3: Isolates of *Trichoderma* spp. and *Purpureocillium lilacinum* from ICIPE and KBL

Depositors name	Place of isolation	Country of isolation	Isolated from
M_2RT_4 (Icipe 700)	Nakuru	Kenya	Maize
F_5S_{21} (Icipe 710)	Loitokitok	Kenya	Onions
F_2L_4 (Icipe 709)	Nakuru	Kenya	Onions
MK_4	Murangá	Kenya	Soil
KLF_2	Kiambu	Kenya	Soil
MR_2	Murangá	Kenya	Soil

3.3.2 Assessment of pineapple root colonization by *Trichoderma* isolates

The four isolates of *Trichoderma* spp. (M2RT4, F5S21, F2L4 and MK4) were tested for colonization of pineapple roots under greenhouse conditions at ICIPE. Pineapple cv. Smooth Cayenne crowns were established in 10 L pots containing autoclaved soil for three weeks to allow roots to develop. The rooted crowns were then removed from the soil mixture with roots intact, the soil washed off with tap water and the roots immersed in a 250 ml suspension containing 1×10^8 spores per ml of *Trichoderma* spp. for 8 h for

each isolate; the controls were immersed in distilled water for 8 h. Each treatment was replicated four times and arranged in a complete randomized design. The rooted treated crowns were then planted into pots containing a 10 L of 2:1:1 mixture of autoclaved soil, sand and manure. Two weeks after inoculation the plants were removed from the soil mixture with roots intact and rinsed with tap water to remove the soil. Leaves and roots sections were aseptically removed and cut into 1cm length pieces under a laminar flow hood. These were surface sterilized using 0.5 % NaOCl, rinsed first in 70% alcohol and then in distilled water. For each plant, five pieces each of leaves and roots were separately placed 4 cm apart onto potato dextrose agar (PDA) plates and incubated at 26 ±1°C for 10 days. Mycelial growth was assessed between days 2 to 10. Fungal colonization was recorded by counting the number of pieces that showed the presence of inoculated *Trichoderma* fungi according to Koch's postulate for each piece.

3.3.3 Mass production of the fungal antagonists

Inocula for all fungal isolates were multiplied using rice. Rice grains were washed with water, surface dried using a paper towel and 2 kg placed in Milner bags (autoclavable bags), before autoclaving at 121°C for 50 minutes. The sterilized rice grains were inoculated with pure cultures of each of the antagonistic fungi in separate bags. The bags were massaged from the outside to evenly distribute the inoculum over all the rice grains. The inoculated rice was incubated at 25 ±1°C for 21 days. The bags were shaken on alternate days to encourage uniform colonization by the fungus. The bags were then opened up to allow the rice and the conidia to dry for seven days, before using to make spore suspensions. A 0.1 g sample of conidia was placed in universal bottles with 10 ml

sterile distilled water containing 0.05 % Triton X-100 and allowed to vortex for 5 min to produce homogenous conidial suspensions.

The spore concentration was estimated using a haemocytometer (Hausser Scientific, Horsham, Pennsylvania, USA) and adjusted to 3×10^6 and 1×10^8 spores per ml for *P. lilacinum* and *Trichoderma* spp., respectively, through dilution. To assess the viability of the fungus, 100 µL of conidial suspension for each isolate was inoculated on plates of potato dextrose agar (PDA). A sterile microscope cover slip (2 x 2 cm) was placed on top of the agar in each plate before incubation. The inoculated plates were incubated for 24 h at 25°C. The percentage conidial germination was assessed by counting the number of germinated conidia per 100 in one randomly selected field of view. Conidia were considered as germinated when germ tubes exceeded half of the diameter of the conidium. The percent germination of over 95 % is recommended (Parsa *et al*., 2013).

3.4 Evaluation of the efficacy of fungal antagonists on *Meloidogyne* spp. under greenhouse conditions

3.4.1 Treatments and treatments application

The treatments were as follows:

- T0: No fungal no nematode treatment (negative control),
- T1: 3000 IJ2s only (nematode inoculated control)- no fungal treatment,
- T2: 1.0×10^8 spores/ml MK_4 (*Trichoderma* spp.) + 3000 IJ2s,
- T3: 1.0×10^8 spores/ml M_2RT_4 (*T. asperellum* isolate) +3000 IJ2s,
- T4: 1.0×10^8 spores/ml F_5S_{21} (*T. atroviride* isolate) +3000 IJ2s,
- T5: 3.0×10^6 spores/ml KLF_2 (*P. lilacinum*) + 3000 IJ2s,

- T6: 3.0 $\times 10^6$ spores/ml MR_2 (*P. lilacinum*) +3000 IJ2s.

Pineapple (cv. Smooth Cayenne) crowns were established in 10 L pots containing autoclaved soil for three weeks to allow roots to develop. The rooted crowns were then removed from the soil with roots intact and the soil gently rinsed with tap water. They were then surface sterilized using 0.5 % NaOCl for 30 seconds, and rinsed with 70 % alcohol for 2 minutes, and then in distilled water for 5 minutes. For the *Trichoderma* isolates biocontrol treatments (T2, T3 and T4), the roots were totally immersed in the 1×10^8 spores per ml conidial suspension for 8 h; for the controls (T0 and T1) and treatments (T5 and T6) that required *P. lilacinum*, the roots were dipped in distilled water for 8 h. The rooted crowns were then planted in pots containing a 10 L, 2:1:1 mixture of autoclaved soil, sand and manure in a greenhouse. After two weeks, three 2-cm deep holes were made around the stem of each plant and a suspension containing one- to five-day-old second-stage IJ2s of *Meloidogyne javanica* inoculated into the holes using a pipette except T0*;* and the holes were covered with the same soil. In treatments (T5 and T6) that required *P. lilacinum*, this was applied one day after nematodes inoculation; 2 cm deep fallows were made around the plants and a 120 ml suspension containing 3.0×10^6 spores/ml applied. Thus the positive controls (T1) were treated with one- to five-day-old second-stage IJ2s only; negative controls (T0) were treated with water only.

The experiment was undertaken in a greenhouse for 90 days after nematode inoculation; the plants were irrigated with clean tap water as needed. A completely randomized experimental design was used and each treatment was replicated six times. The experiment was repeated once in time following the same procedure with a total of 84 pots/ plants in the full experiment.

3.4.2 Disease measurement and data collection

After 90 days of nematode inoculation, plants were gently uprooted, the roots excised from the plant and the soil gently rinsed under running tap water. The roots were then surface sterilized using 1.5 % NaOCl, rinsed first in 70 % alcohol and then in distilled water, dabbed dry with a paper towel and root fresh weights recorded.

The effect of *Trichoderma* isolates and *P. lilacinum* isolates on *Meloidogyne* spp. was determined by scoring for galling index from each plant on a 1-5 scale: 1 -no galling; 2-slight; 3 – mild; 4 -moderate and 5 - severe (Coyne *et al*., 2014). The number of galls on each root system was counted and the nematode density estimated by counting the number of egg masses and eggs under a stereo microscope at ×400 magnification from a representative sample of 5 g chopped up roots from each plant (Holbrook *et al*., 1983; Shurtleff and Averre, 2000). To facilitate counting of egg masses the roots were first stained with phloxine B, which stains the gelatinous matrix pink-red increasing egg mass visibility (Coyne *et al*., 2014). Eggs were extracted from galled pineapple roots by cutting into small pieces and blending in 0.6 % NaOCl for 30 seconds (Stetina *et al*., 1997). The contents were then poured onto nested 75 and 25 µm pore sieves. Eggs collected on the 25- µm-pore sieve were counted under a stereo microscope (×400 magnification). Percentage reduction in the number of galls was computed using the formula below (Oclarit and Cumagun, 2009):

$$\text{Percentage Reduction} = \frac{\text{Number of galls (+ve control)} - \text{Number of galls (Treated)}}{\text{Number of galls (+ve control)}} \times 100$$

3.4.3 Statistical analyses

The data on survey of nematode occurrence in Delmonte and Kakuzi commercial pineapple farms was subjected to two-way analysis of variance (ANOVA) to investigate the effect and interactions between nematode populations at the two farms and the different ages of pineapple. General Linear Model procedures were used to analyse and distinguish nematode genera occurrence and distribution on the sites and the different ages. The data on evaluation of the efficacy of biocontrol agents on *Meloidogyne* spp. under greenhouse conditions was subjected to one way analysis of variance (ANOVA). These statistics were performed in R software version 3.2.3 (R Core Team, 2015) and the means of treatments that were significantly different at $P \leq 0.05$ were separated using Tukey-HSD.

CHAPTER FOUR

4.0 RESULTS

4.1 Survey and nematode genera identified

A total of eight (8) genera of plant parasitic nematodes (PPNs) were identified and recorded from the soils and root samples collected from Delmonte and Kakuzi commercial pineapple fields (Table 4.1). The plant parasitic nematode genera identified included *Helicotylenchus* spp., *Hoplolaimus* spp. (spiral), *Meloidogyne* spp. (root knot), *Pratylenchus* spp. (lesion), *Tylenchus* spp., *Filenchus* spp., *Rotylenchus* spp. and *Aphelenchoides* spp. (Plate 4.1). From the survey, non plant parasitic nematodes were also identified from both soils and root of the pineapple fields. They included bacterial feeders, omnivores and predators as shown in plate 4.1.

Table 4.1: Plant parasitic nematodes genera isolated from soils and roots of pineapple in Delmonte and Kakuzi farms.

Order	Sub-order	Family	Genus
Tylenchida	Tylenchina	Hoplolaimidae	*Helicotylenchus*
			Hoplolaimus
			Rotylenchus
		Pratylenchidae	*Pratylenchus*
		Meloidogynidae	*Meloidogyne*
		Tylenchidae	*Tylenchus*
			Filenchus
Aphelenchida	Aphelenchina	Aphelenchoididae	*Aphelenchoides*

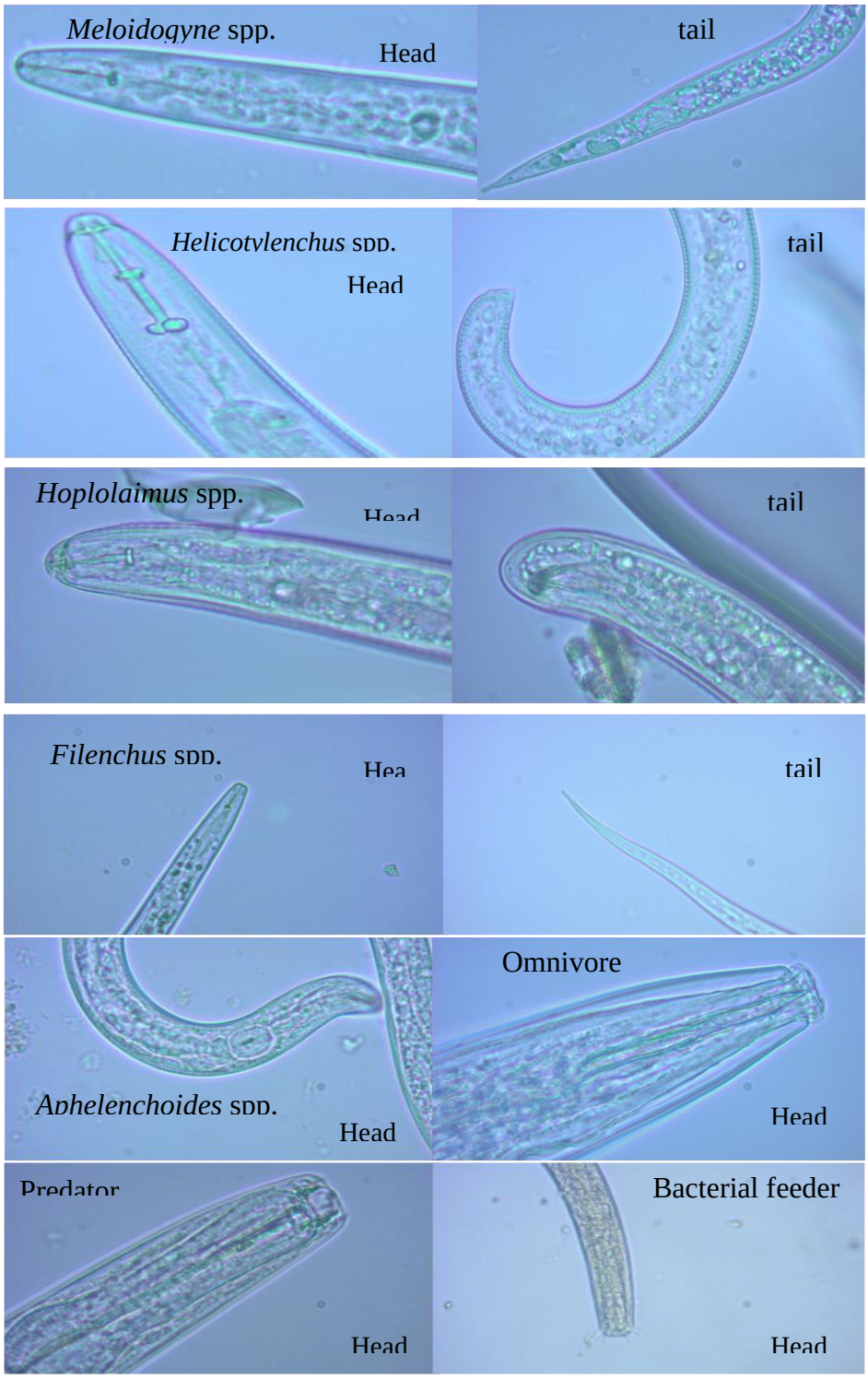

Plate 4.1: Plant-parasitic nematodes and non plant-parasitic nematodes on pineapple in Delmonte and Kakuzi farms (x 1000 Mg) (Photographs courtesy Kiriga, 2017).

The most commonly found PPNs in soils and roots of pineapple from the two farms were *Helicotylenchus* spp., *Meloidogyne* spp., *Tylenchus* spp. and *Aphelenchoides* spp. in all roots and soil samples. *Helicotylenchus* spp. and *Meloidogyne* spp. were found in all the fields of all ages and in high numbers both in Kakuzi and Delmonte farms. *Pratylenchus* spp. and *Rotylenchus* spp. were only recorded in Delmonte farm. *Filenchus* spp. were recovered from all fields in Kakuzi, while in Delmonte they were observed only in fields at 36 months.

4.1.1 Population density of nematode genera in pineapple soils

Plant parasitic nematodes were detected in soil samples of Delmonte and Kakuzi commercial pineapple farms at varying densities. In Delmonte soils, high population densities of *Helicotylenchus* spp., *Tylenchus* spp., *Aphelenchoides* spp. and *Meloidogyne* spp. (15.4%, 11.0%, 7.9% and 7.4% respectively) were recorded, while very low population densities were recorded for *Pratylenchus* spp., *Filenchus* spp. and *Rotylenchus* spp. (0.5%. 0.5% and 0.3%) (Table 4.2). In Kakuzi soils, high population densities of *Aphelenchoides* spp., *Helicotylenchus* spp., *Meloidogyne* spp. and *Filenchus* spp. (9.7%, 9.6%, 7.2% and 5.6%) were recorded while *Tylenchus* spp. (4.8%) had the least population densities (Table 4.3). The percentage proportion of *Meloidogyne* spp. was almost similar in Delmonte (7.4%) and Kakuzi (7.2%). The percentage proportion of *Helicotylenchus* spp. was higher in Delmonte (15.4%) than in Kakuzi (9.6%). The percentage proportion for the plant parasitic nematodes was slightly higher in Delmonte (43.1%) than in Kakuzi (36.8%) (Table 4.2 and 4.3).

Table 4.2: Population densities of nematodes associated with soil samples from Delmonte farm, Kenya.

Nematode genus	Mean average population/100ml soil	Population (%)
Helicotylenchus spp.	2847	15.4
Meloidogyne spp.	1370	7.4
Pratylenchus spp.	97	0.5
Rotylenchus spp.	50	0.3
Filenchus spp.	97	0.5
Tylenchus spp.	2026	11.0
Aphelenchoides spp.	1457	7.9
Non PPNs	10490	56.9
Total	18434	

Non PPNs- Non-Plant Parasitic Nematodes; %- Percentage

Table 4.3: Population densities of nematodes associated with soils samples from Kakuzi farm, Kenya.

Nematode genus	Mean average population/100ml soil	Population (%)
Helicotylenchus spp.	1130	9.6
Meloidogyne spp.	850	7.2
Tylenchus spp.	560	4.8
Filenchus spp.	660	5.6
Aphelenchoides spp.	1140	9.7
Non PPNs	7440	63.2
Total	11780	

Non PPNs- Non-Plant Parasitic Nematodes; %- Percentage

4.1.2 Population densities of nematode genera in pineapple roots

Root samples from Delmonte fields had high populations of *Meloidogyne* spp., *Tylenchus* spp., *Aphelenchoides* spp. and *Helicotylenchus* spp. while *Filenchus* spp., *Pratylenchus* spp. and *Hoplolaimus* spp. population densities observed were very low (Table 4.4). In Kakuzi also high populations of *Meloidogyne* spp., *Tylenchus* spp. and *Aphelenchoides* spp. were recorded on the pineapple roots while low populations of *Filenchus* spp., *Pratylenchus* spp. and *Helicotylenchus* spp., were recorded (Table 4.5). The percentage proportion of *Meloidogyne* spp. was higher in Delmonte (29.6%) than in Kakuzi (16.6%). The total population for PPNs was higher in Delmonte than Kakuzi root population resulting to 76.0% and 58.1% respectively whereas the percentage proportion for non-

plant parasitic nematodes was higher in Kakuzi (41.9%) than Delmonte (24%) (Table 4.4 and 4.5).

Table 4.4: Population densities of nematodes associated with roots samples from Delmonte farm, Kenya

Nematode genus	Mean average population/5g roots	Population (%)
Helicotylenchus spp.	387	10.2
Hoplolaimus spp.	3	0.08
Meloidogyne spp.	1127	29.6
Pratylenchus spp.	17	0.4
Filenchus spp.	37	1.0
Tylenchus spp.	850	22.3
Aphelenchoides spp.	477	12.5
Non PPNs	913	24.0
Total	3811	

Non PPNs- Non- Plant Parasitic Nematodes; %- Percentage

Table 4.5: Population densities of nematodes associated with roots samples from Kakuzi farm, Kenya

Nematode genus	Mean average population/5g roots	Population (%)
Helicotylenchus spp.	130	2.7
Meloidogyne spp.	790	16.6
Pratylenchus spp.	130	2.7
Tylenchus spp.	750	15.7
Filenchus spp.	440	9.2
Aphelenchoides spp.	530	11.1
Non PPNs	2000	41.9
Total	4770	

Non PPNs- Non- Plant Parasitic Nematodes; %- Percentage

4.1.3 Occurrence of PPNs in soils at different stages (ages) of the pineapple crop in Delmonte and Kakuzi farms

The results from Table 4.6, show that *Helicotylenchus* spp. were present in almost all the fields of different ages in Kakuzi and Delmonte farms except in fields at 24 months of Kakuzi farm. Compared to Kakuzi farm, Delmonte recorded significantly higher ($P \leq 0.05$) numbers of *Helicotylenchus* spp. in the older months namely 8, 11, 24 and 36 months while the population was significantly higher ($P \leq 0.05$) in Kakuzi farm at 0, 3 and 5 months fields than Delmonte farm. *Meloidogyne* spp. was ubiquitous in all the fields at all ages and the populations increased with age from 3 to 36 months in Delmonte farm while the population in Kakuzi farm increased with age up to 11 months and the

number started decreasing up to 36 months. There was significantly higher population of *Meloidogyne* spp. ($P \leq 0.05$) in soils at 36 months (910 nematodes) of Delmonte farm, compared to other months.

Rotylenchus spp. and *Pratylenchus* spp. were encountered in very low populations in fallow field and 36 months respectively, of Delmonte farm. In all the fields, *Tylenchus* spp. recorded a significant higher population in Delmonte than Kakuzi farm. However, the highest number occurred in Delmonte pineapple fields at 24 months with a mean population of 703 nematodes per 100 ml soil. *Aphelenchoides* spp. was also ubiquitous in all the ages in both farms. *Filenchus* spp. occurred in all the fields of Kakuzi and was only recorded in fields at 36 months of Delmonte farm (Table 4.6**)**.

Table 4.6: Population of plant parasitic nematodes associated with pineapple soils at different stages of the pineapple crop in Delmonte and Kakuzi farms (per 100 ml soil).

Age	***Helicotylenchus***		***Meloidogyne***		***Pratylenchus***		***Rotylenchus***		***Filenchus***		***Tylenchus***		***Aphelenchoides***	
of	Delmonte	Kakuzi	Delmonte	Kakuzi	Delmonte	Kakuzi	Delmonte	Kakuzi	Delmonte	Kakuzi	Delmonte	Kakuzi	Delmonte	Kakuzi
crop														
0	290cA	330.0aA	86.7bA	30.0dB	43.3aA	0.0aB	46.7aA	0.0aB	0.0bB	50.0cdA	123.3eA	70.0abB	103.3dB	280.0aA
3	20eB	180.0bA	16.7cdB	100.0bcA	0.0bA	0.0aA	0.0bA	0.0aA	0.0bB	30.0dA	250.0bcA	10.0cB	313.3aA	70.0cB
5	83.3dB	200.0abA	53.3bcB	110.0bcA	0.0bA	0.0aA	0.0bA	0.0aA	0.0bB	90.0abA	250.0bcA	110.0abB	130.0cdA	130.0bA
8	416.7bcA	30.0cB	6.7dB	150.0bA	0.0bA	0.0aA	0.0bA	0.0aA	0.0bB	90.0abA	340.0bA	120.0aB	280.0aA	120.0bB
11	273.3cA	200.0abB	130.0bB	290.0aA	0.0bA	0.0aA	3.3bA	0.0aA	0.0bB	130.0aA	146.7deA	90.0abB	293.3aA	90.0bcB
24	773.3abA	0.0dB	166.7bA	100.0bcA	0.0bA	0.0aA	0.0bA	0.0aA	0.0bB	70.0bcA	703.0aA	240.0aB	186.7bB	380.0aA
36	990.0aA	190.0bB	910.0aA	70.0cB	53.3aA	0.0aB	0.0bA	0.0aA	96.7aA	100.0abA	213.3cdA	20.0bcB	150.0bcA	70.0cB

Means within columns shows **s**ignificant differences of nematode genera among the ages of the crop and are followed by small letters; means within rows shows **s**ignificant differences of each nematode genus between the two farms and are followed by capital letters. Means followed by the same letter(s) are not significantly different ($P \leq 0.05$).

4.1.4 Occurrence of PPNs associated with pineapple roots at different crop age in Delmonte and Kakuzi farms

Helicotylenchus spp. occurred in low populations in Kakuzi and was only recorded in 11 and 24 months (20 and 110 nematodes per 5g of root sample respectively) (Table 4.7). At 24 months, *Helicotylenchus* spp. occurred in both farms with significantly higher populations ($P \leq 0.05$) occurring in Kakuzi than Delmonte farm. In Delmonte farm, *Helicotylenchus* spp. was recorded in all other 6 fields except in fields at 11 months with the highest populations of 320 nematodes per 5g root sample occurring at 36 months.

Significantly higher ($P \leq 0.05$) populations of *Meloidogyne* spp. were encountered in Kakuzi than Delmonte farm at 5, 8 and 24 months. The populations at 11 months had no significant difference between the two farms. At 36 months, significantly higher populations ($P \leq 0.05$) were recorded in Delmonte than Kakuzi farm. *Meloidogyne* spp. was absent in fields at 8 months of Delmonte farm. The population increased with age in Delmonte with the highest mean population recording at 36 months (680 nematodes per 5g root sample). In Kakuzi the populations increased up to 24 months (490 nematodes per 5g root sample) and the number decreased in 36 months (100 nematodes per 100 ml soil).

Pratylenchus spp. was rarely recorded in the two farms as they were only detected at 11 and 24 months in Kakuzi farm and 36 months in Delmonte farm. In Kakuzi farm *Filenchus* spp. occurred in all fields except in fields at 11 months while in Delmonte farm they only occurred in fields at 36 months. The populations were higher in older fields (24 and 36 months) compared to the fields with younger crop (5 and 8 months). *Tylenchus*

spp. occurred in all fields in both farms. In Delmonte farm, a significantly higher number of *Tylenchus* spp. was recorded in fields at 24 months compared to other fields. *Aphelenchoides* spp. were recovered from all fields in both farms.

Table 4.7: Population of plant parasitic nematodes associated with pineapple roots at different stages of the pineapple crop in Delmonte and Kakuzi farms (per 5g of root sample).

Age of crop	***Helicotylenchus***		***Meloidogyne***		***Pratylenchus***		***Hoplolaimus***		***Filenchus***		***Tylenchus***		***Aphlenchoides***	
	Delmonte	Kakuzi	Delmonte	Kakuzi	Delmonte	Kakuzi	Delmonte	Kakuzi	Delmonte	Kakuzi	Delmonte	Kakuzi	Delmonte	Kakuzi
5	6.7dA	0.0cA	13.3dB	60.0cA	0.0bA	0.0cA	0.0 aA	0.0aA	0.0bB	20.0cA	103.3bA	50.0cB	30.0bA	20.0eA
8	16.7bcA	0.0cB	0.00eB	80.0bcA	0.0bA	0.0cA	0.0aA	0.0aA	0.0bB	150.0bA	53.3cB	100.0bA	26.7bB	130.0bA
11	0.00eB	20.0bA	40.0cA	60.0cA	0.0bB	30.0bA	0.00aA	0.0aA	0.0bA	0.0dA	106.7bA	40.0cB	266.7aA	30.0dB
24	43.3bB	110.0aA	393.3aB	490.0aA	0.0bB	100.0aA	3.33aA	0.0aA	0.0bB	300.0aA	553.3aA	50.0cB	133.3aA	100.0cB
36	320.0aA	0cB	680.0aA	100.0bB	16.7aA	0.0cB	0.00aA	0.0aA	36.7aB	280.0abA	33.3cB	200.0aA	20.0bB	250.0aA

Means within columns shows **s**ignificant differences of nematode genera among the ages of the crop and are followed by small letters; means within rows shows **s**ignificant differences of each nematode genus between the two farms and are followed by capital letters. Means followed by the same letter(s) are not significantly different ($P \leq 0.05$).

4.2 Morphological and molecular identification of *Meloidogyne* spp.

Meloidogyne spp. second stage juveniles were found to have morphological features such as long, narrow tapering tail with terminus ending in a finely rounded tail, cuticular constrictions along the hyaline terminus, distinct stylet knobs and basal bulb (plate 4.1). The perineal pattern had low dorsal arch and double lateral lines that were the main characteristic features (Fig. 4.1 i & ii; Appendix 2).

The specific SCAR primers Fjav/Rjav (*M. javanica*) (Zijlstra *et al*., 2000) gave consistent results and the products were readily amplified from DNA of individual females (Fig. 4.2). The MI-F/MI-R *M. incognita*-specific SCAR primers (Meng *et al*., 2004), JMV *M. hapla* primers (Wishart *et al*., 2002) and MK7-F/Mk7-R *M. enterolobii* primers (Tigano *et al*., 2010) gave no amplification products from single females (Fig. 4.3). Whilst the first stage of the key enables all the *M. javanica* products to be amplified, it was possible to confirm the identification of the nematode species in the pineapple plantations by use of the second step (Fig. 4.2 and Fig. 4.3).

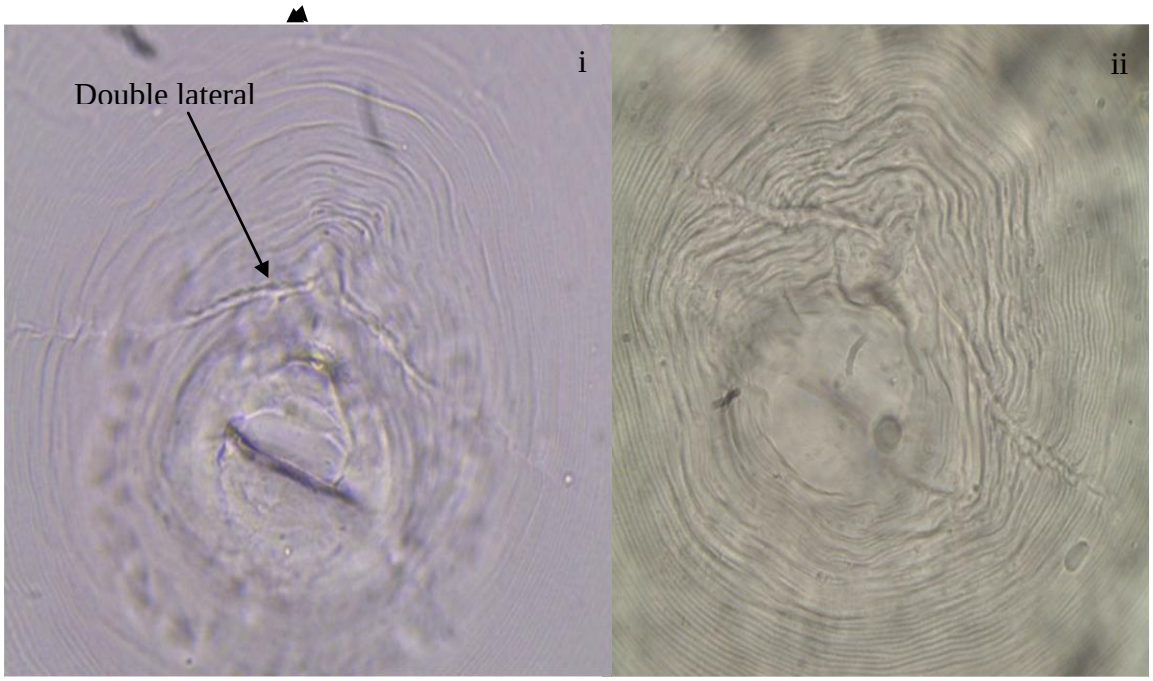

Figure 4.1:i amd ii: Perineal pattern from mature live egg-laying *M. javanica* females (×1000 Mg).

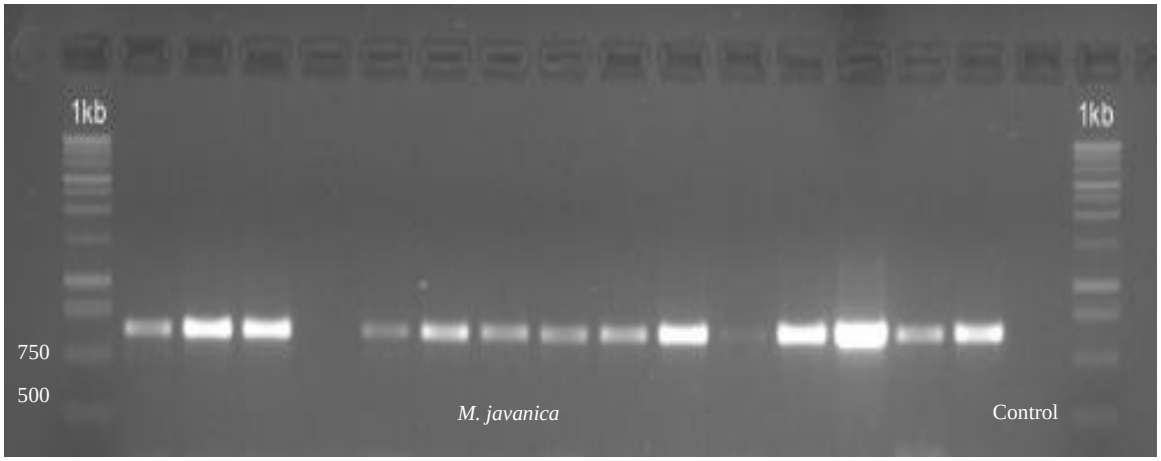

Figure 4.2: PCR products (720 bp) obtained from amplification of DNA from single females of M. javanica from pineapple farms using Fjav / Rjav *M. javanica* specific primers of (1-kb ladder).

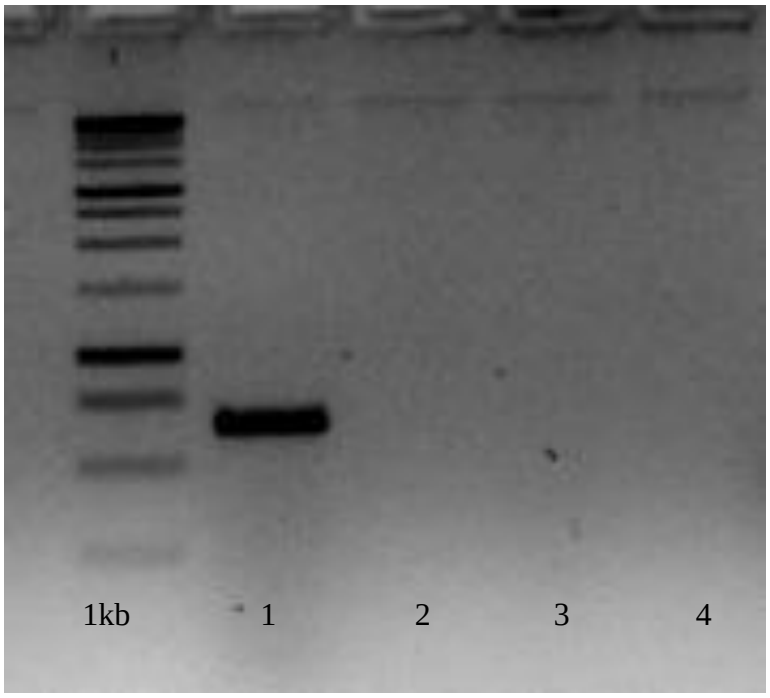

Figure 4.3: DNA amplification using species specific primers for; (1) *M. javanica*, (2) *M. incognita*, (3) *M. enterolobii* and (4) *M. hapla*.

4.3 Colonization of pineapple roots by *Trichoderma* isolates

The number of roots (Fig. 4.4) and leaves (Fig. 4.5) sections colonized by *T. asperellum* M_2RT_4, *T. atroviride* F_5S_{21}, and *Trichoderma* sp. MK_4 was statistically similar at $P \leq 0.05$ but significantly greater than for *T. harzianum* F2L4 which on average had a mean score of 0.5 and 1.25 for roots and leaves, respectively (Fig. 4.3a and 4.3b). Hence isolate F2L4 (*T. harzianum*) was not further tested as a biocontrol agent.

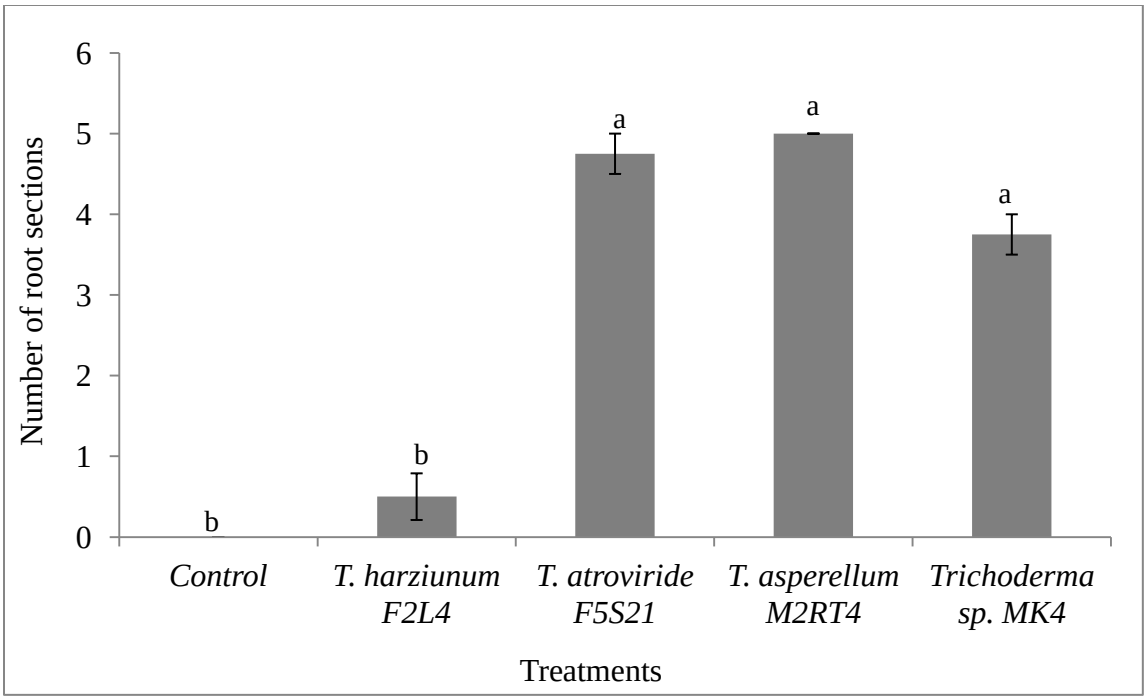

Figure 4.4: Number of pineapple root sections colonized by the *Trichoderma* isolates. (Bars with same letter (s) indicate means are not significantly different ($p < 0.05$)).

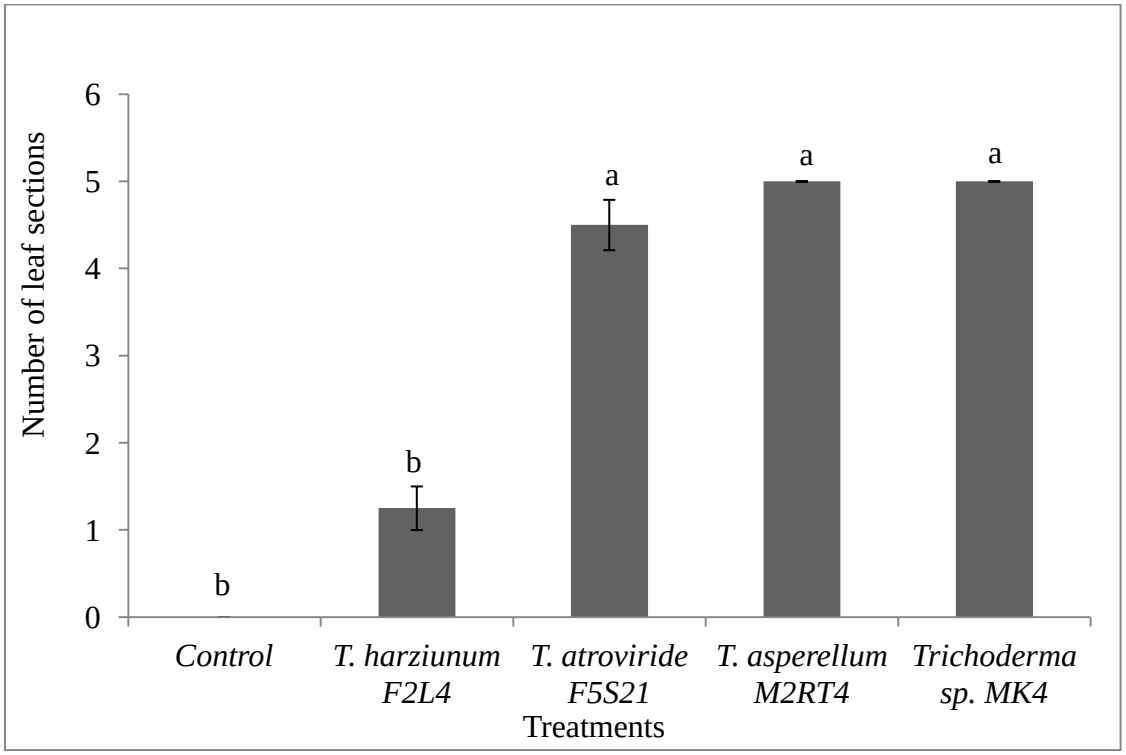

Figure 4.5: Number of pineapple leaf sections colonized by the *Trichoderma* isolates. (Bars with same letter (s) indicate means are not significantly different ($p \leq 0.05$)).

4.4 Effect of Bio control agents on *Meloidogyne* spp. affecting pineapple

4.4.1 Effect of bio control agents on root fresh weight

The two experimental sets were statistically similar ($P \leq 0.05$) on the effect of the fungal antagonists and so results were combined for analysis. Fresh root weight of positive control (13.7 ± 0.9) was significantly ($P \leq 0.05$) lower than in all other treatments (Fig. 4.6). Fresh root weights of plants treated with *T. asperellum* M2RT4 (26.1 ± 1.1) was similar to that of *Trichoderma* sp. MK4 (22.6 ± 0.7) but significantly ($P \leq 0.05$) greater than that of the negative control; the negative control was similar to *T. atroviride* F5S21 and the two *P. lilacinum* isolates (KLF2 and MR2) (Fig. 4.6).

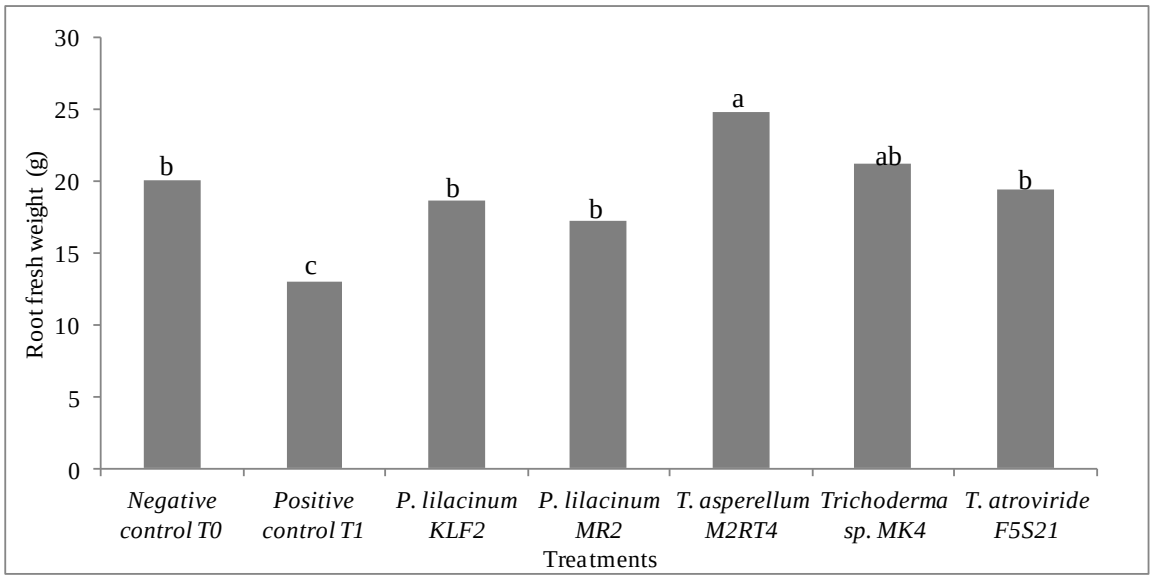

Figure 4.6: Effect of soil treatments with *Trichoderma*, *P. lilacinum* isolates and *Meloidogyne* spp. on root weight of pineapple plants 90 days after nematode inoculation. Bars with different letter (s) indicate significantly different means ($P \leq 0.05$). Mean values (n=12) pooled from two experiments repeated in time.

4.4.2 Effect of BCAs on root galling index

The analysis revealed significant difference in galling index among the different treatments ($F_{6, 35}$ = 40.6, P ≤ 0.001). Galling index was moderate on plants treated with *T. atroviride* F_5S_{21} (3.8 ± 0.17) and the positive control (3.7 ± 0.21), and significantly greater (P ≤ 0.05) than on plants treated with *Trichoderma* spp. MK_4 and the *P. lilacinum* isolates KLF_2 and MR_2 (Table 1). Galling index in *T. asperellum* M_2RT_4 treated plants (1.8 ± 0.17) was slight and significantly less (P ≤ 0.05) than for the other *Trichoderma* isolates. There was no galling in the negative control (Fig. 4.7).

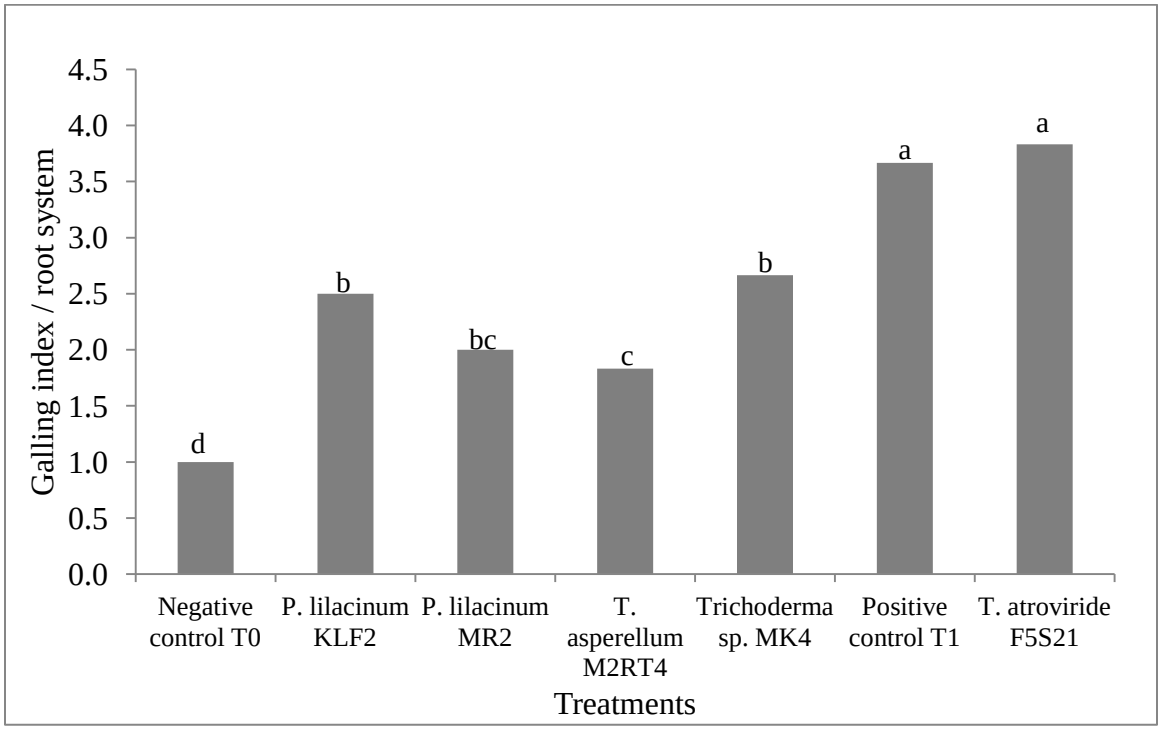

Figure 4.7: Effect of soil treatments with *Trichoderma*, *P. lilacinum* isolates and *Meloidogyne* spp. on the galling index 90 days after nematode inoculation. Bars with different letter (s) indicate significantly different means (P ≤ 0.05). Mean values (n=12) pooled from two experiments repeated in time.

4.4.3 Effect of BCAs on number of galls

Number of galls showed significant variances amongst different treatments ($F_{6, 35}$ =692, P< 0.001). The number of galls per 5 g root sample treated with *T. atroviride* F_5S_{21} (125.8 ± 10.0) was significantly ($P \leq 0.05$) greater than for the positive control. These were also significantly more ($P \leq 0.05$) than for the other BCA treatments. The number of galls for the *T. asperellum* M_2RT_4 treatment (15 ± 1.0) was significantly less ($P \leq 0.05$) than other BCA treatments except *P. lilacinum* MR_2 treated plants which had no significant difference ($P \leq 0.05$) . *P. lilacinum* MR_2 treated plants had a similar ($P \leq 0.05$) number of galls with *P. lilacinum* KLF_2. There were no galls in the negative control (Fig. 4.8).

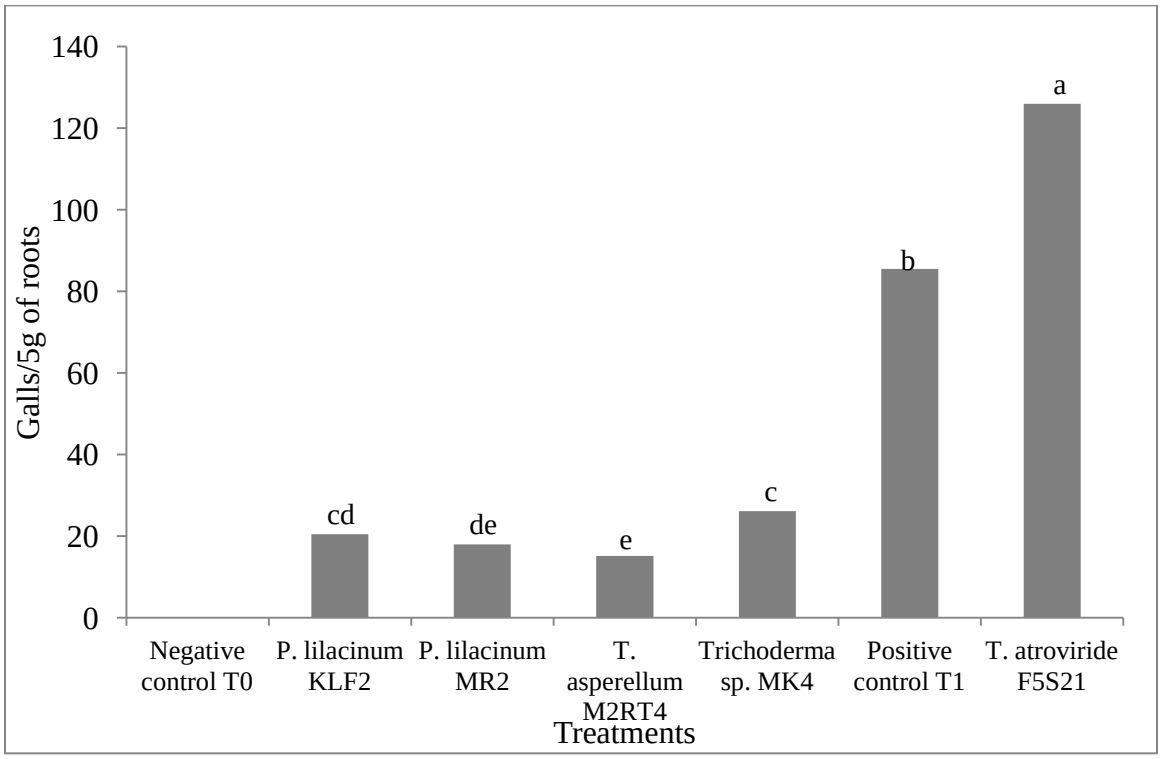

Figure 4.8: Effect of soil treatments with *Trichoderma*, *P. lilacinum* isolates and *Meloidogyne* spp. on number of galls 90 days after nematode inoculation. Bars with different letter (s) indicate significantly different means ($P \leq 0.05$). Mean values (n=12) pooled from two experiments repeated in time.

4.4.4 Effect of BCAs on egg mass and egg counts;

Significant differences were seen in the number of egg masses among the different treatments ($F_{6, 35}$ =573.9, $P \leq 0.01$. The number of egg masses per 5 g root sample on plants treated *T. atroviride* F_5S_{21} (231.7± 11.0) was similar to the positive control (209.2 ± 8.6) but significantly greater than all the other treatments. The number of egg masses on plants treated with *T. asperellum* M_2RT_4 was significantly less ($P \leq 0.05$) compared with other BCA treatments. *Trichoderma* spp. MK_4 and *P. lilacinum* KLF_2 treated plants had a similar number of eggmasses, which was less ($P \leq 0.05$) than on the positive control. There were no egg masses in the negative control (Fig. 4.9 A). The number of eggs on plants treated with *T. atroviride* F_5S_{21} (25400 ± 1470.20 per 5 g) was significantly higher ($P < 0.05$) than for the positive control T1 (20232 ± 758.54); these were also significantly greater ($P < 0.05$) than for all the other BCA treatments. The number of eggs per 5g of root sample with *T. asperellum* M_2RT_4 (1443 ± 80.15) was significantly less ($p < 0.05$) than for all other BCA treatments. *Trichoderma* sp. MK_4, *P. lilacinum* isolates (KLF_2 and MR_2) had significantly fewer ($P < 0.05$) eggs than the positive control. There were no eggs in the negative control (Fig. 4.9 B).

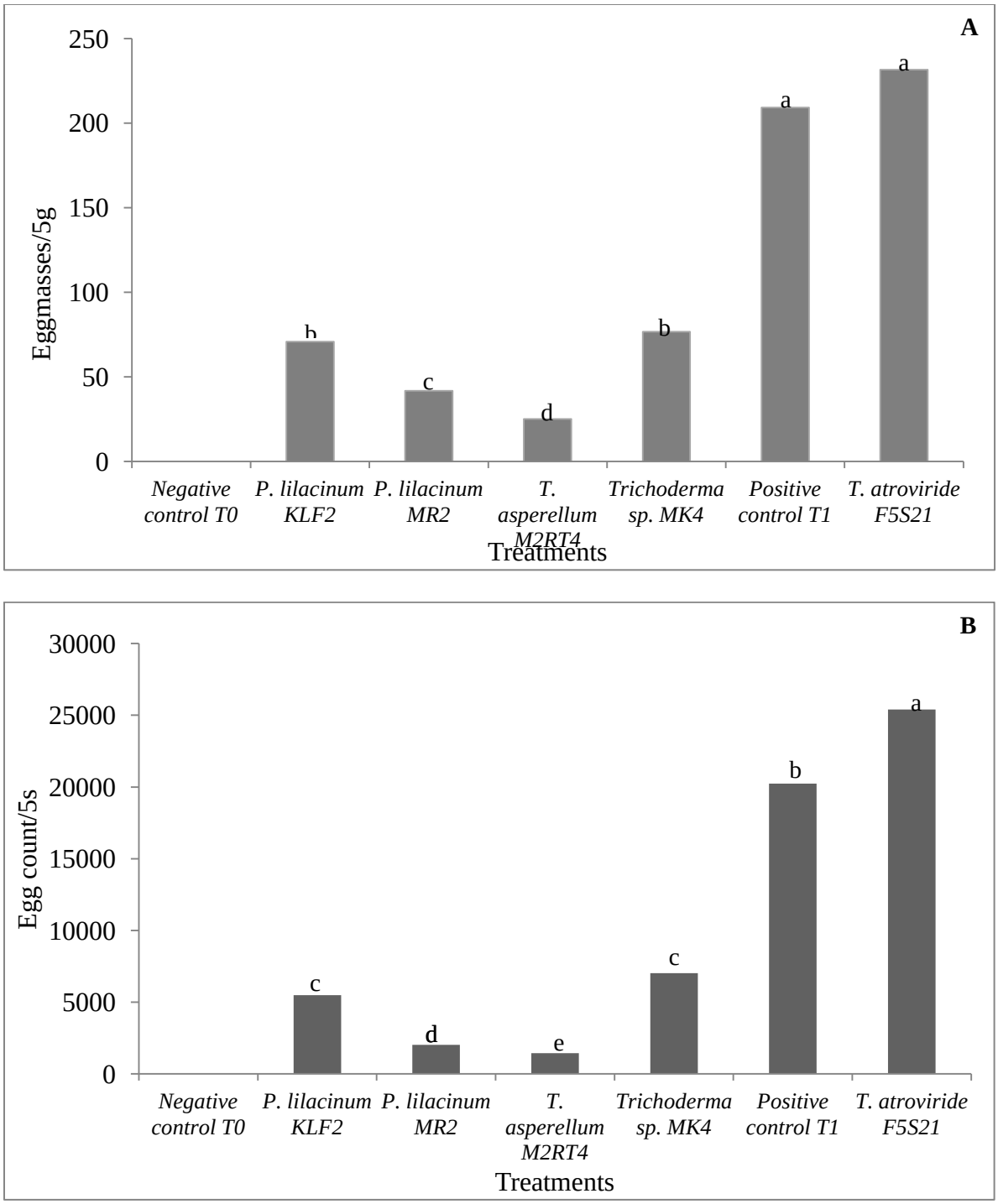

Figure 4. 9: A and B: Effect of soil treatments with *Trichoderma*, *P. lilacinum* isolates and *Meloidogyne* spp. on eggs and egg mass number 90 days after nematode inoculation. Bars with different letters indicate significantly different means ($P \leq 0.05$). Mean values (n=12) pooled from two experiments repeated in time.

4.4.5 Percentage gall reduction/increase

On average, the number of galls was reduced by 82.6% and 69.8% by application of *T. asperellum* M_2RT_4 and *Trichoderma* isolate MK_4 respectively. The number of galls was reduced by 79.1% and 75.6% by the application of *P. lilacinum* MR_2 and KLF_2 respectively. However, application of *T. atroviride* F_5S_{21} led to 46.5% more galls (Fig. 4.10).

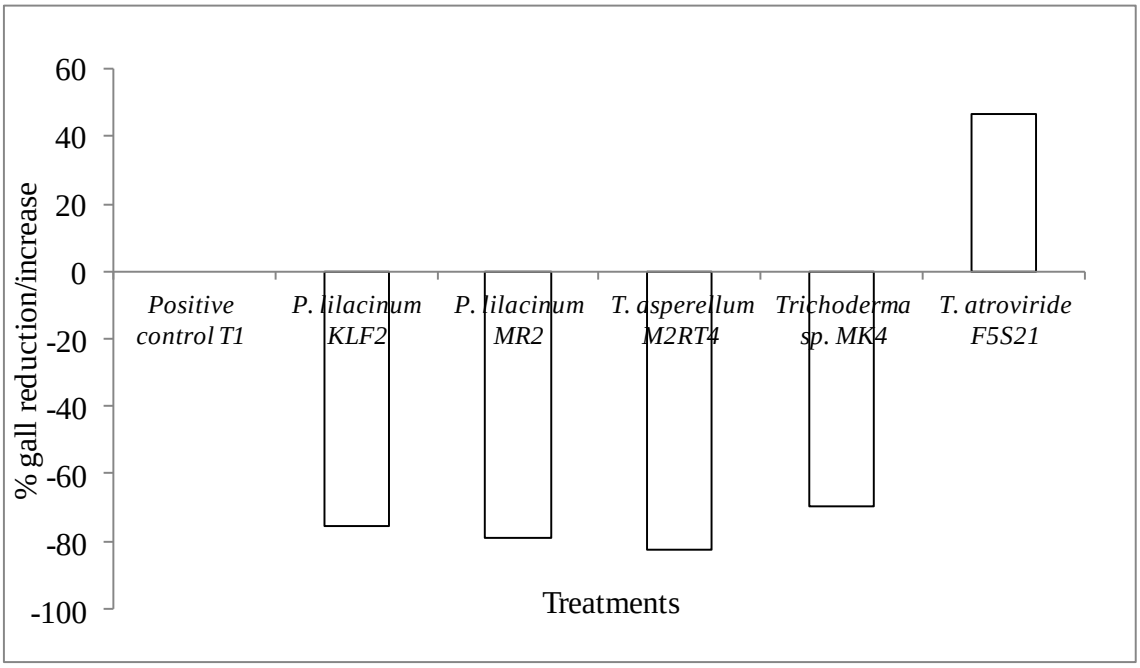

Figure 4.10: Percentage gall reduction / increase by *Trichoderma* and *P. lilacinum* isolates on pineapple. Mean values (n=12) pooled from two experiments repeated in time.

CHAPTER FIVE

5.0 DISCUSSION

5.1 Occurrence and distribution of PPNs in pineapple farms

The results of this study demonstrated that different genera of nematodes are widespread in different fields of Delmonte and Kakuzi farms. Extraction of the diverse genera from the soil and root samples in all the seven fields of Delmonte and Kakuzi farms confirms that pineapple is a host to PPN. The widespread occurrence and distribution of PPN in pineapple fields have been recorded to contribute to low pineapple production (Asopa, 2003). Migratory stage juveniles are the infective stage of most PPN genera (Abad *et al*., 2003) and the size of their initial population in the soil essentially determines the level of damage to plants. If the level of migratory juveniles is reduced during or prior to crop growing, it is likely that the initial number of nematodes establishing in crops would be minimized (Khan *et al*., 2006a).

The higher population of almost all PPNs at fallow stage than at 3 months in the two plantations was explained by the fact that the sampling was done before fumigation or treatment with nematicide was done. At 3 months the population of PPNs was significantly less than the fallow due to the application of the fumigants before planting. At 3 months, the pineapple plant is not yet well established (Rohrbach and Apt, 1986) and the densities may remain low for several months after planting (Stirling and Nikulin, 1993). The low densities were also associated with nematodes not getting enough food to enhance their reproduction.

The population of plant parasitic nematodes was higher in soils and roots of Delmonte farm than of Kakuzi farm. This could be associated with soil fumigation with 1, 3-dichloropropene during fallow stage and intensive monocultural practice in Delmonte farm. Sipes *et al.* (2005) found out that the two practices combined created a favourable soil environment for the parasites to survive and reproduce. Heavy use of toxic chemicals by Delmonte farm during fallow period reduced the parasitic nematodes initially but the practice also gave room for the nematodes to reproduce at higher rates since the crop cannot be fumigated once in the field (Sipes *et al.*, 2005). Toxic fumigants used in Delmonte farm could also be killing the beneficial organisms (e.g. nematophagous fungi and bacteria) in the soils that could be naturally suppressing the nematodes (Viaene *et al*., 2006) hence the high population of PPNs. The higher numbers of PPNs in soils, over 37%, in both farms indicated unhealthy soils. The two farms employ fallow of at most 6 months and Rohrbach and Apt (1986) indicated that shorter fallow periods could contribute to increasing problem of PPNs.

The results of this study demonstrated that *Meloidogyne* spp. are common nematodes in Delmonte and Kakuzi pineapple plantations. According to Babatola (1985) and Stirling (1993), *Meloidogyne* spp. are among the most important nematode pests of pineapple worldwide. *Meloidogyne* spp. pose major challenges in their management due to their endoparasitic nature, ability to attack a wide range of crops and their short life cycles which enables them to reproduce fast and form multiple generations within a short time (Manzanilla-Lopez and Starr, 2009). The population density of *Meloidogyne* spp. in both sites was higher in roots than soils due to their endoparasitic nature (Curtis *et al*., 2009). However higher populations found in Delmonte than Kakuzi farm could be explained by

the differences in age (Delmonte farm is older than Kakuzi farm) and the more toxic chemical used by Delmonte at fallow stage as explained above. Population density of *Meloidogyne* spp. in Kakuzi farm increased up to 11 months where the population densities started to decrease up to 36 months. Sipes *et al*. (2005) reported that the population increase of *Meloidogyne* spp. happens up to a certain level in time where the population densities may decrease or remain the same with no significant decrease occurring during the remaining part of the crop cycle until the crop is destroyed and the field fallowed. According to Sipes *et al*. (2005), the population increase of *Meloidogyne* spp. on pineapple is slow compared to other host crops. Studies have demonstrated that nematophagous fungi and bacteria increase under perennial crops and may control some PPNs (Stirling, 2011); it was speculated that these beneficial organisms could be killed by the toxic chemical used in Delmonte.

The spiral nematodes (*Helicotylenchus* spp.), were found in large numbers in the pineapple soils of Delmonte and Kakuzi and has been reported to commonly occur in soils where pineapple is grown (Nath *et al*., 1997; Costa *et al*., 1998; Sipes *et al*., 2005). Since this nematode has a wide host range, its presence in large numbers was probably influenced by cropping history such as monocropping for an extended period thus predisposing pineapple to higher densities. *Helicotylenchus* spp. has also been implicated to be economically important in pineapples (Babatola, 1985; 1986; Ko and Schmitt, 1993). Higher numbers were detected in older fields of Delmonte pineapple soils than in younger months. The presence of *Helicotylenchus* spp. in association with roots of the commercial pineapple, since they are ectoparasites, was associated with the nature of the pineapple roots whose main roots are partly suberised on the outermost layer of the bark

(Bernards, 2002). The nematodes can penetrate and hide in these corky cells. High numbers of nematodes near roots does not always guarantee their parasitism, because various free living nematodes similarly congregate in the rhizosphere where food is most abundant. Similarly, dead rootlets, killed by fungi or other agents, soon are invaded by non-parasitic forms (Linford *et al*., 1949).

Pratylenchus spp. was only detected in low numbers in roots samples of Delmonte and Kakuzi farms. Their low occurrence in the roots could be attributed to the ability of the pineapple to resist or tolerate these nematodes attack (Cook and Evans, 1987). The lesion nematode, particularly *Pratylenchus brachyurus* have been found associated with pineapple root system (Stirling, 1993) and are reported to cause major production losses (Raski and Krusberg, 1984). In Nigeria, *Pratylenchus* spp. was among the genera which were associated with poor yield and reduced sucker production (Babatola, 1985).

Other nematode genera associated with pineapple in Delmonte and Kakuzi pineapple plantations were *Tylenchus* spp. and *Aphelenchoides* spp. that occurred in large numbers and at high frequencies in the soil and root samples examined. The abundance of these nematode species in soil has been widely reported but their economic importance is yet to be established (Bafokuzara, 1996; Sharma and Amabile, 1999). Although there is need to conduct more research on such PPNs that are found in large numbers in association with pineapple, high population density of a particular PPN does not always guarantee that the nematode causes damage to the plant (Sipes *et al.*, 2005). *Rotylenchus* spp. and *Hoplolaimus* spp. were found in small numbers in both farms and research has indicated that they are of limited or unknown pathogenicity on pineapple (Sipes *et al*., 2005). Their

presence and occurrence in pineapple could probably form an additional pressure of parasitism and pose a threat to pineapple production.

5.2 Colonization of roots by *Trichoderma* spp.

Three of the four isolates of the *Trichoderma* spp. tested successfully colonized the pineapple roots and leaves endophytically and this study is the first to report such a finding on pineapple. The F2L4 isolate of *T. harzianum* did not meet these criteria though whether this was related to the soil substrate used, incompatibility with pineapple roots, or the strain tested remains unknown. *Trichoderma* spp. have been widely demonstrated to colonize the roots of other plant species endophytically (Yedidia *et al*., 1999; Sharon *et al*., 2001; Dababat and Sikora, 2007) but rates of fungal growth can differ markedly between strains within a species (Ahmad and Baker, 1987).

Successful colonization of the rhizosphere by *Trichoderma* and its potential as a biocontrol agent are dependent on its ability to compete for nutrients and space (Howell, 2003; Harman, 2006). Most successful strains not only colonize the roots, but can also penetrate the epidermis, access the cortex, and even enhance plant development (Harman and Kubicek 1998, Yedidia *et al*., 1999). To colonize the host the fungus should be compatible with both the host cultivar and the soil substrate (Al-Hazmi and TariqJaveed, 2016). Van Damme *et al*. (2005) and Dababat *et al*. (2006) recommended that the *Trichoderma* sp. needs to be applied before the introduction of the crop to enhance maximum colonization once seedlings are transplanted. The endophytes colonize the same root tissues as sedentary plant-parasitic nematodes making them competent biological control agents if applied at the right time (Siddiqui and Shaukat, 2003).

5.3 Biocontrol of *Meloidogyne* spp.

All the biocontrol agents tested significantly increased the root fresh weight of the pineapple when compared to the *M. javanica* treated plants. Their ability to increase the root weight was attributed to the competence of the fungus in promoting root establishment. Several studies have proved that *Trichoderma* spp. and other endophytic fungi are able to improve the growth of the plant (Spiegel and Chet, 1998; Pandey *et al*., 2003; Sharon *et al*., 2007; Lamovšek *et al*., 2013). Root weight increased significantly when the tomato seedlings were inoculated with *T. harzianum* BI at planting compared to nematode controls (Naserinasab *et al*., 2011). Pre-colonization of the seedlings with *Trichoderma* (T-203) during their growth in the nursery significantly improved fresh weight of *Trichoderma*-treated plants (Sharon *et al*., 2001) compared to the non treated plants.

Similar results were obtained by other researchers with *P. lilacinum* on its effect on plant growth. *Purpureocillium lilacinum* significantly improved the growth of tomato plants inoculated with 2000 juveniles of *M. javanica* (Ganaie and Khan, 2010). *Bradyrhizobium* and fungus *P. lilacinum* biocontrol agents resulted in increased plant growth on black gram plants (Bhat *et al*., 2012) by increasing mineral uptake of plants thus enabling the plant escape the damage from pathogens (Kiewnick and Sikora, 2006; Siddiqui, 2006). However, Oclarit and Cumagun (2009) found that the weight of tomato roots treated with *M. incognita* alone was significantly higher than those of plants treated with *P. lilacinum*. Lowest weight recorded in infested control of this study was probably due to the presence of galls which led to poor establishment of the roots; severe infection leads to stunted

root system and the plants are altered in the uptake of water and nutrients (Sipes *et al*., 2005).

This study demonstrated suppression of root galling on pineapple by two *Trichoderma* isolates; *T. asperellum*-M_2RT_4 and *Trichoderma* sp. MK_4. Different isolates of *Trichoderma* have been variously reported to significantly reduce the root galling (Spiegel and Sharon, 2005; Sharon *et al*., 2001). Similar results were obtained in other studies done on *Trichoderma* spp. where different isolates reduced root galling of *M. javanica* on tomatoes (Spiegel and Chet, 1998) and *M. incognita* on chickpea in both field and pot experiments (Pandey *et al*., 2003). *Trichoderma harzianum* had significant reduction on galling indices of root-knot nematode *M. javanica* on tomato plants in a greenhouse experiment (Sharon *et al*., 2001, Sahebani and Hadavi, 2008). Simultaneous treatment of the soil at transplanting with *Trichoderma* spp. and *M. incognita* resulted in nematode galling reduction of up to 19.5 %. According to Sharon *et al*. (2007), tomato roots that were pre-treated and colonized by *T. asperellum*-203 demonstrated significantly fewer galls compared to the roots that were not treated.

Trichoderma isolates, *T. asperellum*-M_2RT_4 and *Trichoderma* sp. MK_4, reduced egg mass and egg production thus effectively suppressing damage by *M. javanica* while *T. atroviride* F_5S_{21} did not. Suitability as a biocontrol agent needs to consider the broader range of characteristics than the ability to colonize roots. Howell (2003) found that strains of *T. koningii*, which were outstanding root colonizers, showed little or no biocontrol activity. Findings by Sharon *et al.* (2001) demonstrated the nematicidal activity of *T. asperellum* (T-203) against *M. javanica*. Naserinasab *et al*. (2011) reported that *T. harzianum* BI reduced egg masses, eggs number of *M. javanica* on tomato in a pot

experiment in the greenhouse. Windham *et al.* (1989) reported a reduction in egg production by *M. arenaria*, following soil treatments with T-12 isolate of *T. harzianum* and T-8 isolate of *T. koningii* preparations in maize plants. Sharon *et al.* (2007) who demonstrated the parasitism of egg masses, eggs and J2s inside them by a strain of *T. atroviride* contradicted the findings of this study where F_5S_{21} a strain of *T. atroviride* in this study increased the egg mass and egg production. Our results showed that some strains once they colonize the plant rhizosphere they suppress the immunity of the plant making the host more susceptible to pathogens (Howel, 2003).

Trichoderma atroviride-F5S21 had a negative effect on *M. javanica* affecting pineapple although it was found to colonize their roots endophytically. Al-Hazmi and TariqJaveed (2016) suggested the need to understand the compatibility of the fungal isolate with the host and the soil, which is crucial in establishment and survival of the *Trichoderma* spp. It is essential that the biocontrol isolate persists in the soil, readily colonizes the environment and efficiently establishes on the host plant (Harman, 2000, Howell *et al*., 2000) to enable its competence as a successful biocontrol agent (Singh and Singh, 2009). However, strains of *T. koningii* were found to be outstanding root colonizers but showed little or no biocontrol activity. Thus there is need to consider a broader range of characteristics beyond the ability to colonize roots (Howell, 2003). Endophytic *Trichoderma* spp. have previously been shown to suppress the damage caused by nematodes, in part by preventing nematode penetration (Lamovšek *et al*., 2013). Similarly, the nematicidal activity of *T. asperellum* (T-203) (Sharon *et al*., 2001) and *T. atroviride* have been confirmed (Sharon *et al*., 2007), demonstrating that strain, target pest species and host need to be compatible.

The two isolates of *P. lilacinum* (MR_2 and KLF_2) in our study reduced root galling, egg mass and egg production, reflecting results similar to those reported by Ganaie and Khan (2010) on tomatoes, when applied 10 days prior to introduction of *M. javanica* inoculum. Results similar to this study have also been reported by different researchers on effect of *P. lilacinum* on *Meloidogyne* spp. (Bonants *et al*., 1995; Spiegel and Chet, 1998; Sharon *et al*., 2001; Kienwick and Sikora, 2006). Jatala *et al*. (1986) reported parasitism on eggs and other developmental stages of several nematode species by *P. lilacinum*. *Purpureocillium lilacinum* alone reduced the galls in tomato by 53% compared to the *M. javanica*-inoculated control (Khan *et al*., 2006a) in pot trials. *Purpureocillium lilacinum* is considered a good rhizosphere competitor (Mukhtar *et al.*, 2013) and its suppressive effect has been reported to reduce *M. incognita* and *M. javanica* in soil and roots of tomato (Lara *et al*., 1996; Siddiqui *et al*., 2000). Kienwick and Sikora (2006) demonstrated increased biocontrol efficacy of *P. lilacinum* 251 on *M. incognita* in tomato when applied before planting, combined with a seedling drench and second application. According to Rodriguez-Kabana *et al*. (1984), isolates of *P. lilacinum* differ widely in their biocontrol capacity and ability to establish in soil, emphasising the need to establish compatibility with the local specific circumstances.

CHAPTER SIX

6.1 CONCLUSIONS

Significantly high occurrence of plant parasitic nematodes was observed in the fields of Delmonte and Kakuzi commercial pineapple farms. This indicates that pineapple is a potential host to PPN pests. *Meloidogyne javanica* was the most dominant species in pineapple production in these farms with higher populations found in older fields. *Helicotylenchus* spp. was also common in Delmonte and Kakuzi pineapple farms. The widespread distribution of these and other plant parasitic nematodes in Delmonte and Kakuzi farms is disturbing and could have a negative impact on yields in pineapple production.

This study indicates that both *Trichoderma* spp. and *P. lilacinum* isolates had direct and indirect effects on nematode reproduction (eggs and egg masses) and host response (host growth and root galling). These results demonstrate significant suppressive effects of isolates *Trichoderma asperellum* (M_2RT_4) (ICIPE), *Trichoderma* isolate MK4 and the *P. lilacinum* isolates (KLF_2 and MR_2) (KBL) against *M. javanica* on pineapple. *Trichoderma* spp. colonized the roots of pineapple endophytically. This study is, to our knowledge, the first to report the endophytic colonization of *Trichoderma* spp. on pineapple, and its consequent suppression of root knot nematodes on the crop.

The results of this study provide strong support for exploring further the use of fungal biocontrol agents for nematode management on pineapple under field conditions. Therefore, these fungal isolates represent a new tool for an integrated management program for *Meloidogyne* spp.

6.2 RECOMMENDATIONs

The study makes the following recommendations;

The results can be used to improve on surveillance methods/ procedures through describing the distribution and prevalence of nematodes already present as well as enabling management and cost benefit decisions.

Having established that *Meloidogyne* spp. and *Helicotylenchus* spp. occurs in commercial pineapple farms of Kenya, there is need to determine the possible economic damage caused by association of such high population densities with pineapple plants. More research is also needed to evaluate the damage threshold of these nematodes in pineapple and the amount of economic damage they cause in the commercial pineapple plantations.

There is also need for enhancing awareness of these pests to pineapple producers mainly because; (i) sometimes the host may be heavily infected without developing typical big galls on the roots and ii) The above-ground symptoms are not easy to differentiate from those caused by other factors and hence the problem become hard to recognize.

The results can help design better management systems of nematodes which are more environmentally sensitive options for combining with other management methods towards more sustainable pineapple production systems.

The study recommends longer fallow systems and monitoring of crop age as this could help reduce the problem of plant parasitic nematodes

REFERENCES

Abad, P., Favery, B., Rosso, M.N. and Castagnone-Sereno, P. (2003). Root-knot nematode parasitism and host response: molecular basis of a sophisticated interaction. *Molecular Plant Pathology* 14, 217-224.

Abawi, G.S. and Widmer, T.L. (2000). Impact of soil health management practices on soilborne pathogens, nematodes and root diseases of vegetable crops. *Applied Soil Ecology* 15, 37-47.

Adam, M., Phillips, M., and Blok, V. (2007). Molecular diagnostic key for identification of single juveniles of seven common and economically important species of root-knot nematode (*Meloidogyne* spp.). *Plant Pathology* 56(1), 190-197.

Ahmad, J.S., and Baker, R. (1987). Rhizosphere competence of *Trichoderma harzianum. Phytopathology* 7, 182-189.

Akhtar, M. (1998). Biological control of plant parasitic nematodes by neem products in agricultural soil. *Applied Soil Ecology* 7, 219-223. ISSN 0105-8568

Akhtar, M. and Malik, A. (2000). Roles of organic soil amendments and soil organisms in the biological control of plant-parasitic nematodes: a review. *Bioresource Technology* 74, 35-47.

Al-hazmi, A.S. and TariqJaveed, M. (2016). Effects of different inoculum densities of *Trichoderma harzianum* and *Trichoderma viride* against *Meloidogyne javanica* on tomato. *Saudi Journal of Biological Sciences* 23(2), 288–292

Ashraf, M.S. and Khan, T.A. (2010). Integrated approach for the management of *Meloidogyne javanica* on eggplant using oil cakes and biocontrol agents. Archives of *Phytopathology* and *Plant Protection* 43, 609-614.

Asopa, V.N. (2003). Competitiveness in pineapple canning industry Hawaii international conference on business. Hawaii USA, *Honolulu,* pp. 1-3.

Atkins, S.D., Clark, I.M., Pande, S., Hirsch, P.R. and Kerry, B.K. (2005). The use of real-time PCR and species-specific primers for the identification and monitoring of *Paecilomyces lilacinus*. FEMS *Microbial Ecology* 51, 257–264.

Aubertot, J.N., West, J.S., Bousset-Vaslin, L., Salam, M.U., Barbetti, M.J. and Diggle, A.J. (2006). Improved resistance management for durable disease control: A case study of phoma stem canker of oilseed rape (*Brassica napus*). *The European Journal of Plant Pathology* 114, 91-106.

Babatola, J.O. (1985). Diseases and Pests of fruits and their control in Nigeria: In proceedings of the national workshop on fruit production in Nigeria held at Federal Agriculture Coordinating Unit (FACU), Ibadan, pp. 120-131.

Backman, P.A., Wilson, M. and Murphy, J.F. (1997). Bacteria for biological control of plant diseases. In: Rechcigl N.A. and Rechcigl J.E. (Eds.). Environmentally safe approaches to crop disease control. Boca Raton, SL, USA, *CRC Press* (*Lewis Publishers*), pp. 95-109.

Bafokuzara, N.D. (1996). Incidence of different nematodes on vegetable and fruit crops and preliminary assessment of yield loss due to *Meloidogyne* spp. in Uganda. *Nematologia Brasileira* 20, 32-43.

Bartholomew, D.P., Paull, R.E., and Rohrbach, K.G. (2003). The Pineapple: Botany, production and uses. Wallingford, UK. *CABI Publishing*, Pp. 1-301.

Bernards, M.A. (2002). "Demystifying suberin". *Canadian Journal of Botany* 80 (3), 227–240.

Bert, W., Leliaert, F., Vierstraete, A. R., Vanfleteren, J. R. and Borgonie, G. (2008). Molecular phylogeny of the Tylenchina and evolution of the female gonoduct (Nematoda : Rhabditida). *Molecular Phylogenetics and Evolution* 48, 728–744.

Bhat, M.Y., Wani, A.H. and Fazal, M. (2012). Effect of *Paecilomyces lilacinus* and plant growth promoting rhizobacteria on *Meloidogyne incognita* inoculated black gram, *Vigna mungo* plants. *Journal of Biopesticides* 5(1), 36-43.

Bishop, A.H., Gowen, S.R., Pembroke, B., and Trotter, J.R. (2007). Morphological and molecular characteristics of a new species of *Pasteuria* parasitic on *Meloidogyne ardenensis*. *Journal of invertebrate pathology* 96(1), 28-33.

Bonants, P.J.M., Fitters, P.F.L., Thijs H., DenBelder, E., Waalwijik, C. and Henfling, J. (1995). A basic serine protease from *Paecilomyces lilacinus* with biological activity against *Meloidogyne hapla* eggs. *Microbiology* 141, 775 -784.

Brand, D., Roussos, S., Pandey, A., Zilioli, P.C., Pohl, J. and Soccol, C.R. (2004). Development of a bionematicide with *Paecilomyces lilacinus* to control *Meloidogyne incognita*. *Applied biochemistry* and *biotechnology* 118, 81–88.

Cavalcante, U.M.T., Warumby, J.F., Bezerra, J.E.F., and Moura, R.M. (1984). Nematóides associados aos abacaxizeiros no Estado de Pernambuco. *Nematologia Brasileira* 8, 39–45.

Caveness, F.E. (1965). End of Tour Progress report on the nematology project. Ministry of Agriculture and Natural Resources Western Region, Nigeria and the United States of America Agency for International Development. *USAID/NIGERIA* pp. 47.

Collange, B., Navarrete, M., Peyre, G., Mateille, T. and Tchamitchian, M. (2011). Root-knot nematode (*Meloidogyne*) management in vegetable crop production: the challenge of an agronomic system analysis. *Crop Protection* 30(10), 1251-1262.

Cook, R. and Evans, K. (1987). Resistance and tolerance. In: Brown R. H. and Kerry B.R. (Eds.), principles and practice of nematode control in crops. *Sydney*, *Academic*, pp. 179-231.

Coppens d'Eeckenbrugge, G., Sanewski, G.M., Smith, M.K., Duval, M.F., Leal, F. (2011). Ananas. In: Kole, C. (Eds.), wild crop relatives: Genomic and breeding resources, Tropical and Subtropical fruits. *Berlin*, *Springer*, pp. 21–41.

Correa, V.R., Mattos, V.S., Almeida, M.R.A., Santos, M.F.A., Tigano, M.S., Castagnone-sereno, P. and Carneiro, R.M.D.G. (2014). Genetic diversity of the root-knot nematode *Meloidogyne ethiopica* and development of a species-specific SCAR marker for its diagnosis. *Plant Pathology* 63, 476–483.

Costa, D.C. and Matos, A.P. (2000). Nematoses. Embrapa: Mandioca e Fruticultura Tropical, Cruz das Almas – Ba,Circular 2333.

Costa, D.C., Sanches, N.F. and Santos, J.M. (1998). Levantamento de fitonematóides associados ao abacaxizeiro. *Revista Brasileira de Fruticultura* 20, 392–396.

Coveca, D.P. (2002). Comision veracruzana de comercializacion agropecuaria. Gobierno del Estado de Veracruz, México.

Coyne, D.L., Fourie, H.H., and Moens, M. (2009). Current and future management strategies in resource-poor farming. In: Perry R.N., Moens M., and Starr J.L. (Eds.). Root-Knot Nematodes. Wallingford UK, *CAB International*, pp. 444-447.

Coyne, D.L., Nicol, J.M. and Claudius-Cole, B. (2014). Practical plant nematology: a field and laboratory guide. 2nd edition. SP-IPM Secretariat, International Institute of Tropical Agriculture (IITA), Cotonou, Benin.

Cuadra, R., Ortega, J., Morfi, O.L., Soto, L., Zayas, M.D.l.A., Perera, E. (2008). Effect of the biological controls Trifesol and Nemacid on root-knot nematodes in sheltered vegetable production. *Revista de Protección Vegetal* 23, 59-62.

Curtis, R.H.C., Robinson, A.F. and Perry, R.N. (2009). Hatch and host location. In: Perry R.N., Moens M., and Starr J.L. (Eds.). Root-Knot Nematodes. Wallingford, UK, *CAB International*, pp. 139-155.

Dababat, A.A. and Sikora, R.A. (2007). Use of *Trichoderma harzianum* and *Trichoderma viride* for the biological control of *Meloidogyne incognita* on tomato. *Jordan Journal of Agricultural Sciences* 3, 953–961.

Dababat, A.A., Hauschild, R., and Sikora, R. (2006). Comparative effect of *Trichoderma* spp. and the mutualistic endophyte *Fusarium Oxysporum* 162 on *Meloidogyne Incognita* infestation of tomato, 58th international symposium on crop crotection, Ghent University, (Abstract).

Daramola, F. and Afolami, S. (2014). Studies on the distribution of plant-parasitic nematodes associated with pineapple in Delta, Imo and Cross River states of Nigeria. *Australian Journal of Basic and Applied Sciences* 8 (7), 248-256.

Davide, R.G. and Zorilla, R.A. (1985). Evaluation of a fungus *Paecilomyces lilacinus* for the biological control of root-knot nematodes *Meloidogyne incognita* on okra as compared with nematicide Isazofos. *Philippine Agricultural Scientist* 68, 493–500.

Davies, K.G., Laird, V. and Kerry B.R. (1991). The motility, development and infection of *Meloidogyne incognita* encumbered with spores of the obligate hyperparasite *Pasteuria penetrans*. *Nematology* 14, 611–18

De Waele, D. and Elsen, A. (2007). Challenges in tropical plant nematology. *Annual Review* of *Phytopathology* 45, 457-485.

Diepens, N.J., Pfennig, S., Van den Brink, P.J., Gunnarsson, J.S., Ruepert, C. and Castillo, L.E. (2014). Effect of pesticides used in banana and pineapple plantations on aquatic ecosystems in Costa Rica. *Journal of Environmental Biology* 35, 73-84.

Duncan, L.W. (1991). Current options for nematode management. *Annual Review of Phytopathology* 29, 469-90

Eisenback, J.D. and Hirschmann, H. (1980). Morphological comparison of *Meloidogyne* males by electron microscopy. *Journal of Nematology* 12, 23- 32.

Elad, Y. and Freeman, S. (2002). Biological control of fungal plant pathogens. In: Esser K. and Benett J.W. (Eds.), The Mycota a comprehensive treatise on fungi as experimental systems for basic and applied research. Germany, *Springer Press*, pp. 92-109.

FAOSTAT, 2015. http://faostat3.fao.org/browse/Q/*/E. (accessed 28.03.17).

Ganaie, M.A. and Khan, T.A. (2010). Biological potential of *Paecilomyces lilacinus* on pathogenesis of *Meloidogyne javanica* infecting tomato plant. *European Journal of Applied Sciences* 2, 80-84.

Gaspard, J.T., Jaffee, B.A. and Ferris, H. (1990). *Meloidogyne incognita* survival in soil infested with *Paecilomyces lilacinus* and *Verticillium chlamydosporium*. *Journal of Nematology* 22, 176–181

Gautam, A., Siddiqui, Z.A. and Mahmood, I. (1995). Integrated management of *Meloidogyne incognita* on tomato. *Nematologia Mediterranea* 23, 245-247.

Gheysen, G. and Fenoll, C. (2002). Gene expression in nematode feeding sites. *Annual Review of Phytopathology* 40, 191-219.

Gianessi, L.P., Silvers, C.S., Sankula, S. and Carpenter, J. (2002). Plant biotechnology; current and potential. impact for improving pest management in US agriculture. An analysis of 40 case studies. National Centre for Food and Agricultural Policy (NCFAP). Washington D.C, 20036, 2-8.

Goswami, B.K. and Mittal, A. (2004). Management of root-knot nematode infecting tomato by *Trichoderma viride* and *Paecilomyces lilacinus*. *Indian Phytopathology* 57, 235-236.

Goswami, B.K., Pandey, R.K., Rathour, K.S., Bhattacharya, C. and Singh, L. (2006). Integrated application of some compatible biocontrol agents along with mustard oil seed cake and furadan on *Meloidogyne incognita* infecting tomato plants. *Journal of Zhejiang University Science* 7, 873-875.

Government of Kenya, (2014). Republic of Kenya Murang'a County first County integrated development plan 2013-2017. http://www.kpda.or.ke/documents/CIDP/Murang%27a.pdf (accessed 28.03.17).

Government of Kenya, (2015). County government of Kiambu physiographic and natural conditions. http://www.kiambu.go.ke/about/physiographic-natural-conditions (accessed 28.03.17).

Hallman, J., Davies, K.G. and Sikora, R. (2009). Biological control using microbial pathogens, endophytes and antagonists. In: Perry R.N., Moens M., Starr J.L. (Eds.). Root-Knot Nematodes. Wallingford, UK, *CAB International*, pp. 380-411.

Harman, G.E. (2000). Myths and dogmas of biocontrol: Changes in perceptions derived from research on *Trichoderma harzianum* T-22. *Plant Diseases* 84, 377-391.

Harman, G.E. and Kubicek C. P. (1998). *Trichoderma* and *Gliocladium*, Vol. 2 Enzymes, Biological control and Commercial Applications. Taylor and Francis, London,UK.

Harman, G.E., Howell C.R., Viterbo A., Chet I. and Lorito M. (2004). *Trichoderma* species - opportunistic, avirulent plant symbionts. *Nature Reviews Microbiology* 2, 43-56.

Hasabo, S.A. and Noweer, E.M.A. (2005). Management of root-knot nematode *Meloidogyne incognita* on egg plant with some plant extracts. *Egyptian Journal of Phytopathology*. 33, 65-72.

Haseeb, A. and Kumar, V. (2006). Management of *Meloidogyne incognita-Fusarium solani* disease complex in brinjal by biological control agents and organic additives. *Annals of Plant Protection Sciences* 14, 519-521.

Hewlett, T.E., Dickson D.W., Mitchell D.J. and Kannwischer-Mitchell, M.E. (1988). Evaluation of *Paecilomyces lilacinus* as a biocontrol agent of *Meloidogyne javanica* on tobacco. *Journal of Nematology* 20, 578–584.

Holbrook, C.C., Knauft, D.A. and Dickson,D.W. (1983). A technique for screening peanut for resistance to *Meloidogyne arenaria*. *Plant Disease* 57, 957–958.

Holland, R.J., Williams K.L. and Khan, A. (1999). Infection of *Meloidogyne javanica* by *Paecilomyces lilacinus*. *Nematology* 1, 131–139.

Hooper, D.J., Hallmann, J. and Subbotin, S. (2005). Methods for extraction, processing and detection of plant and soil nematodes. In: Luc, M., Sikora, R.A. and Bridge J. (Eds.), plant parasitic nematodes in Subtropical and Tropical agriculture. Wallingford, UK. *CAB International*, pp. 53-86.

Horticultural Crops Development Authority (HCDA) (2008). Horticulture data 2005-2007 validation report, Kenya: HCDA, Pp 1-72. http://www.countrystat.org/country/ken/doc/methodology/horticulturevalidation.pdf (accessed 28.03.17).

Howell, C.R. (2003). Mechanisms employed by *Trichoderma* species in the biological control of plant diseases: The history and evolution of current concepts. *Plant Disease* 87, 4-10.

Howell, C.R., Hanson, L.E., Stipanovic, R.D. and Puckhaber, L.S. (2000). Induction of terpenoid synthesis in cotton roots and control of *Rhizoctonia solani* by seed treatment with *Trichoderma virens*. *Phytopathology* 90, 248–252.

http://www.Delmonte.co.ke/ (accessed 03.04.17).

http://www.distancesfrom.com/ke/Delmonte-thika-latitude-longitude-Delmonte-thika-latitude-Delmonte-thika_longitude/LatLongHistory/1215575.aspx? IsHistory= 1andLocationID= 1215575. (accessed 28.03.17).

http://www.naads.or.ug/files/downloads/PINEAPPLE%20PRODUCTION.pdf (accessed 28.03.17).

Hunt, D.J., Luc, M., Manzanilla-López, R.H. and Luc, M. (2005). Identification, morphology and biology of plant parasitic nematodes. In: Luc M.S., Sikora R.A., and Bridge J. (Eds.), plant parasitic nematodes in subtropical and tropical agriculture. Wallingford, UK, *CAB International*, pp 11-52.

Hussain M.A., Mukhtar T., Kayani M.Z. (2011). Efficacy evaluation of *Azadirachta indica*, *Calotropis procera*, *Datura stramonium* and *Tagetes erecta* against root-knot nematodes *Meloidogyne incognita*. *Pakistan Journal of Botany* 43, 197–204.

Jatala, P. (1986). Biological control of plant parasitic nematodes. *The Annual Review of Phytopathology* 24, 453-489.

Jatala, P., Kaltenback, R., Bocangel, M., Devaus, A.J. and Campos, R. (1980). Field application of *Paecilomyces lilacinus* for controlling *Meloidogyne incognita* on potatoes. *Journal of Nematology* 12, 226–227.

Jonathan, E.I. and Rajendran, G. (2000). Biocontrol potential of the parasitic fungus *Paecilomyces lilacinus* against the root-knot nematode *Meloidogyne incognita* in banana. *Journal of Biological Control* 14, 67–69.

Jones, J.T., Haegeman, A., Danchin, E.G., Gaur, H.S., Helder, J., Jones, M.G., Kikuchi, T., Manzanilla-López R., Palomares-Rius J.E., Wesemael, W.M. and Perry R.N. (2013). Top ten plant-parasitic nematodes in molecular plant pathology. *Molecular plant pathology* 14(9), 946-961.

Kariuki, G., Kariuki, F., Birgen, J., and Gathaara, V. (2010). Participatory development, testing and validation of concepts and technologies for site-specific detection and

control of plant parasitic nematodes infecting tomatoes in Mwea, Kenya. Second RUFORUM Biennial Regional Conference on "Building capacity for food security in Africa", *Entebbe, Uganda*, 20-24 September 2010.

Karssen, G., Wesemael, W. and Moens, M. (2013). Root-knot nematodes. In: Perry R. and Moens M. (Eds.). Plant Nematology. Wallingford, UK, *Cambridge*, pp. 73-108.

Kerry, B.R. and Evans, K. (1996). New strategies for the management of plant parasitic nematodes. In: Hall, R. (Eds.), principles and practice of managing soilborne plant pathogens. St. Paul, Minnesota, *The American Phytopathological Society Press*, pp. 134–152.

Kerry, B.R. and Hidalgo-diaz, L. (2004). Application of *Pochonia chlamydosporia* in the integrated control of root-knot nematodes on organically grown vegetable crops in Cuba. *International Organization for Biological Control WPRS* 27, 23-26.

Khan, A., Williams, K.L. and Nevalainen, H.K.M. (2004). Effects of *Paecilomyces lilacinus* protease and chitinase on the eggshell structures and hatching of *Meloidogyne javanica* juveniles. *Biological Control* 31, 346–352.

Khan, A., Williams, K.L. and Nevalainen, H.K.M. (2006a). Control of plant- parasitic nematodes by *Paecilomyces lilacinus* and *Monacrosporium lysipagum* in pot trials. *BioControl* 51, 643–658.

Khan, A., Williams, K.L. and Nevalainen, H.K.M. (2006b). Infection of plant-parasitic nematodes by *Paecilomyces lilacinus* and *Monacrosporium lysipagum. BioControl* 51, 659–678.

Khan, A., Williams, K.L., Molloy, M.P. and Nevalainen, H.K.M. (2003). Purification and characterization of a serine protease and chitinases from *Paecilomyces lilacinus* and detection of chitinase activity on 2D gels. *Protein Expression and Purification* 32, 210-220.

Khan, T.A. and Saxena, S.K. (1997). Effect of root-dip treatment with fungal filtrates on root penetration, development and reproduction of *Meloidogyne javanica* on tomato. *International Journal of nematology* 7, 85-88.

Khan, Z., Kim, S.G., Jeon, Y.H., Khan, H.U., Son, S.H. and Kim, Y.H. (2008). A plant growth promoting rhizobacterium, *Paenibacillus polymyxa* strain GBR-1, suppresses root-knot nematode. *Bioresource Technology* 99, 3016-3023.

Kiewnick, S. and Sikora, R.A. (2006). Biological control of the root-knot nematode *Meloidogyne incognita* by *Paecilomyces lilacinus* strain 251. *Biological Control* 38(2), 179-187.

Kiewnick, S., Lueth P. and Sikora, R.A. (2002). Development of a biocontrol product based on *Paecilomyces lilacinus* (strain 251). *Phytopathology* 92, S41–S42.

Ko, M.P. and Schmitt, D.P. (1993). Pineapple inter-cycle cover crops to reduce plant-parasitic nematode populations. *Acta Horticulturae* 334, 373-382.

Koech, W., Ithinji, G.K., Kibet, L.K. and Ngenoh, E. (2014). Evaluation of allocative efficiency of small scale pineapple (*Ananas comosus*) production in Bureti District, Kenya. *Agricultural Journal* 9(1), 61-67.

Kormelinck, A.G. and Janssen, I. (2012). Business case Pineapple West-Kenya-Wageningen UR Centre for Development Innovation, pp 1-15.

Kumar, D. and Singh, K.P. (2006). Assessment of predacity and efficacy of *Arthrobotrys dactyloides* for biological control of root knot disease of tomato. *Journal of Phytopathology* 154, 1-5.

Kumar, V., Haseeb, A. and Sharma, A. (2009). Integrated management of *Meloidogyne incognita-Fusarium solani* disease complex of brinjal cv. Pusa Kranti. *Annals of Plant Protection Sciences* 17, 192-194.

Lacerda, J.T., Carvalho, R.A., and Oliveira, E.F. (2009). *Cochonilha Dysmicoccus* brevipes: a praga cosmopolita da abacaxicultura. *Tecnologia e Ciências Agropecuárias* 3, 15–21.

Lamovšek, J., Urek, G. and Trdan, S. (2013). Biological Control of Root-Knot Nematodes (*Meloidogyne* spp.): Microbes against the Pests. *Acta agriculturae Slovenica* 263 – 275.

Lara, J., Acosta, N., Betancourt, C., Vincente, N. and Rodrıguez, R. (1996). Biological control of *Meloidogyne incognita* in tomato in Puerto Rico. *Nematropica* 26, 143–152.

Le H.T.T., Padgham J.L. and Sikora R.A. (2009). Biological control of the rice root-knot nematode *Meloidogyne graminicola* on rice, using endophytic and rhizosphere fungi, *International Journal of Pest Management* 55:1, 31-36

Li, J., Todd, T.C., Lee, J. and Trick, H.N. (2011). Biotechnological application of functional genomics towards plant-parasitic nematode control. *Plant Biotechnology Journal* 9, 936–944. doi:10.1111/j.1467-7652.2011. 00601.x

Lichtfouse, E., Navarrete, M., Debaeke, P., Souchère, V., Alberola, C., and Ménassieu, J. (2009). Agronomy for sustainable agriculture: a review *Sustainable Agriculture, Springer*, pp. 1-7.

Linford, M.B., Oliveira, J.M. and Ishii, M. (1949). *Paratylenchus minutes* sp., a nematode parasitic on roots. *Pacific science* 3, 111-119.

Lopez-Lima, D., Carrion, G. and Núñez-Sánchez, Á.E. (2014). Isolation of fungi associated with *Criconemoides* sp. and their potential use in the biological control of ectoparasitic and semiendoparasitic nematodes in sugar cane. *Australian Journal of Crop Science* 8(3), 389-396.

Luc, M., Bridge, J. and Sikora, R.A. (2005). Reflections on nematology in subtropical and tropical Agriculture. In: Luc, M., Sikora, R.A., Bridge. J. (Eds.). Plant parasitic nematodes in Subtropical and Tropical agriculture. Wallingford, UK, *CABI Publishing*, pp. 259-318.

Manso, E.C., Tenente, R.C.V., Ferraz, L.C.B., Oliveira, R.S.O. and Mesquita, R. (1994). Catálogo de Nematoides Fitoparasitos Encontrados associados a Diferentes Tipos de Plantas no Brasil. Centro Nacional de Pesquisa de Recursos Genéticos e Biotecnologia. EMBRAPA-SPI, 1, 354–355.

Manzanilla-Lopez R.H. and Starr J.L. (2009). Interaction with other pathogens. In: Perry R.N., Moens M., and Starr J.L. (Eds.). Root-Knot Nematodes. Wallingford UK, *CAB International*, pp. 223-245.

Manzanilla-López, R.H., Esteves, I., Finetti-Sialer, M., Hirsch, P.R., Ward, E., Devonshire, J., and Hidalgo-Díaz, L. (2013). *Pochonia chlamydosporia*:

Advances and challenges to improve its performance as a biological control agent of sedentary endo-parasitic nematodes. *Journal of Nematology* 45(1), 1–7.

Manzanilla-Lopez, R.H., Kenneth, E. and Bridge, J. (2004). Plant diseases caused by nematodes. In: Chen Z.X., Chen S.Y., Dickson D.W., (Eds.). Nematology: advances and perspectives. Nematode management and utilization. Wallingford, UK, *CAB International*, pp. 637–716.

Martin, F.N. (2003). Development of alternative strategies for management of soilborne pathogens currently controlled with methyl bromide. The *Annual* Review of *Phytopathology* 41, 325-350.

McSorley, R., Stansly, P.A., Noling, J.W., Obreza, T.A. and Conner, J.M. (1997). Impact of organic soil amendments and fumigation on plant-parasitic nematodes in a Southwest Florida vegetable field. *Nematropica* 27, 181-189.

Meng, Q.P., Long, H. and Xu, J.H. (2004). PCR assays for rapid and sensitive identification of three major root-knot nematodes, *Meloidogyne incognita*, *M. javanica* and *M. arenaria*. *Acta Phytopathologica Sinica* 34, 204–210.

Meyer, S.L.F. and Roberts, D.P. (2002). Combinations of biocontrol agents for management of plant-parasitic nematodes and soilborne plant-pathogenic fungi. *Journal of Nematology* 34, 1-8.

Meyer, S.L.F., Roberts, D.P., Chitwood, D.J., Carta, L.K., Lumsden, R.D. and Mao, W. (2001). Application of *Burkholderia cepacia* and *Trichoderma virens*, alone and in combinations, against *Meloidogyne incognita* on bell pepper. *Nematropica* 31, 75-86.

Mitowski, N.A. and Abawi G.S. (2011). Root-knot nematodes." The plant health instructor. http://www.apsnet.org/edcenter/intropp/lessons/Nematodes/Pages/RootknotNematode.aspx (accessed 28.03.17).

Montinola, L.R. (1991). *Pina*. Amon Foundation, Manila, Philippines.

Moyle, R.L. and Botella, J.R. (2014). EST sequencing of *Meloidogyne javanica* infected pineapple root tissues reveals changes in gene expression during root-knot nematode induced gall formation. *Tropical Plant Biology*, *7*(2), 43-52.

Mukhtar, T., Hussain, M.A., and Kayani, M.Z. (2013). Biocontrol potential of *Pasteuria penetrans*, *Pochonia chlamydosporia*, *Paecilomyces lilacinus* and *Trichoderma harzianum* against *Meloidogyne incognita* in okra. *Phytopathologia Mediterranea* 52(1), 66−76.

Naserinasab, F., Sahebani, N. and Etebarian, H.R. (2011). Biological control of *Meloidogyne javanica* by *Trichoderma harzianum* BI and salicylic acid on Tomato. *African Journal of Food Science* 5, 276 – 280.

Nath, R.C., Mukherjee, B., Dasgupta, M.K. and Siddiqi, M.R. (1997). Density, diversity and community structure of plant parasitic nematodes in pineapple plantations of Tripura, India. *International Journal of Nematology* 7, 51-61.

Ndungu, S. (2014). A report on conventional pineapple production in Kenya. Prepared for Swedish Society for Nature Conservation (SSNC), Sweden. http://www.naturskyddsforeningen.se/sites/default/files/conventional_pineaple_production_Kenya.pdf. (accessed 03.04.17).

Nitao, J.K., Meyer, S.L.F. and Chitwood, D.J. (1999). *In vitro* assays of *Meloidogyne incognita* and *Heterodera glycines* for detection of nematode-antagonistic fungal compounds. *Journal of Nematology* 31, 172-183.

Noling, J.W. and Becker, J.O. (1994). The challenge of research and extension to define and implement alternatives to methyl-bromide. *Journal of Nematology* 26, 573-586.

Oclarit, E.L. and Cumagun, C.J.R. (2009). Evaluation of efficacy of *Paecilomyces lilacinus* as biological control agent of *Meloidogyne incognita* attacking tomato. *Journal of Plant Protection Research* 49 (4), 337–340.

Oka, Y. (2010). Mechanisms of nematode suppression by organic soil amendments-A review. *Applied Soil Ecology* 44, 101-115.

Pandey, G., Pandey, R.K. and Pant, H. (2003). Efficacy of different levels of *Trichoderma viride* against root-knot nematode in chickpea (*Cicer arientinum* L.). *Annual Plant Protection Science* 11(1), 96-98.

Parsa, S., Ortiz, V. and Vega, F.E. (2013). Establishing fungal entomopathogens as endophytes: Towards endophytic biological control. *Journal of Visualized Experiments* 74, 1-5.

Pay, E. (2009). The market for organic and fair-trade mangoes and pineapples. Study prepared in the framework of FAO project GCP: RAF/404/GER."Increasing incomes and food security of small farmers in West and Central Africa through exports of organic and fair-trade tropical products. Food and Agriculture Organization of the United Nations (FAO), Trade and Markets Division, Rome, Italy (September).

R Core Team, 2015. R: A language and environment for statistical computing. Vienna, Austria. Available from: *http://www.R-project.org* (accessed 03.04.17).

Rammah, A. and Hirschmann, H. (1990). Morphological comparison of three host races of *Meloidogyne javanica*. *Journal of Nematology* 22(1), 56–61.

Rao, M.S., Reddy, P.P. and Nagesh, M. (1996). Evaluation of plant based formulations of *Trichoderma harzianum* for the management of *Meloidogyne incognita* on egg plant. *Nematologia Mediterranea* 26, 59-62.

Raski, D.J. and Krusberg, L.R. (1984). Nematode parasites of grapes and other small fruits. In: plant and insect nematodes . W.R Wickle. Ed Marcel Decker Inc. New York. pp: 457-506.

Reddy, P.P., Rao, M.S. and Nagesh, M. (1996). Management of citrus nematode, *Tylenchulus semipenetrans* by integration of *Trichoderma harzianum* with oil cakes. *Nematologia Mediterranea* 24, 265-267.

Regaieg, H., Ciancio, A., Raouani, N.H. and Rosso, L. (2011). Detection and biocontrol potential of *Verticillium leptobactrum* parasitizing *Meloidogyne* spp. *World Journal of Microbiology and Biotechnology* 27, 1615-1623.

Renco, M. and Kovacik, P. (2012). Response of plant parasitic and free living soil nematodes to composted animal manure soil amendments. *Journal of Nematology* 44, 329-336.

Rhoades, H.L. (1982). Effect of temperature on survival of *Meloidogyne incognita* in flooded and fallow muck soil. *Nematropica* 12, 33-37.

Rodriguez-Kabana, R., Morgan-Jones, G., Godoy, G. and Gintis, H.O. (1984). Effectiveness of species of *Gliocladium*, *Paecilomyces* and *Verticillium* for control of *Meloidogyne arenaria* in field soils. *Nematropica* 14, 155 – 170.

Rohrbach, K.G. and Apt W.J. (1986). Nematode and disease problems of Pineapple. *Plant Disease* 70, 81 – 87.

Sahebani N. and Hadavi N. (2008) Biological control of the root-knot nematode *Meloidogyne javanica* by *Trichoderma harzianum*. *Soil Biology and Biochemistry*, 40, 2016–2020.

Samuels, G.J., Ismaiel, A., Mulaw, T. B., Szakacs, G., Druzhinina, I. S., Kubicek, C. P., & Jaklitsch, W. M. (2012). The Longibrachiatum clade of *Trichoderma*: a revision with new species. *Fungal diversity* 55, 77-108.

Sanches, N.F. (2005). Manejo integrado da cochonilha do abacaxi. Embrapa: Mandioca e Fruticultura Tropical, Cruz das Almas.

Sanewski, G. and Scott, C. (2000). The Australian pineapple industry. In: Subhadrabandhu, S. and Chairidchai, P. (Eds.). Proceedings of the Third International Pineapple Symposium. *International Society for Horticultural Science*, Pattaya, Thailand, pp 53-55.

Santhosh, J.E., Beena, B. and Ramana, K.V. (2005). Tropical soil microflora of spice-based cropping systems as potential antagonists of root-knot nematodes. *Journal* of Invertebrate *Pathology* 88(3), 218-225.

Sariah, M., Choo, C.W., Zakaria, H. and Norihan, M.S. (2005). Quantification and characterization of *Trichoderma* spp. from different ecosystems. *Mycopathologia* 159, 113–117.

Seinhorst, W. (1966). Killing nematodes for taxonomic study with hot F.A. 4:1. *Nematologica* 12, 178-179.

Sharma, P. and Pandey, R. (2009). Biological control of root-knot nematode; *Meloidogyne incognita* in the medicinal plant; Withania somnifera and the effect of biocontrol agents on plant growth. *African Journal of Agricultural Research* 4(6), 564-567.

Sharma, R.D. and Amabile, R.F. (1999). Plant parasitic nematodes associated with sesame genotypes under cerrado conditions. *Nematologia Brasileira* 23, 84 - 87.

Sharon, E., Bar-Eyal, M., Chet, I., Herra- Estrella, A., Kleifeld, O., and Spiegel, Y. (2001). Biological control of the root-knot nematode *Meloidogyne javanica* by *Trichoderma harzianum*. *Phytopathology* 91, 687-693.

Sharon, E., Chet, I., Viterbo, A., Bar-Eyal, M., Nagan, H., Samuels, G.J., and Spiegel, Y. (2007). Parasitism of *Trichoderma* on *Meloidogyne javanica* and role of the gelatinous matrix. *European journal of plant pathology* 118(3), 247-258.

Shurtleff, M.C. and Averre, III, C.W. (2000). Diagnosing plant diseases caused by nematodes. *The American Phytopathological Society*Press, St. paul, USA, pp 187.

Siddiqui, I.A., Atkins, S.D. and Kerry, B.R. (2009). Relationship between saprotrophic growth in soil of different biotypes of *Pochonia chlamydosporia* and the infection of nematode eggs. *Annals of Applied Biology* 155, 131-141.

Siddiqui, I.A., Qureshi, S.A., Sultana, V., Ehteshamul-Haque, S. and Ghaffar, A. (2000). Biological control of rot-root knot disease complex of tomato. *Plant Soil* 227, 163–169.

Siddiqui, Z.A. (2006). PGPR: Prospective biocontrol agents of plant pathogens. In: Siddiqui, Z.A. (Eds.), biocontrol and biofertilization. Netherlands, *Springer* pp. 111-142.

Siddiqui, Z.A. and Mahmood, I. (1996). Biological control of *Heterodera cajani* and *Fusarium udum* on pigeonpea by *Glomus mosseae*, *Trichoderma harzianum* and *Verticillium chlamydosporium. Israel Journal of Plant Sciences* 44, 49-56.

Siddiqui, Z.A. and Shaukat, S. (2003). Endophytic bacteria: prospects and opportunities for the biological control of plant-parasitic nematodes. *Nematologia Mediterranea* 31, 111-120.

Siddiqui, Z.A. and Shaukat, S. (2004). *Trichoderma harzianum* enhances the production of nematicidal compounds *in vitro* and improves biocontrol of *Meloidogyne javanica* by *Pseudomonas fluorescens* in tomato. *Letters in Applied Microbiology* 38, 169-175.

Sikora, R.A. and Fernandez, E. (2005). Nematode parasites of vegetables. In: Luc M.S., Sikora R.A., and Bridge J. (Eds.), plant parasitic nematodes in subtropical and tropical agriculture. Wallingford, UK, *CAB International*, pp. 319-392.

Sikora, R.A., Niere, B. and Kimenju, J. 2003. Endophytic microbial biodiversity and plant nematode management in African agriculture. In: Neuenschwander, P., Borgermeister, C. and Langewald, J. (Eds.), biological control in IPM systems in Africa. *CAB International*, pp. 179-192.

Singh, H.B. and Singh, D.P. (2009). From biological control to bioactive metabolites: Prospects with *Trichoderma* for safe human food. *Pertanika Journal of Tropical Agricultural Science* 32, 99 - 110

Singh, V., Mawar, R. and Lodha, S. (2012). Combined effects of biocontrol agents and soil amendments on soil microbial populations, plant growth and incidence of charcoal rot of cowpea and wilt of cumin. *Phytopathologia Mediterranea* 51, 307–316.

Sipes, B., Caswell-Chen, E., Sarah, J., Apt, W. (2005). Nematode parasites of pineapple. In: Luc, M., Sikora, R., and Bridge, J. (Eds.). Plant parasitic nematodes in Subtropical and Tropical agriculture. *Egham, UK, CABI Publishing*, pp. 709 – 731.

Spiegel, Y. and Chet, I. (1998). Evaluation of *Trichoderma* spp. as a biocontrol agent against soil-borne fungi and plant-parasitic nematodes in Israel. *Integrated Pest Management Reviews* 3, 169-175.

Spiegel,Y. and Sharon, E. (2005). Mechanisms and improved biocontrol of the root-knot nematodes by *Trichoderma* spp. In: Vanachter A. (Eds.), chemical and non-chemical soil and substrate disinfestation. ISHS *Acta Horticulturae*, pp. 225-228.

Stetina, S.R., McGawley, E.C. and Russin, J.S. (1997). Extraction of root associated *Meloidogyne incognita* and *Rotylenchulus reniformis*. *Journal of Nematology* 29, 209-215.

Stirling, G.R. (1993). Nematodes. In: Broadley, R. H., Wassman, R.C. and Sinclair, E. (Eds.), pineapple: Pests and disorders. Brisbane, *Queensland Department of Primary Industries*, pp. 21-29.

Stirling, G.R. (2011). Biological control of plant-parasitic nematodes: an ecological perspective, a review of progress and opportunities for further research. Springer, *Biological Control of Plant-Parasitic Nematodes*, pp. 1-38.

Stirling, G.R. 1991. Biological control of plant-parasitic nematodes. *Wallingford, UK, CAB International*, pp. 282.

Stirling, G.R. and Nikulin, A. (1993). Population dynamics of plant parasitic nematodes in Queensland pineapple fields and the effects of these nematodes on pineapple production. *Australian Journal of Experimental Agriculture* 33, 197–206.

Stirling, G.R. and Pattison, A.B. (2008). Beyond chemical dependency for managing plant parasitic nematodes. Examples from banana, pineapple and vegetable industries of tropical and subtropical Australia. *Australian Plant Pathology* 37(3), 254-267.

Szczech, M., Rondomanski, W., Brzeski, M.W., Smolinska, U. and Kotowski, J.F. (1993). Suppressive effect of a commercial earthworm compost on some root infecting pathogens of cabbage and tomato. *Biological Agriculture and Horticulture* 10, 47-52.

Tariq, J.A. (2008). Bioantagonistic activity of plant growth promoting rhizobacteria (PGPR) against *Meloidogyne javanica* for the control of root-knot disease of tomatoes. PhD thesis (Plant pathology), Faculty of Agriculture University of Agriculture Faisalabad, Pakistan.

Taylon, A.A., Dropkin, H. and Marin, G.C. (1956). Perineal patterns of the root-knot nematodes. *Phytopathology* 46, 26-34.

Thakur, R.P. (2007). Host plant resistance to diseases: potential and limitations. *Indian journal of Plant protection* 35, 17-21.

Thoden, T.C., Korthals, G.W. and Termorshuizen, A.J. (2011). Organic amendments and their influences on plant-parasitic and free-living nematodes: a promising method for nematode management. *Nematology* 13, 133-153.

Tigano, M., De Siqueira, K., Castagnone-sereno, P., Mulet, K., Queiroz, P., Dos Santos, M., Teixeira, C., Almeida, M., Silva, J. and Carneiro, R. (2010). Genetic diversity of the root-knot nematode *Meloidogyne enterolobii* and development of a SCAR marker for this guava-damaging species. *Plant pathology* 59, 1054-1061.

Trudgill, D.L. and Blok, V.C. (2001). Apomictic, polyphagous root-knot nematodes: exceptionally successful and damaging biotrophic root pathogens. *Annual* Review of *Phytopathology* 39, 53–77.

UNCTAD 2016. Pineapple- An INFOCOMM commodity profile unctad.org/en/PublicationsLibrary/INFOCOMM_cp09_Pineapple_en.pdf. (accessed 28.03.17).

UNEP, (1995). Montreal protocol on substance that deplete Ozone layer. Methylbromide technical option committee, Kenya, Pp. 304. http://www.unep.org.

USAID, (2005). Horticultural development program. United States Agency for International Development, Kenya. http://www.humanitarianinfo.org/niger/uploads/appeal/USAID_Sahel%20Fact%20Sheet%20No.1_16%20Aout%2005.pdf. (accessed 28.03.17).

USDA, (2003). Fruit and tree nuts outlook. Bigger citrus Crop Forecast for 2003/04, http://usda01.library.cornell.edu/usda/ers/FTS-11-21-2003. (accessed 28.03.17).

Van Damme, V., Hoedekie, A. and Viaene, N. (2005). Long-term efficacy of *Pochonia chlamydosporia* for management of *Meloidogyne javanica* in glasshouse crops. *Nematology* 7, 727-736.

Verdejo-Lucas, S., Sorribas, F.J., Ornat, C. and Galeano, M. (2003). Evaluating *Pochonia chlamydosporia* in a double-cropping system of lettuce and tomato in plastic houses infested with *Meloidogyne javanica*. *Plant Pathology Journal* 52, 521-528.

Viaene, N., Coyne, D.L. and Davies, K.G. (2006). Biological and cultural management. In: Perry R.N. and Moens M. (Eds.). Plant Nematology. Wallingford UK, *CAB International*, pp. 383-410.

Waceke, J. (2002). Organic soil amendments: An integrated approach to root-knot nematode management in Okra. *Proceedings of the integrated pest management conference for Sub-Saharan Africa.* Kampala, Uganda, pp. 8-12.

Windham, G.L., Windham, M.T. and William, W.P. (1989). Effect of *Trichoderma* spp. on maize growth and *Meloidogyne arenaria* reproduction. *Plant Disease* 73, 493-495.

Wishart J., Phillips M.S. and Blok V.C. (2002). Ribosomal intergenic spacer: a polymerase chain reaction diagnostic for *Meloidogyne chitwoodi*, *M. fallax*, and *M. hapla*. *Phytopathology* 92, 884–92.

Yedidia, I., Benhamou, N., and Chet, I. (1999). Induction of defense response in cucumber plants (*Cucumis sativus* L.) by the biocontrol agent *Trichoderma harzianum*. *Applied and Environmental Microbiology* 65, 1061-1070.

Zijlstra, C., Donkers-Venne, D.T.H.M. and Fargette M. (2000). Identification of *Meloidogyne incognita*, *M. javanica* and *M. arenaria* using sequence characterised amplified regions (SCAR) based PCR assays.*Nematology* 2, 847–53.

APPENDICES

Appendix 1: Ages of the fields from Delmonte and Kakuzi pineapple farms sampled.

Sample collected	Fields sampled from Kakuzi and Delmonte
Twenty five (25) core soil samples only collected	Fallow (0 months) 3 months
Twenty five (25) core soil and root sub samples collected from each field	5 months 8 months 11 months 24 months 36 months

Appendix 2: Grouping of *Meloidogyne* spp. based upon perineal pattern morphology (Jepson, 1987).