Root knot nematodes (*Meloidogyne incognita*) interaction with selected Asteraceae plants and their potential use for nematode management

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DECLARATION

This dissertation is my original work and has not been presented for the award of a degree in any other University.

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DEDICATION

I dedicate this work to my son Mapalo Mwamba and my wife, Chilufya Musendeka Mwamba, my brother James Mulenga Muloshi, for spiritual and emotional support rendered to me during my studies at the Jomo Kenyatta University of Agriculture and Technology (JKUAT) in Kenya.

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LIST OF ACRONYMS

RKNs	Root Knot Nematodes
GC-MS	Gas Chromatography Mass Spectrometry
SPME	Solid Phase Micro Extraction
J2s	Juvenile stages 2
JKUAT	Jomo Kenyatta University of Agriculture and Technology
ICIPE	International Center of Insect Physiology and Ecology
HIPVs	Herbivore-Induced Plant Volatiles
VOCs	Volatile Organic Compounds

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ABSTRACT

The southern root knot nematode (*Meloidogyne incognita*) is one of the highly polyphagous species of many cultivated plants. The losses associated with RKNs in small holder vegetable farms are not quantified but may range between 30-100% in tropical and subtropics. Application of broad spectrum nematicides is the common method of control and are to be highly toxic to humans and pollute the environment, hence the need for safer alternatives for small scale crop production systems. Biological control especially the use repellent plants is an environmentally safe option. Plants from the Asteraceae family have phytotoxic for nematode management. Investigations were conducted between June 2015 and January 2016 to determine the effect of four different Asteraceae species *viz. Tagetes minuta, Artemisia annua, Bidens pilosa* and *Chrysanthemum cinerariaefolium* on the behavior of *M. incognita*. The responses of Juveniles to odours from intact roots of the four asteraceous plant species were investigated. In addition, identification and quantification of volatile components present the in intact roots of Asteraceae plants. was also evaluated. Dual-choice soil olfactometer assays and gas chromatography-mass spectrometry (GC-MS) analysis were conducted in the laboratory. Root volatiles of 4 asteraceous plants antagonized the behavioral responses of *M.incognita* juveniles (repellence) ($P = 0.05$). Using the GC-MS analysis 59 volatile organic compounds were identified from intact roots of the Asteraceae plants; 30 from *Tagetes minuta*, 32 detected in *Artemisia annua*, 21 from *Bidens pilosa* and 12 from *Chrysanthemum cinerariaefolium* respectively. These findings suggest that the four asteraceous plants are repellent to *M. incognita*. Products from these plants may thus be incorporated in an integrated nematode management program for smallholder vegetable farming systems.

CHAPTER ONE

1.0 INTRODUCTION

Plant parasitic nematodes are microscopic organisms that cause significant damage to most plant species and are widely spread. The annual global losses associated to plant parasitic nematodes are estimated at 14% for most economically important crops such vegetables, fruits and nonedible field crops amounting to over $80 billion annually (AGRIOS, 2005). In the tropics and subtropics the crop losses due to nematodes is 14.6% compared to 8.8% in temperate regions (Nicol, *et al*., 2011). Plant parasitic nematodes are small in size (300 to 1000 micrometers) and are composed of several genera. Of the many genera so far described, ten have been found to be of economic importance in Agriculture with *Meloidogyne* spp. (root-knot nematodes) on top of the list (Jones *et al*., 2013). The grouping plant parasitic nematodes is based on the damage they cause to their hosts and scientific knowledge such as species novelty. Plant parasitic nematodes cause damage by establishing a parasitic relationship with their host plants thereby transforming vascular cells into giant / or multinucleate feeding from which they exploit nutrients and water (Hajra *et al*., 2015; Gheysen & Mitchum, 2011)

Plant parasitism is mainly a feeding and survival mechanism and usually manifested in different ways in plant parasitic nematodes(Luc, *et al*, 2005) There are over 90 characterized species of economic importance in the *Meloidogyne* sp group (Lamovšek, *et al*., 2013) and these are obligate endoparasites that are capable of infecting nearly every species of higher plants in a wide range of geographical distribution (Adegbite, 2011; Favery, *et al*., 2016). The estimated global yield losses for arable crops associated with RKNs are estimated between 5-43% in the tropical and subtropical areas (Surendra, *et al*., 2014). There are four major *Meloidogyne* spp. that are common in the tropics viz., *M. incognita, M. javanica, M. arenaria,* and *M. hapla* (occurring in the temperate regions) of which *M.incognita* is the most destructive among the species causing yield losses of up to 16% (Perry, *et al*., 2009). In Africa *M. incognita, M. javanica, M. arenaria* are the most

dominant species and their presence and damage on smallholder tomato production in Kenya specifically in Mwea area has been documented (De Waele & Elsen, 2007). The damage is mainly due to the feeding on the plant roots through establishment of a permanent feeding site that results in the formation of giant cells which culminates into disruption of water, plant nutrient uptake as well as translocation of photosynthetic products (Bartlem, *et al*, 2014).

Currently, control of RKNs is mainly through use of chemical nematicides, but alternative management options are required due to the recent public concerns and restrictions regarding use of nematicides because of the impact they pose on environment and human health (Chellemi, 2014). It has also been observed that the increased use of agrochemicals (fumigation of soil and rootstocks), synthetic fertilizers have resulted in environmental pollution, pest resistance, resurgence and poisoning of food sources for humans and livestock (Russell & Kranthi, 2009). De-registration of several chemical nematicides and the phasing out of methyl bromide due to its high toxicity among pesticides available on the market for pest-control has in the recent past necessitated for new methods of controlling nematodes that are ecologically sound and safe for the environment (Chitwood, 2003; Zhuo *et al*., 2010; Sellés *et al.,* 2012; Aballay *et al.,* 2009). Alternative nematode control options include the naturally occurring and economically sound bio-pesticides that are easy to integrate in a farming system (Koul, *et al*, 2008; Rasmann, *et al*, 2012a).

Higher plant species possess phytochemical compounds antagonistic to plant parasitic nematodes including root knot nematodes. The compounds have a broad spectrum mode of action consisting mainly monoterpenes and sesquiterpenes (Chitwood, 2002). Monoterpenes and sesquiterpenes have a good repellence effect in many instances when used as essential oils (Nerio, *et al*., 2010). For instance, several plant extracts of tobacco (*Nicotiana tabacum* L), neem (*Azadirachta indica*, clove (*Syzygium aromaticum* L), Sunn hemp (*Crotalaria* spp.) and sweet flag (*Acorus calamus* L) have nematicidal effect on root knot nematodes (Wiratno *et al*., 2009; Hildalgo-Diaz & Kerry, 2007).

In the recent past some plant species from Asteraceae family have been reported to have potential for use in the management of root knot nematodes in the soil rhizosphere and these could offer an ecologically sound control option as a substitute to chemical nematicides (Gershenzon & Dudareva , 2007; Hussain, *et al*., 2011; Sadia, *et al*., 2013; Silva, *et al*. 2014). Asteraceae plant species with nematicidal properties have been tested for their potency in the management of root knot nematodes (Tsay *et al*., 2004). The essential oils and other secondary metabolites extracts from these plants have direct effects to RKNs especially on the nematode egg hatchability and juvenile stage development (Perez, *et al*., 2003; Gong *et al*., 2013; Hooks, *et al*., 2010).

Plants produce an array of volatile organic compounds when attacked by herbivory pest and these volatiles act as repellents, attractants as well as inducement of biological functions such as indirect defense for herbivores (Baldwin, *et al*., 2002; Holopainen & Blande, 2012). Plants use both internal and external signals to establish their systemic responses to enemy attack. Recently resistance / antagonism to herbivores has been associated to induced airborne cues (volatile organic compounds, VOC) and is connected in the context of 'communication' among independent individual intact plants (Baluska & Ninkovic, 2010). The VOCs can either be induced as a result of plant attack or produced naturally while in other instances these cues can be headspace or from the roots below in the soil rhizosphere. However, studies to establish chemical defense of roots from Asteracaeae plant species despite reports of these plants having agricultural and pharmacological properties have not been exploited for use in integrated crop protection. It is also known that volatiles molecules influence host seeking behavior in feeding activity of root knot nematodes (Rasmann, *et al*., 2012), but it is not clear whether these volatile organic compounds play a major role in nematode repellence status in most Asteraceae plants.

This study therefore aims at investigating the role of volatile components from Black jack (*Bidens pilosa*), Pyrethrum *(Chrysanthemum cinerariaefolium)*, African marigold

(*Tagetes minuta*.) and sweet wormwood *(Artemisia annua)* in the management of root knot nematodes and to identify and quantify these components.

1.1 Statement of the problem

Root knot nematodes (genus *Meloidogyne*) are important economic pests of many cultivated crops and currently chemical nematicides used for control are being phased out due to their to high toxicity (Lamovšek *et al.*, 2013). Root Knot Nematodes reduce yield and quality of agricultural products consequently causing economic losses to the agricultural industry. Globally losses associated to root knot nematodes which are a major constituents of plant parasitic nematodes are as high as $157 billion dollars annually (Singh, *et al.*, 2015), while in Africa and Kenya it has been difficult to quantify economical losses associated to root knot nematodes even though their detection and distribution has been reported (Onkendi, *et al.*, 2014). Losses due root knot nematodes are associated with damage the cause which results in poor root development, delayed maturity, toppling, reduced yields and quality of export crop produce, high costs of production due to nematode management, hence loss of income (Dropkin, 1955; Plant Protection Act, 2005). To combat these effects new efficient control methods are required for these pathogens.

The current available pesticides phased such as methyl bromide, 1,3 dichloropropene, carbofuran, and ethylene dibromide have been used in vegetable production for management of nematodes, however they are slowly being phased out while others have been restricted due to high toxicity , hazardous to the user, harm on environment (long soil persistence) and beneficial non-target organisms (Joseph, *et al.*, 2012). Nematicides used in horticulture for control of nematodes are usually very toxic and broad spectrum mainly applied as fumigant or non-fumigant, however they high volatility and long persistence in the soil and environment (Chitwood, 2001;Gowen, 1992).Chemical control using nematicides is slowly becoming extinct from the market due to their toxicity and effects on humans, livestock and environment hence the need to invest in safer agricultural practices for management root knot nematodes (Collange *et al.*, 2011). Concerns over chemical pesticides in respect of groundwater contamination, residues on food, and development of resistance to pests has prompted for safer management alternatives such

as biological control (Hussaini, 2014) and avoidance of chemical pesticides/fungicides have been proposed to avert associated environmental problems (Mukerji & Ciancio, 2007). Concerns about the ill health cases reportedly associated with those of pesticides application has been reported by Food and Agriculture Organization, FAO (2008) and it was noted by World Health Organization that about 1 million people suffer from pesticide poisoning with at least 20,000 death cases (WHO, 2006). This is in line to increasing food production for the increasing human population but with emphasis on the use of naturally occurring bio-pesticide that are cost effective , environmentally smart as an alternative to chemical inputs that harm to the environment and usually costly (Reddy & Saravanan, 2013).

Integrated pest management option using naturally occurring bio-pesticides is eminent considering the effects of agrochemicals continues to inflict on the global environment and humanity (Oerke & Dehne, 2004).

1.3 Justification

Root knot nematodes are important pests of many cultivated plants and known to cause yield losses of over 5-43% globally and 25-50% for smallholder farmers in developing countries (Taylor & Sasser, 1978). Nematicides such 1,2-dibromo-3-chloropropane (DBCP) as well as Methyl bromide have been used in control of root knot nematodes and have shown be potent in controlling nematodes compared to non-soil fumigants however their toxicity has caused developed countries to phase them out as of 2005 since their introduction in the 1950's (Giannakou, *et al.*, 2002). These nematicides are now being phased out because of their cost, hazardous nature and high toxicity to non-target organisms as well as environment and ozone layer depletion (Quénéhervé, 1993; Clean air act, 1990). In Africa the use of Methyl bromide has been on decreasing trend with some countries phasing the use of the product for crop protection. The aggregate consumption of Methyl bromide has been reducing in most African countries with a steady progress of phasing out the product in the region reaching advanced stage consumption stands at 7.6 %, down from 10 % in 2010 and from 20 % in 2006 (UNEP, 2012)

Therefore, there is need to venture into plants based sustainable biological avenues as alternatives to chemical control for management root knot nematodes. Plants considered antagonistic to *Meloidogyne* species have been evaluated from Asteraceae family in rotation, intercrop or organic soil amendments and have been able to suppress nematode populations(Tsay *et al.*, 2004). Asteraceae family has 23,000 species with majority having been used as nematicides and for pharmaceutical purposes due to the alellochemicals they posess (Sadia *et al.*, 2013). The use of Asteraceae plants for nematode management has been tried (Tsay *et al.*, 2004) and some plants from this family have shown to offer nematicidal activity / repellence over *Meloidogyne incognita*. These plants includes *Gaillardia pulchella, Tagetes erecta, Tithonia diversifolia, or Zinnia elegans*. The use of essential oils extracted from *Chrysanthemum coronarium* flowerheads also exhibited

strong nematicidal activity in vitro and in growth - chamber experiments at different concentration (Perez *et al*., 2003).

The known mechanisms for suppression of plant parasitic nematodes by marigold has been documented and includes acting as a poor host, improving nematode-antagonistic microorganisms, or by "dead-end" and or trap crop (Hooks *et al*., 2010b). However little is known on whether root diffusate usually play a big role in the suppression and behavior of nematodes towards these plants.

Asteraceae plants contain chemical components distributed broadly among plant organs such as seeds, flowers, pollen, leaves, stems and roots, or sometimes found in just one or two of such organs and act as defense compounds against pest or diseases by volatilization, leaching, decay and exudation (Haig, 2008). In vitro and planta experiment results have also shown that essential oils of *Chrysanthemum oficinalis* and *Chrysanthemum suffruticosa* and organic amendments from Asteraceae may serve as nematicides in controlling RKNs (Perez *et al*., 2003). Asteraceae plants have been known to have biopesticide effect when used as cover crops, intercrops, essential oils and extracts (Song, *et al*., 2014). The use of Asteraceae plant species and those from other families, in nematode control have been studied and this includes incorporation as cover crops, crop rotation intercropping, use of their extracts as essential oils and green manuring (Grimme, *et al*., 2007;Kayani, *et al*., 2012a; Khalaj,*et al*, 2013). The merits regarding the use of antagonistic plants is that they have been found to be environmentally friendly apart from offering the suppressive effect on plant parasitic nematodes and this is option comparatively better to synthetic agro-chemicals that continue to pause harm to mankind and the environment (Osei. *et al*., 2011)

Despite all this about Asteraceae plants, there is a knowledge gap on chemical defenses in most plants that are perceived as non-hosts of root knot nematodes though knowledge on induced plant volatiles released as signals following attack by herbivores to aggregate the natural enemies have been studied (Bezemer & Van Dam, 2005; Ali *et al*., 2012; Rasmann & Turlings, 2008). Despite tritrophic interactions being difficult to study in a

subterranean environment, olfaction has shown to play an important role in the host-seeking process, with parasitic nematodes following a chemical trail toward host associated odours (Dillman *et al.*, 2012). Volatile components from tomato roots have been shown to be highly attractive to *M. incognita* juveniles (Murungi, *et al*, 2015; unpublished data). However little is known concerning what olfactory cues that influence the behavior root knot nematodes that elicit non-host/repellant properties. It is thus important to understand how these volatiles interact either individually or in combination with tomato in influencing nematode behavior and volatile compounds eliciting the responses.

1.4 General Objective

To investigate root volatile components, present in Asteraceae plants associated with repellence for management of root-knot nematodes (*Meloidogyne incognita*)

1.4.1 Specific objectives

1. To determine the behavioral responses of root-knot nematodes to root odors of *Bidens pilosa*, *Chrysanthemum cinerariaefolium*, *Tagetes minuta*. and *Artemisia annua*
2. To identify and quantify the volatile composition in root odours of *B. pilosa*, *C. cinerariaefolium*, *T. minuta*. and *A. annua*

1.5 Hypotheses

1. Intact root odours from Asteraceaea plants do not influence behavioral response of root knot nematodes

2. Volatile compounds present in intact root odours of different Asteraceaea plants do not vary in quality and quantity

CHAPTER TWO

2.0 REVIEW OF LITERATURE

2.1 Distribution of root knot nematodes

Global distribution of nematodes varies greatly based on species, some having a cosmopolitan trend like *Meloidogyne* spp while others are confined to specific geography especially more from the tropical climate than temperate regions or are highly host targets like *Nacobbus* spp (Nicol, *et al.*, 2011b; Perry, *et al.*, 2009b). Root knot nematodes are widely distributed worldwide than any other genus of plant parasitic nematodes and attributed to biological and favourable environmental conditions (Adegbite, 2011; Pmas, 2013; Sasser, 1977). The parasites are capable of surviving in the tropics, subtropics and temperate climates over a wide geographical range where they parasitize nearly all higher plants species (de Almeida-Engler, *et al.*, 2011). Differential presence of root knot nematode has been reported in terms of distribution; *M. incognita* Chitwood, *M. hapla* Chitwood and *M. arenaria* Chitwood have an even presence around the globe while *M. javanica* Chitwood is mainly localized to warmer climates as described by Chitiwood from *Meloidogyne exigua* Goldi (Tiwari, *et al.*, 2009). Previous reports also indicate that *M. incognita* and *M. javanica* occur worldwide (Luc, *et al.*, 2005) while in recent developments *Meloidogyne minor* Karssen *et al* was discovered and reported in Netherlands, Ireland, Portugal, United Kingdom Chile and the United States of America (Wesemael, *et al.*, 2014). However because this species was recently described, information about its geographical distribution is yet to be studied extensively (Prior , 2015). *Meloidogyne enterolobii* a tropical and subtropical species has also been reported in Brazil, Venezuela, China, Cuba, France, Guatemala, Puerto Rico, Martinique, Malawi, Senegal, South Africa, Switzerland, Trinidad and Tobago, United States, West Africa (Ivory Coast and Burkina Faso) and recently in Kenya on parasitizing African nightshade vegetables (Chitambo, *et al.*, 2015; Rito, *et al.*, 2015).

In order to understand the distribution and host range of RKNs it is important have clear geographical mapping and host range information that is essential in determining the nematode damage potential. This data is available and is updated regularly on CABI website (http://www.cabi.org/dmpd). However the abundance of root knot nematodes in the soil is non-consistence making it difficult to have reliable actual numbers for statistical distribution (Manzanilla-lópez & Hunt, 2005).

2.2 Host plants

Root knot nematodes are widespread polyphagous endoparasites and present a serious growing threat to agriculture development because ability of surviving nearly on any host including weeds (Truong, *et al.*, 2015; Rich, *et al.*, 2010). *Meloidogyne minor* was found parasitizing golf field and has also been detected in potato fields in Netherland and United Kingdom. In the northern part of Europe species such as *Meloidogyne naasi* is a known pest of cereals, *Meloidogyne hapla* is parasite carrot, beet, kale, parsnips and ornamentals with *Meloidogyne chitiwood* attacks carrots, potato, wheat, maize and other monocotyledons and dicotyledons. *Meloidogyne fallax* was found infecting potatoes carrots and monocotyledon and dicotyledonous plants (Nischwitz *et al.*, 2013).

Root knot nematodes are capable of infecting of over 2000 host plants icluding all major field crops, vegetables, ornamental plants, fruit trees and some weed species with ability of compatible interaction of up 8 weeks in the roots (Dubreuil, *et al.*, 2011).The host range of root knot nematodes is so vast that it is difficult to find a common landscape and garden as non-hosts while it has been found that vegetables, bedding plants, shrubs as well as trees are all susceptible hosts (Olsen, 2000). Nematodes have evolved complicated strategies for exploiting almost all plant species by establishing and maintaining a close relationship for the purposes of survival and satisfying their nutritional needs (Lohar & Bird, 2003).

2.3 Host Location

Host finding for root knot nematodes is a sophisticated process.In most cases they use chemical emitted by the host (Chaisson & Hallem, 2012; Ashton, *et al.*, 1999). Nematodes use neurons found in the amphidial channel for detection of aqueous chemo-attractants and repellants while with the aid of the wing cells-flattened amphidial neurons they are able to detect volatile odorants. Research has shown that chemotaxis act as guiding principle of root knot nematodes to specific feeding sites of their host and this is triggered by array of chemicals emitted by host plant roots (Reynolds, *et al.*, 2011). Recently studies on induced volatile organic compounds shows that entomopathogenic nematodes responds very well to chemical cues produced by roots of young seedlings of maize when attacked by herbivores (Rasmann, *et al.*, 2005). However intact plant roots also produce volatiles that could either be attractants or repellents and might be very important in modifying or influencing behavior of nematodes. Host finding is very important to the survival and multiplication of nematodes because they must locate a suitable host for feeding (Teillet, *et al.*, 2013).

2.4 Life cycle of Root Knot Nematodes

Root knot nematodes are obligate endoparasites that complete their life cycle inside the host plant feeding for survival and reproduction (Caillaud, *et al.*, 2008) The first life cycle of *Meloidogyne* spp was described by (Muller, 1883) and later many other scientists followed (Sasser & Carter, 1985, Harnett & Kennedy, 2001).The life cycle consists of egg, four juvenile stages and adult stage (Wesemael, *et al.*, 2014) (Fig. 1). The development of the first stage larvae happens within the egg where first molting takes place and this is followed by a second juvenile stage (J2) that infect plant roots after seeking the host within the soil surrounding the root (Noling, 2009). The ability of *Meloidogyne* spp. to survive is enhanced by several physiological and biochemical adaptations, which includes delayed embryogenesis, quiescence and diapause, and lipid reserves that prolong viability until the Juveniles is attained and invades a host (Perry, *et al.*, 2009).

The short lived males are vermiform and motile in nature and they move in the soil to copulate with females for reproduction. Males are not important as female root knot nematodes are capable of reproducing asexually. Adult female nematodes deposit eggs in a gelatinous protective matrix, closer to the outside of root surface. A single female nematode has capacity to lay over 500 to 1500 eggs during its life cycle which lasts for 3 months (Tiwari *et al.*, 2009). Optimal conditions must prevail for egg laying and includes adequate moisture and warm temperatures. The duration of life cycle is from 17 to 57 days depending on the prevailing conditions of the host plant they infest and the environment. After the completion of embryogenesis, the first juvenile stage remains inside the egg up to the time it molts into the second juvenile stage. The juveniles from the second stage hatches freely in the soil in search of a suitable host plant where it will feed for survival. The second Juveniles are well equipped for parasitism of their host and will move to feed by means of a stylet, a retractable mouth part adapted for piercing and feeding (Tiwari *et al.*, 2009). The second juvenile enters the root becomes sedentary and grows thick, feeds on the surrounding cells by inserting its stylet and saliva secretion. With this they are capable of manipulating key aspects of plant biology and are able to hijack host-cellular development to establish a feeding site (Curtis, 2007). Nematodes develop into the J3 and J4 stages as they advance feeding on the giant cells and galling manifests as a response to their feeding while they emerge as adults to lay eggs.

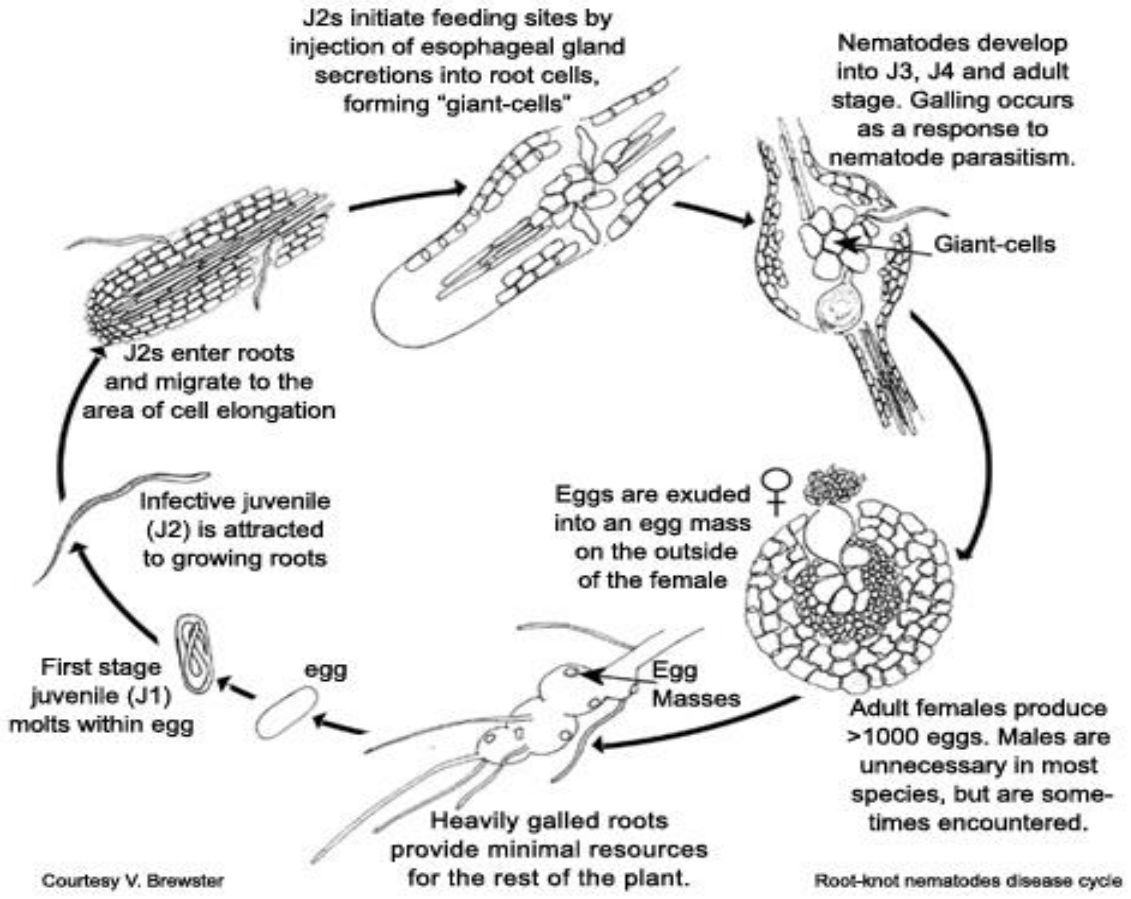

Figure 1: Life cycle of root-knot nematode (*Meloidogyne spp*.) courtesy of V. Brewster (Mitowski and Abawi, 2011)

2.5 Symptoms of root knot nematodes

Root knot nematodes are known to cause non-epidemic diseases which leads to slow decline in yields and this spreads gradually and steadily year by year. The damage caused by root knot nematodes can be visible from above ground and below ground with signals of root galling on most hosts as a major associated symptom (Kofoid, 1998). The above-ground symptoms observed on infected plants are similar to those produced on plants with a damaged and malfunctioning root system. Symptoms include: (i) suppressed shoot growth and accompanied by decreased shoot-root ratio; (ii) nutritional deficiencies showing in form of chlorosis; (iii) temporary wilting in water stress periods or during midday, even in the advent good soil moisture; and (iv) reduced plant yields (Perry & Moens, 2006). The other symptoms includes yellowing and premature leaf shedding with severe stunted growth depending on nematode populations in the field (Duncan, 1991) and sharp decline in plant fruiting (Bird, 1974). The infestation of RKNs results in temporal wilting of plants due to disturbance on the uptake of nutrients, water and plant metabolic activities and this has a serious impact on yield (Murukesan, 2008).

The below-ground symptoms include galls or knots on the roots or tubers of numerous crops as a result of plant response to RKNs which occurs as result of feeding on the vascular tissues (Bird, 1974 & Duncan, 1991). The vascular tissue of many plants are often targeted and plays a major role in pathogen and host plant interactions where an intimate relationship is maintained (Favery *et al.*, 2016). This kind of invasion leads to a striking rear- arrangements of the host vascular system during root-knot nematode infestation of plant roots where they induce permanent feeding sites leading to 'giant cells' that facilitate changes in vascularization, resulting in the giant cells being encaged within a network of de novo formed xylem and phloem cells (Bartlem, *et al.*, 2014). The generated galls (giant cells) on plant roots causes physiological disturbance and further delay plant growth due to poor sink – source relationship resulting in poor yields (Soltani, *et al.*, 2013; Barcala & Cabrera, 2015).The galls vary in size from pin-head to large size due to different levels root knot infestations on host plant and at times they may coalesce

to form large secondary galls (Mohiddin & Khan, 2014). The size of galls also depends upon the host plants and nematode species involved (Agroalimentària, 2008). A clear and typical symptom of root knot nematode infestation produce 'thick-root' appearance; the egg masses or females are concealed inside the root tissue. Other than galling, several other symptoms in the form of forking of tap roots as in carrot or beet and pimple-like tubercles on tubers in potato and pods (groundnut) are also manifested (Kofoid, 1998; Hemeng, 1978) .

2.6 Management of Root Knot Nematodes

2.6.1 Antagonistic plants

Antagonistic plants are those that are considered to produce toxic substances to target organisms when the crop is growing or after its incorporation into the soil (Kayani, *et al.*, 2012). This strategy is used in nematode management and its approach relies mainly on pre plant cover crops, green manuring or intercropping. Marigold, neem, sun hemp, partridge pea, asparagus among other have been studied and used as antagonistic plants for nematode management (Adekunle, 2011;Farooq, *et al.*, 2011; Kosma, *et al.*, 2011). Sun hemp is usually cultivated as a cover crop for direct seeding, intercrop or as a soil amendment and is highly considered as an antagonistic plant for nematodes including root knot nematodes (Wang, *et al.*, 2002). Some studies conducted also revealed that population densities of *M. incognita* were affected by previous cover crops of *Crolatalia juncea* (Wang, *et al.*, 2004). Marigold has also been used in the suppression of plant parasitic nematodes when used as a cover crop and is known to produce allelopathic compounds known as α-terthienyls a Sulphur compound that is known to play a role in nematode silencing (Hooks, *et al.*, 2010). These compounds have been detected in the roots and leaves of actively growing plants and it is believed that these chemicals like the case in marigold are triggered by sequential events in the marigold roots as nematodes penetrate and move through the root tissues (Wang, 2007). However these Sulphur related compounds are not volatiles but fall in the non-volatiles category or contact group. Other studies regarding the use of different varieties of marigold (*Tagetes* spp) have been done

for RKNs (*Meloidogyne incognita*) suppression in tomato. Results from such study showed that *Tagetes* spp was effective in reducing the root galling and infestation as well as improving tomato yield (Ploeg, 2002). Similar studies conducted by Siddiqui & Alam (1987) also reported a success story regarding for marigold when it was used as waste bye product.

2.6.2 Chemical control

Chemical control is one of the oldest methods of controlling nematodes. Nematicides are chemicals used in the field of agriculture to mitigate the negative effects of plant parasitic nematodes on plant health and subsequently on crop productivity and/or quality (Becker, 2014). These chemicals are applied to the soil as fumigants when wet and is ploughed thoroughly very well for better incorporation (Araya & Mario, 2004) These chemicals have a correlated effect on soil type with regards to control as they are known to respond differently to soil type. These fumigants, besides controlling nematode, also help in controlling other soil-borne pathogens and weeds as they are most non-selective. Chemicals such as carbofuran, carbosulfan, fenamiphos have been tried in controlling *M. incognita* and have shown good results in the treatment of seeds when dressed and soil fumigation (Khan, 2004).

2.6.3 Cultural methods

Cultural control is one of the broadest method for management of RKNs and involves cropping systems, fallowing , solarization, use of organic amendments, intercropping , age of transplantable nurseries, altering dates of planting, removal of infected plants and burning of crop residues (Khan, *et al*., 2014). Because nematicides are Slowly being phased, alternative agronomic practices required to solve the nematode problems are encouraged as options for nematode control (Collange *et al*., 2011). Cultural practices are used in many countries where crop rotation is highly adopted for management of nematodes among the many methods that are available (Sharma & McDonald, 1990). Organic soil amendments have been reported to have nematicidal properties in field vegetable trials (Ozores-Hampton, *et al.*, 2012), thus increasing crop yields significantly

(Duponnois, *et al.*, 2001). It works by increasing the level of nutrient supply and improves the soil structure thereby increasing nitrogen availability and consequently improving plant health (Tabarant, *et al.*, 2011). Other cultural practices in the control of root knot nematodes include an incorporation of cover crops as fallows in rotations although their effects on nematodes is decimal and requires prolonged application (Baginsky, *et al.*, 2013). It important to note that only few or a combination of these techniques may work for nematode suppression.

2.6.4 Crop rotation and cropping sequences

Adoption of crop rotation is one of the effective approaches for root knot nematode management especially in annual crops. Root knot nematodes usually survive in the soil either in the form of eggs or second stage juveniles feeding on host plants. Crop rotation in combination with manure showed potency in reducing the nematode population in vineyards (Baginsky *et al.*, 2013). In the absence of a host plant and or when you are alternating with non-host in the field, the populations of root knot nematode juveniles have been observed to decline due to starvation, desiccation and heat if weeds or other hosts (Widmer, *et al.*, 2002). It is important to rotate crops unsuitable for nematode infection/antagonistic in nature, so as to reduce their growth. Sunhemp (*Crotalaria juncea*) and pasture grasses have been used in rotation to reduce or manage root knot nematodes (Noling, 2009). Crop rotation however, has low practical significance for established perennial crops, such as trees and vines, because once these crops are established and can persist at that particular site for a long period of time. Certain antagonistic crops and coupled with other cropping systems with Brassicas (lettuce and broccoli) when used as cover crop and green manure are known to produce general biocides called glucosinolates that have been associated with reduction of root knot nematodes population (Collange *et al.*, 2014; Monfort *et al.*, 2007).

2.6.5 Phytotherapy and organic amendments

Plant parts, their products, extracts and certain other effective amendments have also been reported to possess nematicidal properties. The use of such materials has merits over other methods due to their availability, low cost, being pollution free and their capacity to improve soil fertility. The use organic amendments in the control of root knot nematodes have been exploited and there is evidence of root knot nematode suppression through reduced multiplication and reduced diseases as evidenced by enhanced plant growth parameters (Parihar, *et al.*, 2012). The most common organic amendments like animal manures; poultry litter, crop residues, farm yard manures (FYM) and other concentrated organic amendments like neem cake are the typical examples of soil amendments used for management of root knot nematodes. Liquid organic manure has been found potent in controlling root knot nematodes and promoted plant health in tomatoes (Noling, 2011). Despite organic amendments being environmentally acceptable, large quantities are required per unit area which renders the strategy largely inapplicable in large scale farming.(Mateille, *et al.*, 2007).

2.5.6 Resistance breeding

Breeding for nematode resistance involves the same basic principles implored when breeding for resistances against plant pathogens of pyramiding resistance genes of interest so as to create new cultivar with wide and strong resistance to *Meloidogyne* species (Dong, *et al.*, 2008). Resistance breeding must be combined with desirable agronomic and horticultural traits of recommended cultivars. It is believed that two homozygous genes have been linked to condition nematode resistance in carrot and it poised that using this model of duplicate recessive epistasis may explain the reaction of resistant varieties and nematode in the derived crosses (Wang & Goldman, 1985). Breeding advancement has also revealed that several root knot nematode resistant genes have been discovered in different plants. The candidate nematode resistant gene called Tfg-Mi was discovered and isolated from a resistant fenugreek line Giza 3 a root knot nematode gene from leguminosae by degenerate PCR amplification method and in combination with Race

Amplification of cDNA ends (RACE) technique (Abbas, *et al.*, 2008). The developed lines by this technique have shown resistance to root knot nematodes *M. javanica* and *M. incognita*. Resistance (R) genes are assumed to detect Avr-Avirulent gene specific molecules for activation of defense against root knot nematodes in resistant plants (Hammond-Kosack & Jones, 1997). The recognition of nematodes, mediated directly or indirectly by plant R proteins through nematode secreted effectors evokes a resistance response, which is referred to as effector-triggered immunity (Hogenhout & Bos, 2011)

2.5.7 Biological control

Biological control of plant parasitic nematodes is achieved by use of antagonistic organisms, conservation of local antagonistic or combination of the two in soil by either naturally occurring or introduction techniques (Sikora, 1992). Single isolates of bacterial endophytes isolated from African marigold *Tagetes erecta* and *T. patula* have been tested as biocontrol agents and have shown efficacy in controlling nematodes using endoroot derived bacteria (Sturz & Kimpinski, 2004). Naturally plant growth promoting rhizobacteria play a critical role in the control soil pathogens and is indigenous to the soil environment and rhizosphere (Siddiqui, 2005). The control of plant parasitic nematodes by biological control agents requires inundation of bio formulations in the soil for satisfactory control. Nematophagous fungi have been suggested for control of root knot nematodes as an antagonist trap and endoparastic fungi (Pendse, 2013). *Paecilomyces lilacinus* which is also marketed in Kenya has been used in the control of nematodes as it has ability to effectively parasitize these plant pathogens consequently reducing the egg hatching and increasing root knot mortality (Anastasiadis, *et al.*, 2008). *Pochonia chlamydosporia* var. *chlamydosporia* a nematophagous fungus has been studied extensively as a biological agent against plant parasitic nematode. It is one of the most studied and effective biological control agent for the nematode genera such as *Globodera, Heterodera, Meloidogyne, Nacobbus* and just recently *Rotylenchulus* (Manzanilla-Lopez, *et al.*, 2013).

Pochonia clamydospora fungus has also been reported to on reduce population densities of *M. javanica* in tomato and lettuce grown in green houses consequently stabilizing yields (Verdejo-Lucas, *et al.*, 2003) Recently arbuscular mycorrhizal fungi was capable of safeguarding plants against most soil borne pathogens inclusive of RKNs although the mechanisms of their antagonism still remain unknown (Vos, *et al.*, 2012). Other studies have shown that *Trichoderma harzianum* BI is successful fungi that is capable of infesting nematode eggs and juveniles with ability to significantly reduce root knot nematode (*M. javanica*) under greenhouse conditions (Sahebani & Hadavi, 2008). Bacteria particularly *Pseudomonas aeruginosa* was reported to have an impact on tomato growth and reduced galling (Shankar, *et al.*, 2011)

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study site

Laboratory studies were carried out at the International Centre of Insect Physiology and Ecology (*icipe*) Duduville campus, Nairobi Kenya (1º 16' 60'' S; 36º 49' 0'' E) in the Behavioral and Chemical Ecology Unit.

3.2 Plant materials

Plants of the family Asteraceae including black jack (*Bidens pilosa*), pyrethrum *(Chrysanthemum cinerariaefolium)*, African marigold (*Tagetes minuta*.) and sweet wormwood *(Artemisia annua)* were used in this study. black jack (*B pilosa*) was obtained from JKUAT farm (1530m, 1°.0911'51" S 37 º.0101'91"E,), while pyrethrum *(C. cinerariaefolium),* African marigold (*T. minuta*.) and sweet wormwood *(A. annua)* were collected from Limuru (1608 m, 1°06'28.0"S, 36°38'34.0"E), Juja (1508m, 1º.112'74"72 S 37º 011'74"E) Thika (1505 m, 1°038'8° S, 37°.08'34"E) respectively. *A. annua*, *T. minuta*, and *B. pilosa* were propagated by seed while Pyrethrum (*C. cinerariaefolium*) was propagated using cuttings. The seedlings of *A. annua*, *T. minuta* and *B. pilosa* were transplanted into 2 litre plastic pots (17cm top diameter, 13cm base diameter and 15cm depth) two weeks after germination. Cuttings for *C cinerariaefolium* were directly grown in the pots. Field collected plants conditioned at *icipe* in the greenhouse. Plants were watered once daily on four alternate days in a week with a nutrient solution prepared as described previously by Lambert (Lambert, *et al*., 1992)

3.3 Root knot nematodes

Root knot nematodes (*M. incognita*) were obtained from infected tomato rhizosphere soils collected from Taita Taveta County (3°.3161'14" S, 38°.481'50" E). The nematodes were extracted and isolated using Modified baermann method and later identified using PCR, respectively. The nematodes were reared on 3 weeks old tomato seedlings in the

greenhouse (average temp 23±2 °C, RH 70-80%) at *icipe*. Tomato plants that showed galling symptoms due to RKN infection were selected for nematode extraction. The galled roots were immersed in an aqueous solution of phloxine B (0.15g/lit) for 20 minutes to stain the egg masses then washed with tap water to remove the excess stain three times. The visible egg masses were then extracted from the stained roots with the aid of a stereo microscope (Leica M125, Lieca microsystems, USA). The egg masses extracted were put on culture plates with distilled water and incubated for 48-72 hours in the dark at 23 ˚C to allow hatching. Hatched root knot nematode juveniles were picked with plastic Pasteur pipette from culture plate and placed on nematode counting dish for 5 minutes before counting. Approximately 600 juveniles were counted with the aid of a hand tally counter on the stereo microscope and transferred to the labeled 15 ml falcon tubes using the plastic pasteur for use in bioassays.

3.4 Dual choice soil olfactometer bioassays

The behavioral response of *M. incognita* juveniles to root odors of four different asteraceous plant species *viz*. *B. pilosa*, *C. cinerariaefolium, T. minuta* and *A. annua* were conducted using a two arm soil olfactometer modified from a previously described method by Rasmann, *et al*. (2005). The soil olfactometer constituted of a central release arm (8cm in diameter, 11cm deep) with equally distributed side connectors of 5cm long to the two glass pot connector (5cm top diameter, 11cm depth) in which Asteraceae plants or combinations (Table 1) and source of treatments could be placed. The connecting arms consisted of two detachable parts; all with two plastic screws with rubber seals. The glass pot connectors contained an ultra-fine metal screen preventing the nematodes from reaching the odour source pots. For each experiment, the entire system was filled with sterilized sand (autoclaved at 120˚C for 20 min) weighing 300 grams to about 5 cm from the rim of each pot while the connectors were also filled to complete the glass olfactoctometer setup assembly. Nematodes concentrated to 0.5 to 1 ml were in the middle centre called release arm. Four replicates of each treatment were conducted in a completely randomized design. A total of 600 juveniles were introduced at the nematode

introduction chamber (Plate 3.1) using a Pasteur pipette and were allowed four (4) hours to make a choice between treatments. Thereafter the nematode collection chambers (Plate 3.1) on both ends of respective treatments were disconnected and the sand from both ends placed on a Baermann set-up (Plate 3.3) for 24 hrs. To study the behavior of RKNs towards intact Asteraceae plant root volatiles, a two arm glass olfactometer based on modified nematode six arm glass olfactometer was used developed by Sergio Rasmann *et al* (2005).

Table 1Treatment comparison on response of root knot nematodes to respective root odours

Serial no.	Treatment combinations
1	Asteraceae plants versus Control (sand)
2	Asteraceae plants versus tomato
3	Asteraceae plants + tomato versus Control (sand)
4	Asteraceae plants +tomato versus tomato

The Baermann set-up (plate 3.3) comprised of a paper towel where soil from the different parts of soil duo choice glass olfactometer assay setup connectors were put, a perforated plate where soil in the paper towel was placed and finally a plastic plate filled with water for collection of nematodes moving from paper towel to a water gradient onto the plate. The collecting plate was filled with enough water to ensure that during 24-hour period to ensure nematode survival as they moved from paper towel into the water. Nematodes collected from the plate during 24 hours were poured onto a 27 µm sieve, washed and rinsed thoroughly using a wash bottle. For the purpose of counting nematodes from the extraction a nematode concentrates of 5 ml volume were placed in falcon tubes. The inoculum in the falcon tubes was allowed to settle before counting under the stereo microscope (10×2.5 magnification). Using a pasteur pipette which was immersed at the

bottom of the falcon tube nematodes were drawn and placed on the nematode counting dish for counting. The nematode juveniles were counted using hand tally counter per replicate so as to determine the directional preference of nematodes to respective treatments.

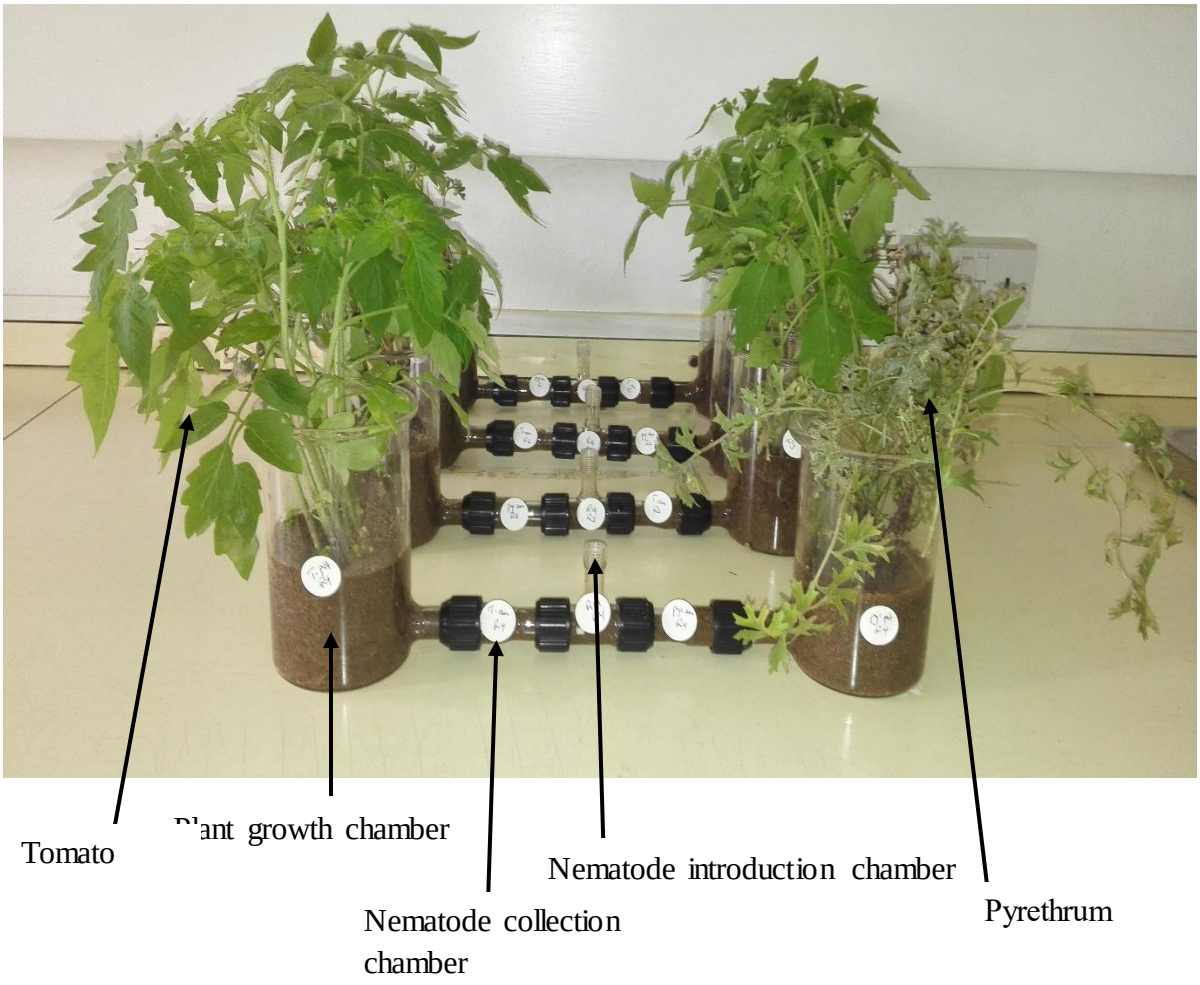

Plate 3.1: dual-choice nematode bioassay setup in the laboratory.

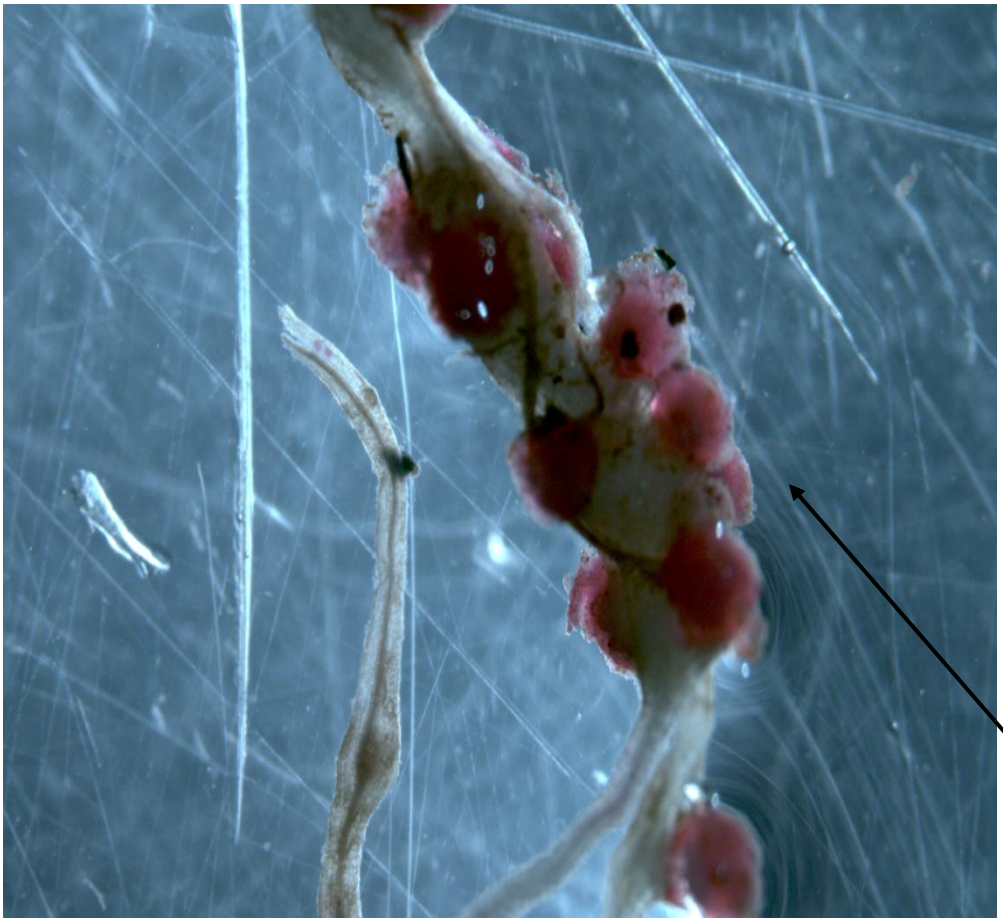

Plate 3.2: Egg masses of nematode infected tomato roots stained with phloxine B

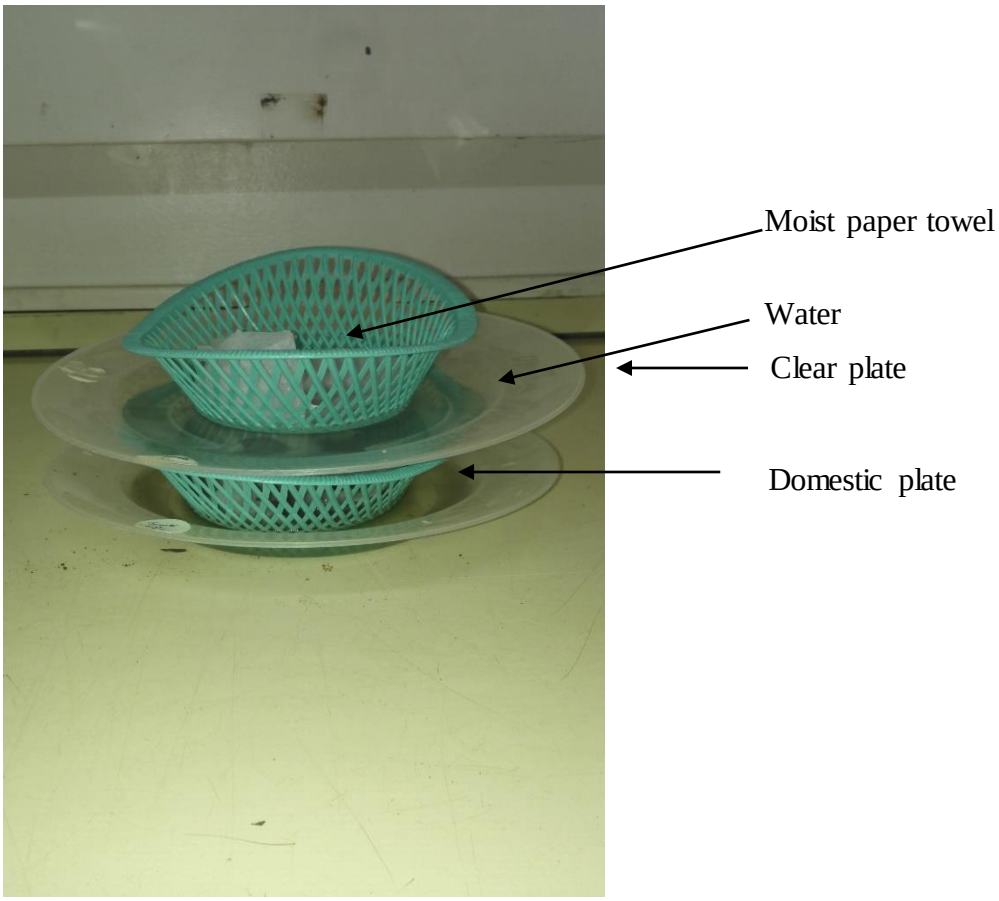

Plate 3.3: A modified Baermann extraction method for Root knot nematode juveniles

3.5 Collection of root volatiles

A total of fifteen plants per species were conditioned for 5 days in glass chambers. Root volatiles were collected using the super Q adsorbent (30 mg, Analytical Research System, Gainesville, Florida, USA) for 24 hours in the laboratory (ex-situ) using a metal probe made of steel inserted in the plant soil root zone in growth glass chambers (Plate 3.4). Clean charcoal filters (activated charcoal) were used to help trap any diffusing volatiles. The adsorbed volatiles in the Super Q filters were eluted with 200 µl dichloromethane (Sigma-Aldrich Corporation 3050 Spruce Street, St. Louis, Missouri 63103 USA) into 1.5 ml auto sampler glass vials and 50 µl for analysis was drawn and the rest of eluate stored at -80°C freezer until use.

3.6 Characterization of volatile components

Gas Chromatography-Mass Spectrometry (GC-MS) analysis was carried on an Agilent Technologies 7890A/5975 C gas chromatograph (www.agilent.com) equipped with a capillary column HP-5 MS (30 m × 0.25mm ID × 0.25 µm film thickness) (Hewlett Packard) and coupled to a 5795C mass spectrometer. One microlitre of each sample was injected in the split less mode, and helium was used as the carrier gas at 1.25 ml min^{-1}. The oven temperature was programmed at 35 °C for 5 min, increased to 280 °C at a rate of 10 °C $min^{-1}$and held under this temperature for 10 min with the total run time of 50 min per sample. The volatile analysis was carried out at 70 eV in the electron impact ionization mode. Compounds identified were confirmed using commercially available synthetic standards analysed similarly and based on comparison with National Institute of Standards and Technology (NIST'08, 05 library data bases).

All the chromatograms were manually integrated by moving the peak detection points so as to allow peak enhancement and manipulation of the baseline to achieve accuracy of individual compound detection. Root volatiles were quantified using internal standards for monoterpenes α-pinene was used while for sesquiterpenoids Carryophllene was

utilized. A linear regression equation for each internal standards ($y = 2e6 - 741414$) and ($y = 2e6 - 3e6$) were developed using the straight line is y = mx + c, where m is the gradient, and y = c is the value where the line cuts the y-axis.while c is the intercept on the y-axis. The regression equations were derived by comparisons with areas to know quantity of the second well defined internal standard that were analysed by GC-MS (α-Pinene and Caryophyllene). Three replicates of the root volatiles for each of the four plant species areas obtained were used for quantification using internal standards.

Quantification of root volatiles from *B pilosa, T minuta, A annua* and *C cinerariaefolium)* was performed using dilution factor of 2 with serial dilutions (50ng/µl, 12.5ng/µl, 6.25ng/µl and 1ng/µl) of authentic standard of (IR)-(+) α-Pinene; 99+%, and (-)-Caryophlyllene; 99+% were prepared and analyzed by GC-MS and their areas used.

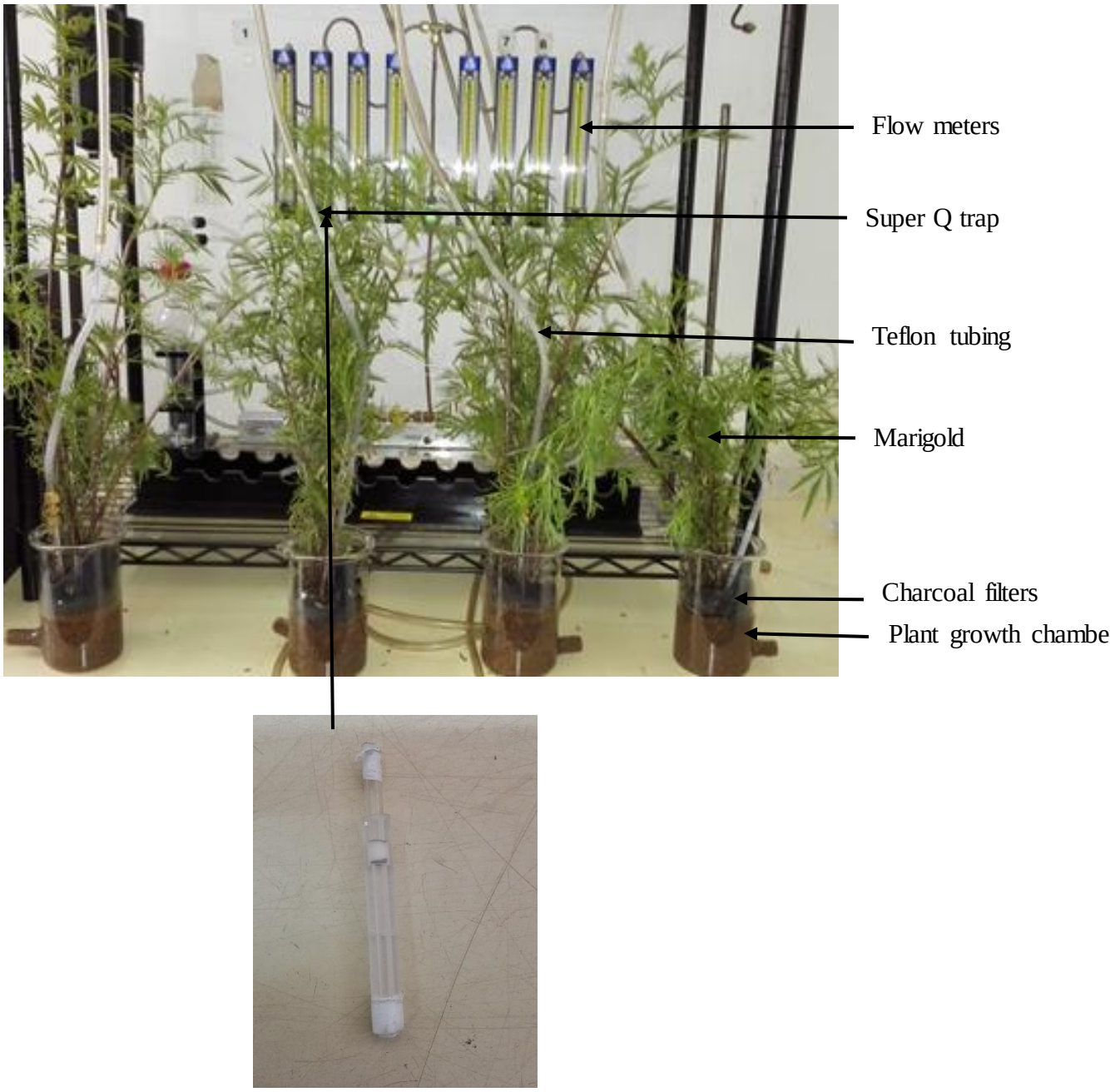

Plate 3.4: A volatile collection system root volatiles using super Q adsorbent with metallic probes.

3.4 Data Analysis

Data obtained from the root knot nematodes duo choice soil olfactometer bioassays on response of nematodes to odours of tested plants were analysed using Chi-square (χ^2) goodness of fit test to study Asterarceae plants root odour discrimination by *Meloidogyne incognita*.

Volatiles from different plants were quantified using an internal standard as described in section 3.6 and the peak areas converted ng/plant/hr. Means and standard errors were calculated using R software by taking into account the replications.

To discriminate the species with the family based on volatile components from the plants, principal component analysis was performed. The amount of volatiles (ng/plant/hr) was log transformed and analysed to generate a visual plot on the basis for evaluation of resemblance and differences on root volatiles they release.

All statistical data analyses were performed using R version 2.15.1 software (R Core Team, 2015).

CHAPTER FOUR

4.0 RESULTS

4.1 Nematode response to root volatiles of Asteraceae plants relative to the control (sand)

M. incognita juveniles avoided the direction with Asteraceae plants tested compared to the control (Sand). Significant differences (*P<0.05*) were observed between treatments (asteraceous plants) and (Sand) Control (Figure 4.1; Table 2).

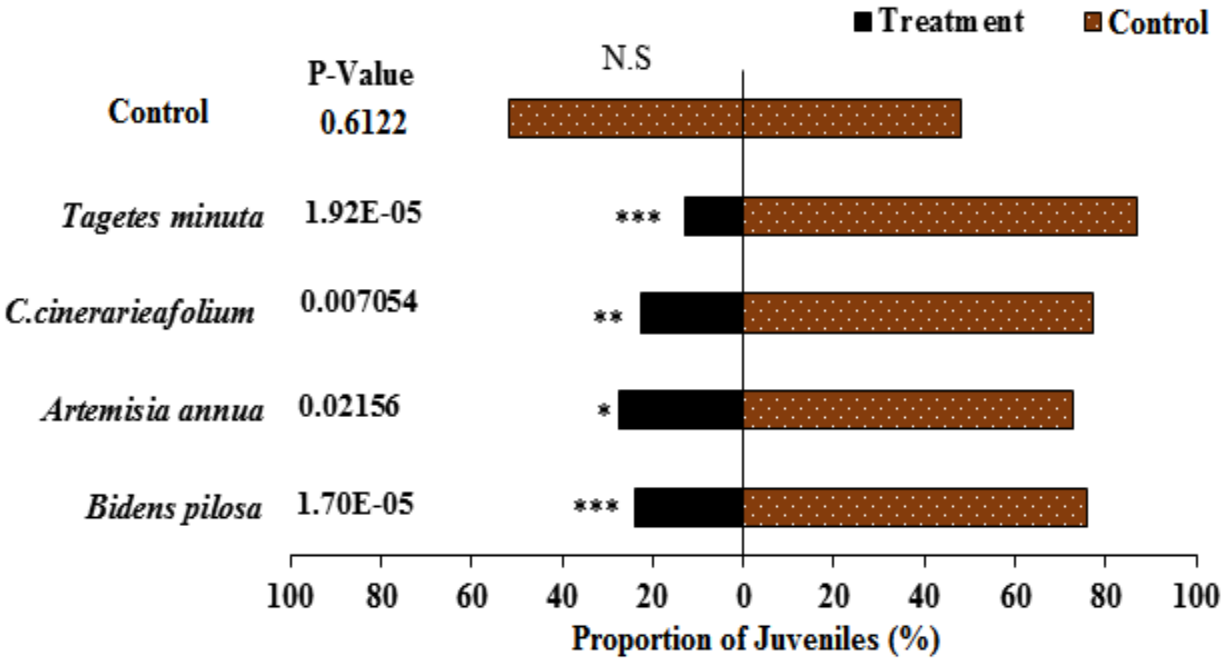

Figure 4.1: Response of M. incognita juveniles to root volatiles of *T. minuta, C. cinerariaefolium, A annua and B. pilosa* compared to a control (sand). *** = significant at P < 0.001, **=significant at P < 0.01 and * = Significant at P < 0.05 for Chi-Square test on equality of proportions of nematode juveniles making choice to root volatiles of Asteraceae plants, tomato and control (Sand) soil olfactometer assays.

4.2: Nematode response to root volatiles of Asteraceae plants relative to tomato

Meloidogne incognita (P<0.05) avoided the direction with volatiles with Asteraceae plants including the control (sand) as compared to those of tomato (host plant) (Figure 4.2; Table 2).

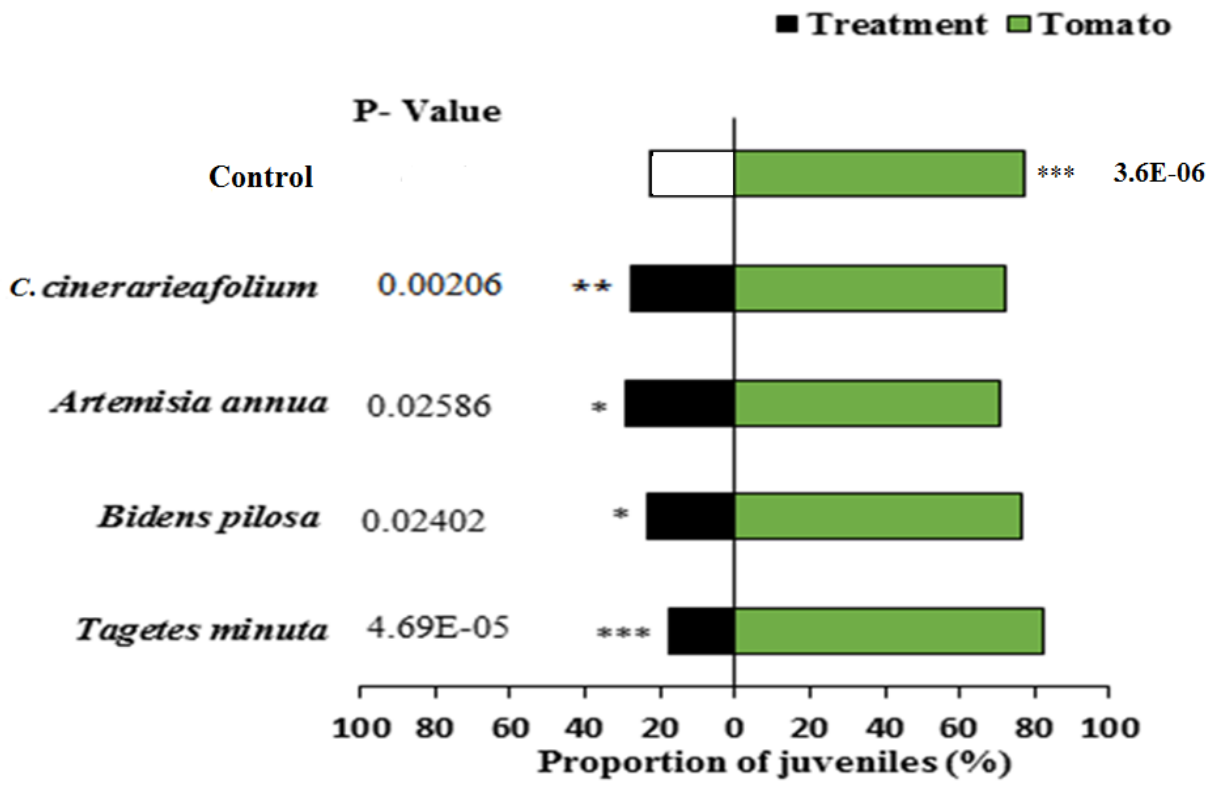

Figure4.2: Response of *M. incognita* juveniles to root volatiles of *T. minuta*, *C. cinerariaefolium*, *A. annua* and *B. pilosa* compared to tomato (host)= significant at $P < 0.001$, **=significant at $P < 0.01$ *and* * = Significant at $P < 0.05$ for Chi-Square test on equality of proportions of nematode juveniles making choice to root volatiles of Asteraceae plants, tomato and control (Sand) soil olfactometer assays.

4.3: Nematode response to root volatiles of Asteraceae plants combined with tomato versus a control (sand)

Meloidogyne incognita juveniles ($P<0.05$) avoided the direction of volatiles with a combination of Asteraceae plants + tomato in preference to tomato alone (Figure 4.3; Table 2). On average 74.5% of the nematode juveniles preferred tomato root volatile relative to Asteraceae plants + tomato combinations while proportions nematode choice to treatment or sand varied according to species.

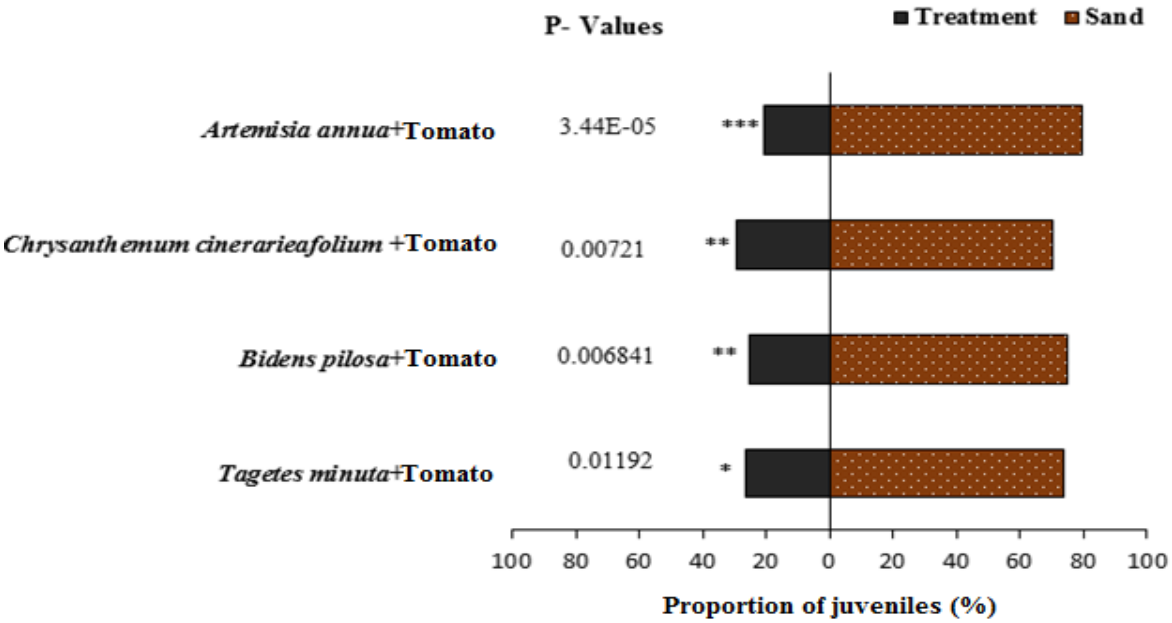

Figure 4.3: Response of *M. incognita* juveniles to root volatiles of asteraceous plants combined with tomato relative to a control (sand).*** = *significant at* $P < 0.001$, **=significant at $P < 0.01$ *and* * = Significant at $P < 0.05$ for Chi-Square test on equality of proportions of nematode juveniles making choice to root volatiles of Asteraceae plants, tomato and control (Sand) soil olfactometer assays.

4.4: Nematode response to root volatiles of Asteraceae plants combined with tomato versus tomato (host plant)

Meloidogyne incognita juveniles ($P<0.05$) avoided the direction with a combination of Asteraceae plants combined with tomato in preference to tomato alone (Figure 4.4; Table 2). On average tomato alone was more preferred 4 times more that the combination of asteraceous plants and tomato.

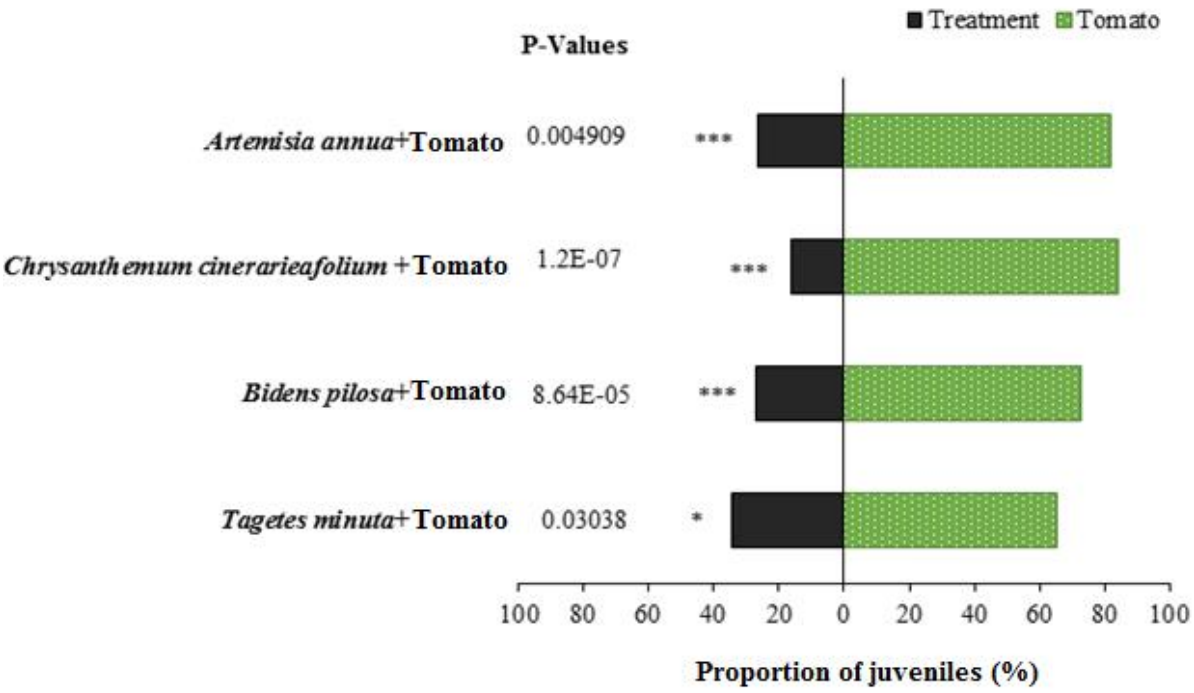

Figure 4.4: Response of *M. incognita* juveniles to root volatiles of asteraceous plants combined with tomato relative to a host plant (Tomato). *** = significant at $P < 0.001$, **=significant at $P < 0.01$ *and* * = Significant at $P < 0.05$ for Chi-Square test on equality of proportions of nematode juveniles making choice to root volatiles of Asteraceae plants, tomato and control (Sand) soil olfactometer assays.

4.5 Chi-square analysis of nematode dual–choice bioassay

The results of the χ^2 indicated that nematodes avoided the direction of all treatment combinations for either a control (sand) or tomato (nematode) host plant. Nematodes (P=0.05) significantly avoided all Asteraceae plants direction relative to the control (sand) and tomato root volatiles

Table 2 Chi- Square analysis for the test of equality of proportions of J2s making a choice on perceived root volatiles relative to the control and tomato.

Treatments	**N**	**n**	**d.f**	**χ2**	**P- value**
Tomat , Sand	135	40	1	21.6	3.6e-06
B. pilosa, sand	74	18	1	18.5	1.70e-05
A. annua, sand	32	9	1	5.2812	0.02156
C. cinerariaefolium, sand	27	6	1	7.2593	0.00705
T. minuta, Sand	37	5	1	18.27	1.92e-05
B. pilosa, Tomato	28	7	1	6.0357	0.01402
A. annua,Tomato	29	8	1	4.9655	0.02586
C. cinerariaefolium, Tomato	51	14	1	9.4902	0.00206
T. minuta, Tomato	44	8	1	16.568	4.69e-05
B. pilosa+tomato , sand	35	9	1	7.3143	0.006841
A. annua + tomato, sand	56	12	1	17.161	3.44e-05
C. cinerariaefolium +.Tomato , Sand	50	15	1	7.22	0.00721
T. minuta + Tomato, Sand	31	8	1	6.3226	0.01192
B. pilosa + tomato,Tomato	20	20	1	15.413	8.64e-05
A. annua + tomato, Tomato	60	16	1	12.15	0.04909
C. cinerariaefolium + tomat , Tomato	66	11	1	15.413	1.20e-07
T. minuta+Tomato, Tomato	48	16	1	4.6875	0.03038

4.1.0 Chemical analysis of Asteraceae plants

4.1.1 Identification and quantification of volatile components in Asteraceae plant species

A total of 59 compounds comprising of Alkanes, Aldehyde, Aromatic, fatty acids, Ketones, esters Monoterpenes and Sesquiterpenes were obtained from the four asteraceous plants. Out of these 30 were from *T minuta*, 32 from *A annua*, 21 from *B pilosa* and 12 from *C cinerariaefolium* respectively (Apendices 4, 5, 6&7). Out of the total of the 59 compounds that identified 2 were Ketones, 4 Alkanes, 23 Monoterpenes, 2 Aldehyde, 2 were Aromatic, 18 Sesquiterpenes, 1 fatty acid, and 6 esters. α-Pinene and Limonene were detected in the roots of all the 4 plants, Camphene and Camphor were detected in *B.pilosa, A.annua* and *C cinerariaefolium*. Δ-3-Carene and Allo–ocimene were detected in roots of *Tagetes minuta , A annua* and *B pilosa* while the rest of the compound were common either in two plant species with the rest confined to specific plant species(Table 3).

Table 3. Abundance of volatiles compounds (ng/plant/hr); identified from root volatiles of four Asteraceae plant species; *Tagetes minuta*, *Artermisia annua*, *Bidens pilosa* and *Chrysanthemum cinerarieafolium*

			Asteraceae plant species			
Peak No.	RT (min)	Compound Name	*T. minuta*	*A. annua*	*B. pilosa*	*C .cinerarieafolium*
1	6.52	Ethyl isovalerate^{Est}	-	-	0.2 ± 0.02	-
2	7.92	2-Hexenal, (E)-^{Ald}	-	0.3 ± 0.04	-	-
3	8.78	Styrene^{Arom}	-	2.0 ± 0.36	-	-
4	8.79	3-Heptanone^{Ket}	2.0 ± 0.19	-	-	-
5	9.01	Nonane^{Alka}	-	-	0.4 ± 0.02	-
6	9.14	Heptanal^{Ald}	0.6 ± 0.08	-	-	-
7	9.63	α -Phellandrene^{Mono}	-	-	1.0 ± 0.11	-
8	9.76	α-Pinene^{Mono}	1.3 ± 0.34	2.4 ± 0.34	13.2 ± 1.49	0.7 ± 0.16
9	10.07	Camphene^{Mono}	-	2.5 ± 0.33	2.3 ± 0.49	0.3 ± 0.05
10	10.62	β-Phellandrene^{Mono}	-	0.5±0.08	3.0±0.39	-
11	10.63	Sabinene^{Mono}	4.6 ± 0.49	-	-	0.4 ± 0.08
12	10.67	β-Pinene ^{Mono}	-	3.4 ± 0.8	6.8 ± 0.46	4.4 ± 0.39
13	10.98	Myrcene^{Mono}	-	0.7 ± 0.19	3.0 ± 0.49	-
14	11.35	Δ-3-Carene^{Mono}	1.9 ± 0.20	-	2.1 ± 0.21	0.6 ± 0.05
15	11.72	Limonene ^{Mono}	30.4 ± 1.09	1.9 ± 0.22	1.0 ± 0.18	0.6 ± 0.10
16	11.76	Cineole<1,8->^{Mono}	-	7.4 ± 0.61	6.2 ± 0.12	-
17	11.87	(E)-β-Ocimene^{Mono}	9.1 ± 0.72	-	0.3 ± 0.06	-

Peak No.	RT (min)	Compound Name	*T. minuta*	*A. annua*	*B. pilosa*	*C .cinerarieafolium*
18	12.06	(Z)-β- Ocimene^{Mono}	0.7 ± 0.06	-	1.0 ± 0.19	-
19	12.17	Dihydro - Tagetone^{Mono}	18.3 ± 1.52	-	-	-
20	12.26	γ –Terpinene^{Mono}	-	6.8 ± 0.91	25.4 ± 1.04	-
21	13.31	1,3,8-p-Menthatriene^{Mono}	0.4 ± 0.07	-	-	-
22	13.46	allo-Ocimene^{Mono}	7.4 ± 0.39	0.5 ± 0.09	0.7 ± 0.06	-
23	13.73	E-Tagetone^{Mono}	32.2±1.89	-	-	-
24	13.74	Camphor^{Mono}	-	1.2 ± 0.22	0.8 ± 0.20	5.5 ± 0.72
25	13.87	Z-Tagetone^{Mono}	33.9 ± 1.97	-	-	-
26	14.52	Methyl salicylate^{Arom}	-	0.7 ± 0.12	0.4 ± 0.09	-
27	14.52	Dodecane^{Alka}	-	1.1 ± 0.34	-	-
28	14.77	Verbenone^{Mono}	2.6 ± 0.39	-	-	-
29	15.11	Z- Ocimenone^{Mono}	63.7 ± 2.11	-	-	-
30	15.22	E-Ocimenone^{Mono}	35.6 ± 8.53	-	-	-
31	15.49	Chrysanthenyl acetate^{Est}	-	-	1.7 ± 0.31	-
32	15.88	Bornyl acetate^{Est}	-	-	3.3 ± 0.34	-
33	15.97	Tridecane^{Alka}	-	1.0 ± 0.01	1.0 ± 0.06	-
34	16.47	Silphiperfol-5-ene^{Sesq}	9.5 ± 0.57	3.5 ± 0.66	-	-
35	16.57	Presilphiperfol-7-ene^{Sesq}	-	1.9 ± 0.38		-
36	16.75	Silphinene^{Sesq}	6.8 ± 0.52	13.5 ± 1.02	-	-
37	16.92	β-Chamigrene^{Sesq}	-	1.3 ± 0.19	-	-
38	17.06	Guaiene<cis-beta->^{Sesq}	-	1.5 ± 0.09	-	-
39	17.25	Modheph-2-ene^{Sesq}	39.8 ± 1.3	14.0 ± 1.04	-	-

Peak No.	RT (min)	Compound Name	*T. minuta*	*A. annua*	*B. pilosa*	*C .cinerarieafolium*
40	17.34	α-Isocomene^{Sesq}	12.4 ± 0.86	-	-	-
41	17.34	β-Elemene^{Sesq}	-	23.8 ± 1.63	-	-
42	17.52	Cyperene^{Sesq}	6.4 ± 0.47		-	-
43	17.62	β-Isocomene^{Sesq}	5.3 ± 0.66	12.1 ± 0.47	-	-
44	17.67	Longifolene-(V4)^{Sesq}	5.2 ± 0.62		-	-
45	17.76	E- Caryophyllene^{Sesq}	4.3 ± 0.42	22.1 ± 0.89	2.1 ± 0.05	-
46	17.87	β-Copaene^{Sesq}	-	1.11 ± 0.11	-	-
47	18.10	β-Farnesene<(E)^{Sesq}	-	47.1 ± 1.71	-	15.7 ± 0.71
48	18.20	α-Humulene^{Sesq}	1.6 ± 0.15	5.9 ± 0.24	-	-
49	18.46	β-Patchoulene^{Sesq}	-	1.9 ± 0.26	-	-
50	18.72	a- Selinene^{Sesq}	-	2.5 ± 0.41	-	-
51	18.73	Bicyclogermacrene ^{Sesq}	4.2 ± 0.41	-	-	-
52	19.32	Dodecanoic acid^{FA}	-	1.6 ± 0.15	-	-
53	19.99	Hexadecanol<n->^{Alc}	-		-	1.9 ± 0.37
54	20.74	Zierone^{Ket}	-	4.2 ± 0.29	-	-
55	20.94	Heptadecane (C17)^{Alka}	-		-	1.6 ± 0.19
56	20.97	Cyclocolorenone<epi->^{Sesq}	-	5.5 ± 0.70	-	-
57	22.29	Isopropyl tetradecanoate^{Est}	-	-	-	2.7 ± 0.31
58	24.28	Isopropyl Palmitate^{Est}	7.6 ± 0.96	-	-	-
59	24.28	Isopropyl hexadecanoate^{Est}	-	3.6 ± 0.28	-	3.5 ± 0.34

Ald - Aldehyde , Alka – Alkanes, Arom – Aromatic, Est – Esters , FA – Fatty acids , Ket – Ketones , Mono- Monoterpenes and Sesq-Sesquiterpenes

4.1.2: Discriminant analysis of volatile composition in Asteraceae plant species

Clusters of Asteraceae plant species based on the intensities of volatiles released were grouped as Principal component 1 and Principal component 2. Black jack and Pyrethrum were very similar clustered together as seen the PCA clusters while Marigold and Artemisia were distantly differentiated.

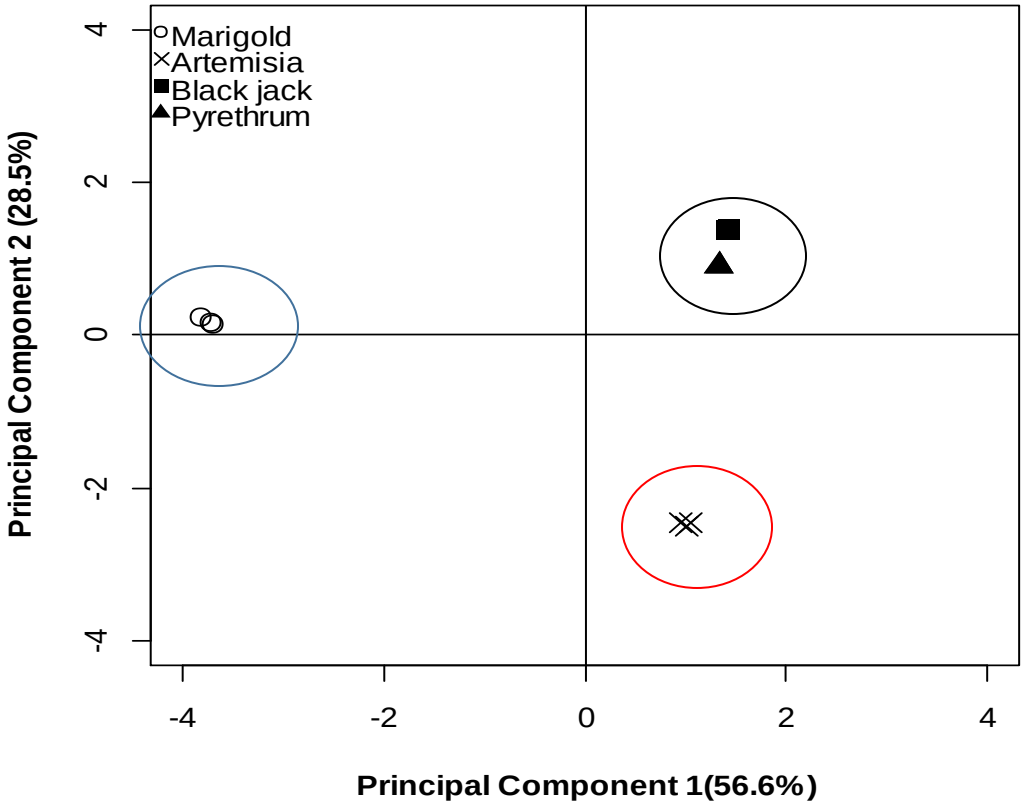

Figure 4.6: Component plot 1 explaining 56.6% of the variation, component plot 2 explains 28.5. %, while the remaining 10 components explain only 14.9%. (Appendix 2)

CHAPTER FIVE

5.0 DISCUSSION

In soil olfactometer nematode bioassays all the four species of Asteraceae plants namely *T. minuta, C. cinerariaefolium, A. annua* and *B. pilosa,* their volatiles from the roots caused avoidance behavior in *M. incognita* when the treatments were compared to the control (sand). On the other hand when the treatments of Asteraceae plants were placed together with tomato (attractant plant) , all the combinations exhibited repellence and more root knot nematodes were observed with the direction of volatiles from an attractant plant (tomato) and the control (sand) under the soil olfactometer assays. In bioassays of Asteraceae plant species relative to the control (sand), the results suggest that these plants may play an antagonistic role in the presence of an attractant plant (tomato). Further confirmation with the tomato relative to sand soil olfactometer assays shows that RKNs are highly attractive to tomato. In the subsequent assays combining the Asteraceae plants with tomato (attractant plant), it was found that these plants indeed were antagonistic to root knot nematodes (*M. incognita*). On the other hand, all treatments with Asteraceae plants combined with the attractant plant (tomato) relative to sand and tomato, the treatments were significantly avoided by *M. incognita* and this was in agreement with the hypothesis that Asteraceae plant species are antagonistic to *M. incognita* in the presence of an attractive plant tomato.

The use of antagonistic plants as botanical pesticides for the management of plant parasitic nematodes has been reported in many studies (Silva *et al.*, 2014; Singh & Prasad, 2014; Umar & Adamu, 2014). Tsay *et al.* (2004) tested and found Asteraceae plant roots were immune or highly resistant to *M. incognita*, while *T. erecta,* a closely related species to *T. minuta* effectively reduced root galling and nematode populations of *M. incognita*. In other studies essential oils extracted from Asteraceae plants were used as repellents in the control of root knot nematodes (*Meloidogyne* spp.) and there was a clear suggestion that essentials oils or main components may serve as nematicides in controlling nematodes

(Duschatzky, *et al*., 2004). Specifically Marigold is one of the most utilized plant species from Asteraceae family in the management of root knot nematodes (Douda, *et al*., 2010). Marigold has been used as cover crop, essential oil extracts, bio-waste, intercrop and has been successful in the management of root knot nematodes (Onkendi *et al*., 2014; Katooli, *et al.*, 2011). *Artemisia* spp. too have also been used in the management of root knot nematodes where it has been used as bio organic waste as well as extracts of its essential oils (Bawa, *et al*, 2014; Pandey, 2005). In other studies use *Chrysanthemum* spp. has been studied regarding their nematicidal activity for the management of root knot nematodes where essential oils, flower heads and organic amendments have been exploited and results have indicated that such plants could serve as potential sources of bio-nematicides (Wiratno *et al*., 2009b; Andrés, *et al*, 2012). In some studies conducted by Pérez *et al*. (2003) on the efficacy of essential oils and organic extracts from Asteraceae plants on nematodes also found that *C. coronarium* a close relative to *C. cinerariaefolium*, all its plant treatments from flower heads, leaves, roots parts were found to be effective and significantly reduced nematode egg hatching / reproduction in vitro and in growth chambers. Some scientific findings by Taba, *et al*. (2008) revealed that *B. pilosa* variety radiata extracts significantly suppressed *M. incognita*. This suppression was attributed to immobilization, lethality, repellence and egg hatching inhibition by extracts of *B. pilosa* plant parts from the leaves, although in another study conducted by Taba, *et al*. (2014) suppression of nematodes by *Bidens pilosa* was found to be more when dried plant chips and plants extracts were mixed and incorporated with the soil. This shows that different parts of the plant may affect nematodes differently.

However mechanisms of nematode suppression remains a controversy as there is no clear explanation as to whether the leaves, stems and roots of Asteraceae plants produce nematode inhibiting compounds differentially (Oka, *et al*., 2000). The findings of this study on nematode behavioral bioassays indicates that Asteraceae plants roots plays a key role in the repellence of *M incognita* juveniles. It is likely that Asteraceae plant roots produce active defensive volatile compounds that repel *M. incognita*.

Volatile compounds from roots of black jack (*B pilosa*), pyrethrum (*C cinerariaefolium*) African marigold (*T minuta*) and sweet wormwood *(A annua)* were identified. Plant volatile organic compounds from showed varied chemical composition in terms of quantity and quality. This was shown by principal component analysis where; principal components 1 gave 56.6% and 28.5% for PCA 2 accounting for about 85.1% variation in reference to detected volatile compounds and composition by quantities and quality. It appears variations observed in Asteraceae species were mainly quantitative and with only few compounds detected in roots of *B.pilosa* and *C. cinerariaefolium* clustered as one while *T minuta* and *A annua* were distantly different due to some associated compound class abundance and compound novelty respectively. Variations at specie or variety level in volatile profile composition of compounds has been reported. Gonzalez-Mas, *et al.* (2011) also found different clusters of citrus varieties when Principal analysis was performed hence it likely that species within the family Asteraceae may have some similar compounds but other compounds are species specific.

The compounds identified from the four Asteraceae plants were mainly monoterpenes and sesquiterpenes which most importantly have been cited literature to have allelopathic, insecticidal, nematicidal and repellant properties (Wichittrakarn, *et al.*, 2013 & Vasudevan, *et al.*, 1997). Monoterpenes and sesquiterpenes were the most abundant compound classes in the four Asteraceae plant roots , these compounds have been reported to possessing repellent properties (Vicidomini, 2011) thus this probably could be reason for *M. incognita* avoidance observed from all bioassay experiments. Chitwood (2002) reported that higher plants have a broad spectrum of active compounds that includes alkaloids, lipids, terpenoids, sesquiterpenoids, diterpenoids, simple and complex phenolics, as well as several other classes that are phyto-toxic to nematodes. Polyacetylenes and Polythienyls which are present in the roots of marigold have been linked to their potency for nematode silencing as well as playing the role of pharmacology (Ntalli & Menkissoglu-Spiroudi, 2011; Cortés-Rojas, *et al*, 2013). Chemistry results of Asteraceae plant roots in this study confirms the presence rich classes of secondary compounds like monocyclic and bicyclic monoterpenes, sesquiterpenes , flavanoides ,

thiophenes and aromatics have been detected(Vasudevan, *et al*., 1997 & Gil, *et al*., 2002). Compound classes of monoterpenes and sesquiterpenes essential oils have been found to possess repellence properties significant for influencing insect behavior (Nerio, *et al*., 2010). Detection of thiophenes in the roots of Marigold (*Tagetes* spp.) and *Chrysanthemum* spp. have been linked to be responsible for reducing the galling in roots for studies where tomato, marigold (*Tagetes* spp.) and *Chrysanthemum* spp. were evaluated alone and as well as combinations with tomato whilst inoculated with nematode juveniles (Hackney & Dickerson, 1975). This is an intercropping aspect of a cropping system where establishing mechanisms of how intercrops works in nematode suppression based on chemical ecology would be very important. These results are in agreement with (Finch & Collier, 2012) who suggested that chemicals from intercrops are capable of repelling herbivores there by providing insect with the choice but more preferably the host and non-host.

These results are also in agreement with (Reynolds, *et al*., 2011) who found that nematodes use the shortest possible route in locating the host much more direct to the source chemo attractant (food source) as the case for tomato alone that attracted more nematodes compared to all other treatments.

However, the mechanisms of Asteraceae plants having nematicidal effect seems to be multi-dimensional where both volatiles and non-volatile compound could be playing a role in nematode suppression or repellence. From the results it is likely that volatile organic compounds could be linked to nematode suppression by Asteraceae plants and thus making the plants species to be non-hosts as reported.

The overall results of this study for all the four different RKNs bioassays indicated that Asteraceae plants have potential in repelling root knot nematodes and this suggests that root chemical defenses may be linked to family/ species-specific adaptation to below ground herbivores (Agrawal, 2004). This entails that it's possible that the Asteraceae plant species evaluated in the bioassays poses phytochemical compounds vital for suppressing of root knot nematodes.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

In conclusion, it was established that Asteraceae plants; *T. minuta, A. annua, B. pilosa* and *C. cinerarieafolium* are important in the management of *M. incognita* by repellence in below ground niches as evidenced by their effect in the behavioral assay experiments. The volatile organic compounds of Asteraceae plants also shows a complex and varied quantitative composition of volatile compounds rich in monoterpenes and sesquiterpenes. This therefore suggests that the candidate Asteraceae plants are potential antagonistics for nematode management in small scale cropping systems.

6.2 Recommendations

1. Evaluating the efficacy of *T. minuta, A. annua, B. pilosa* and *C. cinerarieafolium* plants on RKNs based on the quantity of root volatile components
2. Testing blends and individual components identified in the root volatiles for determine the behavioral responses of *M. incognita*

Design an efficient and cost effective management tool for small holder farming systems.

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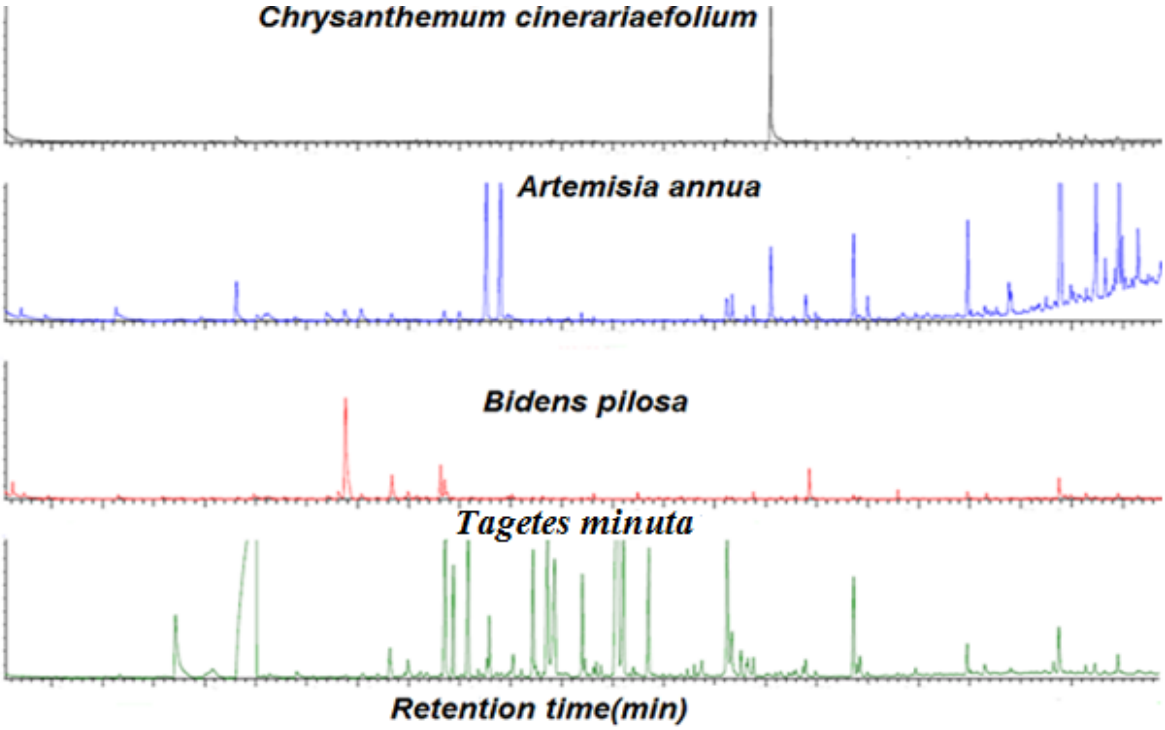
APPENDICES

Overlaid chromatograms

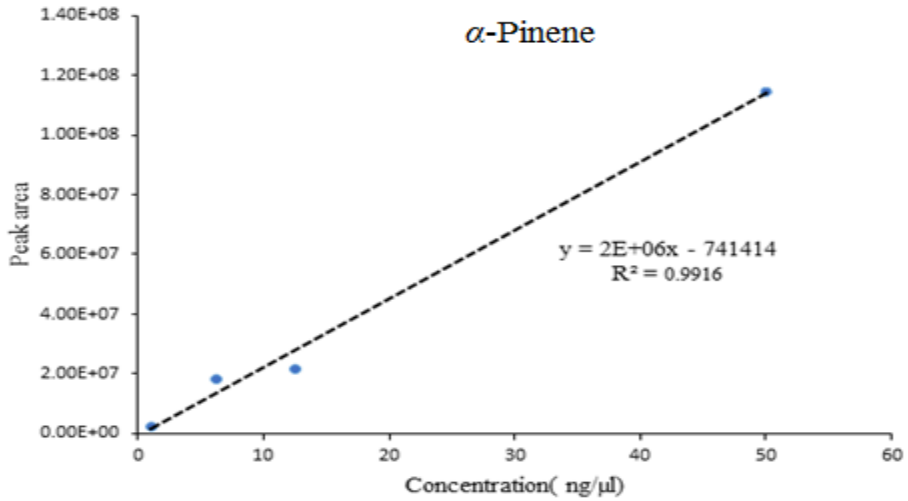


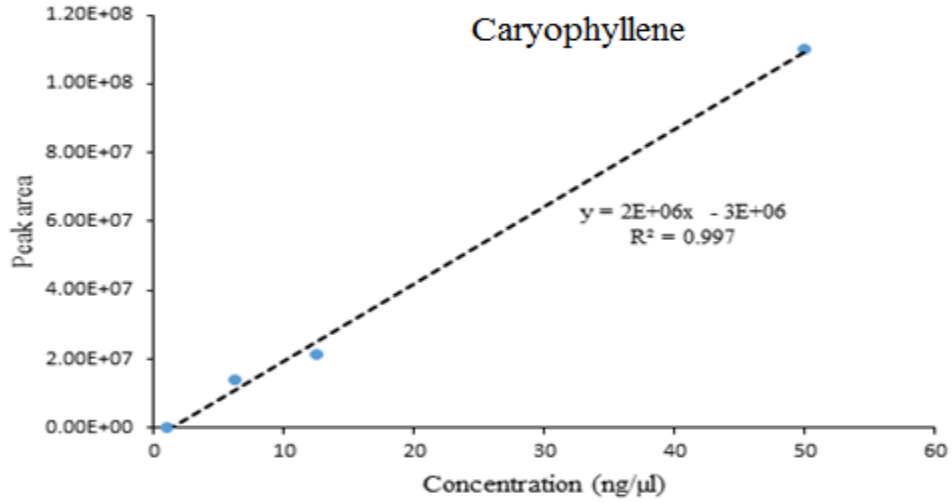
Appendix 1: Overlaid chromatograms of *T. minuta, A. annua, B. pilosa* and *C. cinerarieafolium*

Important principal components table for species analyses

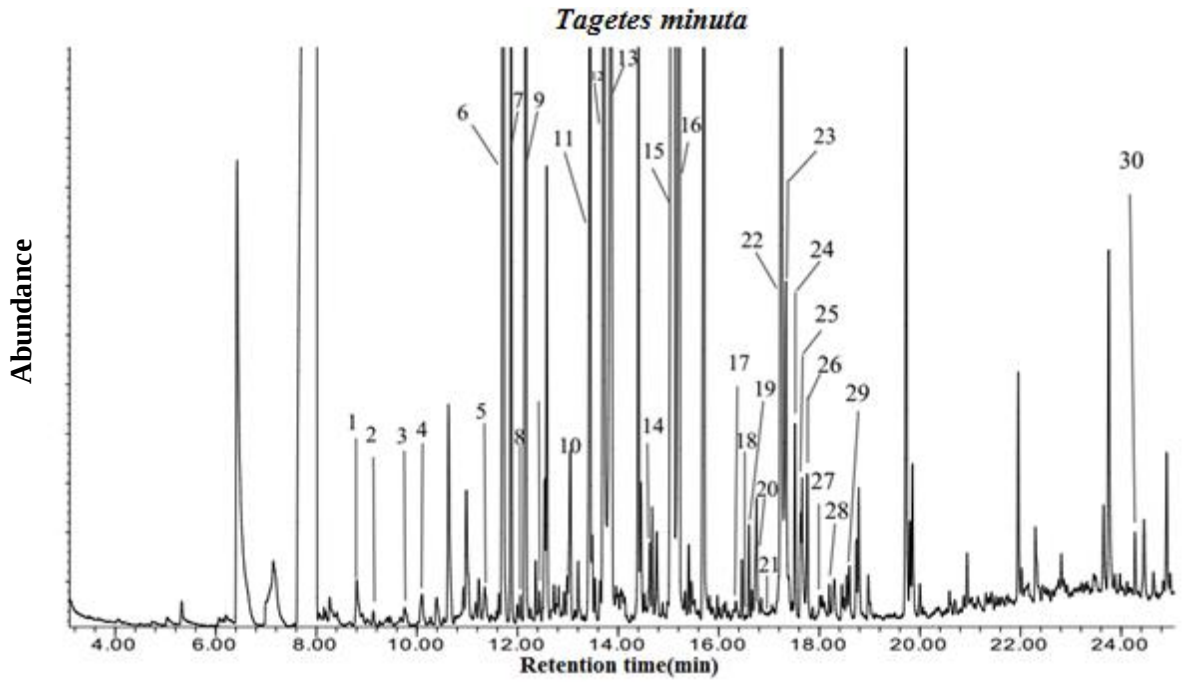
PC's	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9	PC10	PC11	PC12
SD	2.26	1.55	1.06	0.14	0.11	0.10	0.09	0.07	0.05	0.05	0.03	1.7e-16
PV	0.5878	0.2756	0.1295	0.00226	0.00129	0.00122	0.00093	0.00063	0.00036	0.00033	0.00010	0.00e+00
CP	0.5878	0.8634	0.9929	0.99514	0.99643	0.99765	0.99858	0.99921	0.99957	0.99990	1.00000	1.000e+00

Appendix 2: Output for 12 principal components used in discrimination of Asteraceae plant species



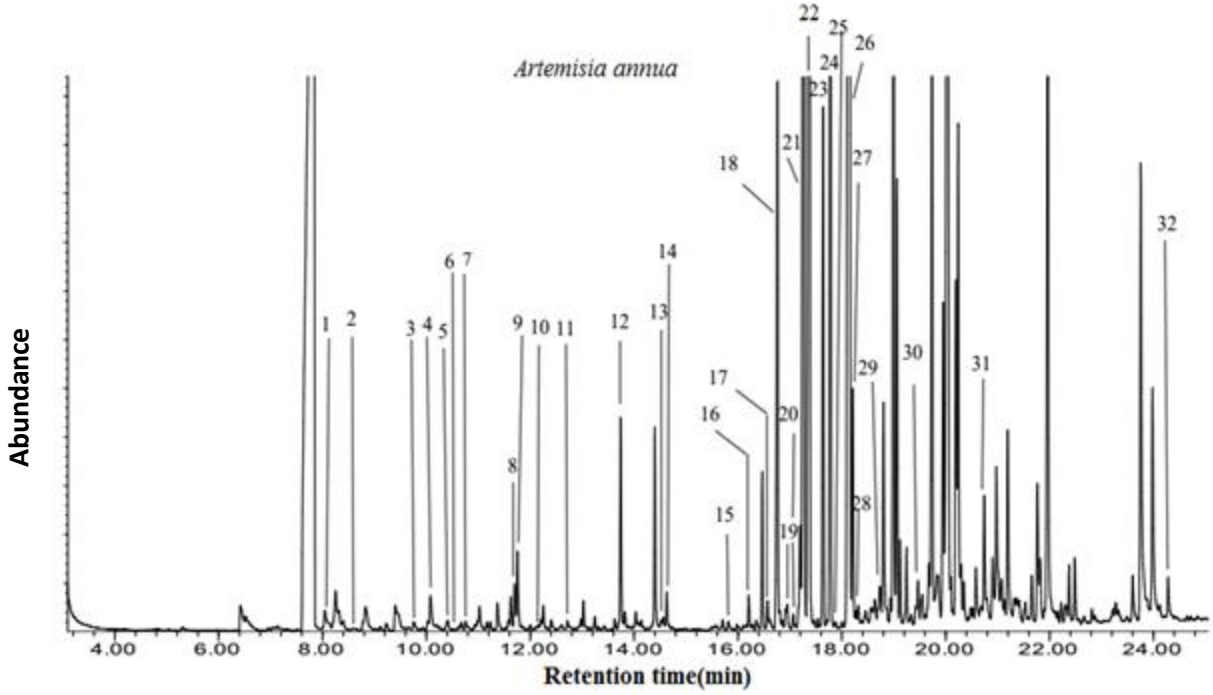


Appendix 3: Calibration curves for α- Pinene and Caryophyllene used in quantification of Volatile organic compounds



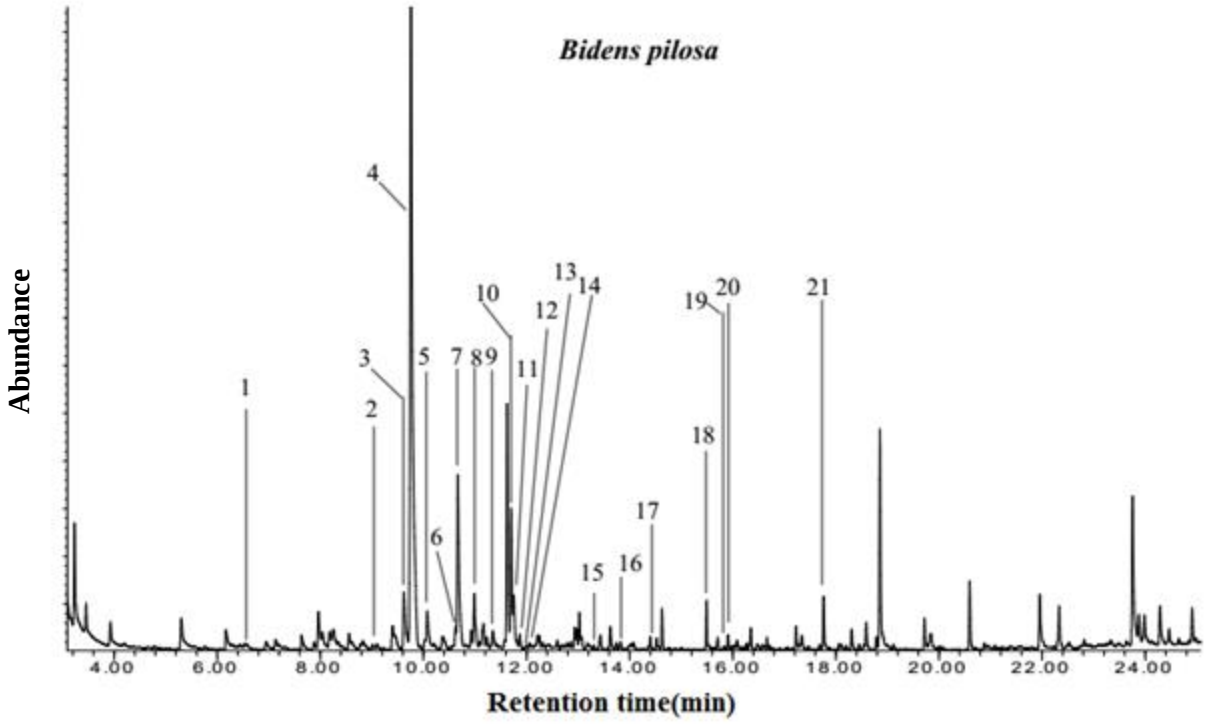
1. 3-Heptanone, **2.**Heptanal, **3.**α-Pinene, **4.**Sabinene, **5.**δ-3-Carene, **6.**Limonene, **7.***β*-Ocimene(E), **8.***β*- Z-Ocimene, **9.**Tagetone<dihydro->, **10.**allo-Ocimene, **11.**1,3,8-p-Menthatriene, **12** E-Tagetone, **13.** Z-Tagetone, **14.** Verbenone, **15.** Z-Ocimenone, **16.** E-Ocimenone, **17.** Silphiperfol-5-ene, **18.** Piperitenone, **19.** Silphinene, **20.** n-Decanoic acid , **21.**Guaiene<cis-beta->, **22.**Modheph-2-ene, **23.** ***α***-Isocomene, **24.**Cyperene,**25.** *β*-Isocomene, **26.** Longifolene-(V4), **27.**(E)-Caryophyllene, **28.** α-Humulene, **29.** Bicyclogermacrene, **30.** Isopropyl Palmitate

Appendix 4: GC-MS chromatogram related to volatile organic compounds related to *T. minuta* (Marigold)



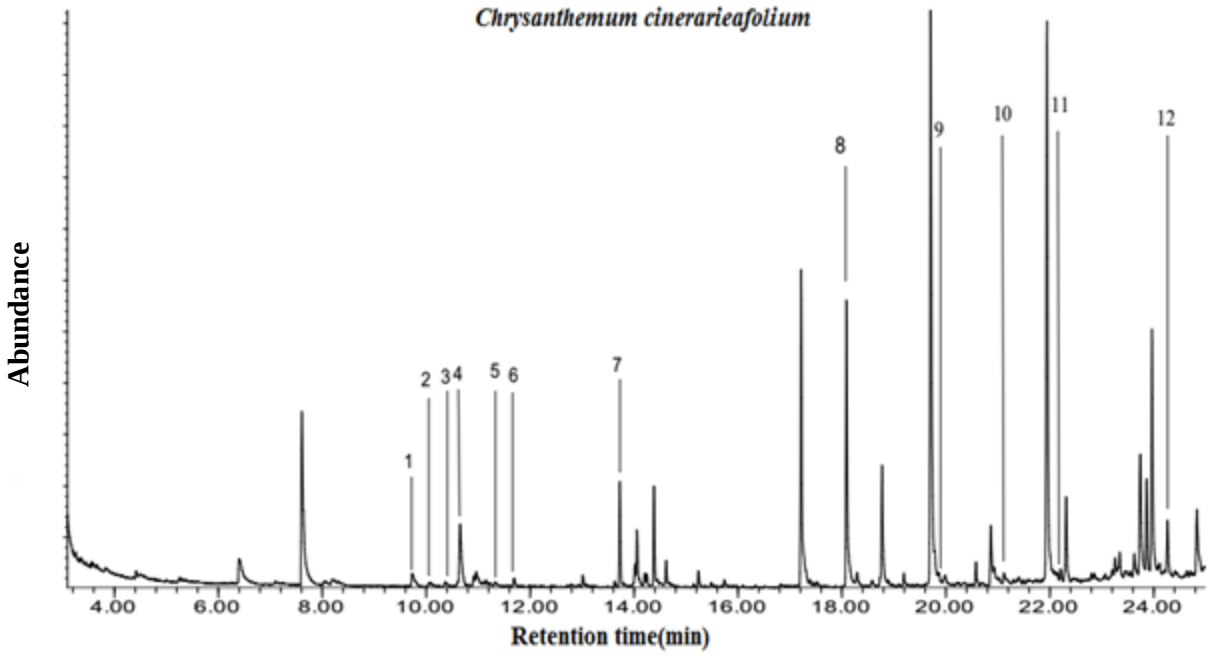
1.2-Hexenal, (E)-, **2**. Styrene, **3** α-Pinene, **4.** Camphene, **5.** *β*-Phellandrene, **6.***β*-Pinene **,7** Myrcene, **8** .Limonene, **9.** Cineole<1,8->,**10.** γ –Terpinene, **11.**Ocimene<neo-allo->, **12.**Camphor, **13**.Methyl salicylate, **14.**Dodecane, **15**. Tridecane, **16.** Silphiperfol-5-ene,**17.** Presilphiperfol-7-ene,**18.** Silphinene,**19**. *β*-Chamigrene, **20.**Guaiene<cis-beta->,**21.** Modheph-2-ene,**22.***β*-Elemene **23.** *β*-Isocomene, 24**.** (E)- Caryophyllene **25.***β*-Copaene, 26**.***β*-Farnesene<(E), **27**.α-Humulene,28**.** *β*-Patchoulene,**29.** a- Selinene, 30**.** Dodecanoic acid,,**31.** Zierone,

Appendix 5: GC-MS chromatogram related to volatile organic compounds related to *A. annua*



1.Ethyl isovalerate ,**2.** Nonane,3**.** α- Phellandrene, **4.** α-Pinene**, 5.** Camphene, **6.beta** Phellandrene, **7.** β-Pinene, **8.**Myrcene, **9.**δ-3-Carene **10.**Limonene, 11. Cineole<1,8>, **12.**β-Ocimene(E),**13.**β-Ocimene<(Z),**14.**γ–Terpinene, **15**.allo-Ocimene, **16**.Camphor, **17.**Methyl salicylate, **18.**Chrysanthenyl acetate<cis->, **19.**Bornyl acetate, **20.**Tridecane, **21.** (E)-Caryophyllene

Appendix 6: GC-MS chromatogram related to volatile organic compounds related to *B. pilosa* (Black jack)



1.α-Pinene, **3.** Camphene, **3.** Sabinene , **4.** β-Pinene, **5.** Δ-3-Carene,**6.** Limonene, **7.** Camphor **8.** β-Farnesene<(E), **9.** Hexadecanol<n->,**10.** Heptadecane (C17), 1**1.** Isopropyl tetradecanoate , **12.** Isopropyl hexadecanoate,

Appendix 7: GC-MS chromatogram related to volatile organic compounds related to *C. cinerarieafolium* (Pyrethrum)