IDENTIFICATION OF SEMIOCHEMICALS MEDIATING ROOT-KNOT NEMATODE (*Meloidogyne incognita*) - PEPPER *(Capsicum annum)* INTERACTIONS

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DECLARATION

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

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DEDICATION

With deepest appreciation, I dedicate this thesis to my husband Joseph and our son Ryan for their love, prayers and great support that have been my utmost inspiration during my endeavor for this degree. I dedicate also to my siblings Emma, Kevin and Jemmimah for their encouragement and my father, Kenneth Kihika for motivating me always to aim for the highest possible achievement.

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ABBREVIATIONS AND ACRONYMS

ANOVA	Analysis of Variance
AVRDC	Asian Vegetable Research and Development Centre (World Vegetable Centre)
cv	Cultivar
GC/MS	Gas Chromatography/Mass Spectrometry
icipe	International Centre of Insect Physiology and Ecology
LC-QToF-MS	Liquid Chromatography-Quadruple Time of Flight Mass Spectrometry
MF	Molecular Formula
m/z	Mass to Charge Ratio
pg	Picogram
PPNs	Plant Parasitic Nematodes
RKNs	Root- Knot Nematodes
SEM	Standard Error of the Mean

ABSTRACT

Root-knot nematodes (RKNs) are economically important polyphagous group of highly adapted obligate plant parasites. They pose a substantial threat to crop production globally due to losses caused in a wide range of agricultural crops. To curb infestations of RKNs, several mitigation measures have been deployed to control these parasites but with minimal success. Alternative integrated strategies are therefore needed for management of these pests. A new strategy being explored at *icipe* focuses on understanding the mechanisms of host location in order to contribute to development of alternative environmentally friendly methods. In this study, it was hypothesized that infective second stage juveniles (J2s) of *Meloidogyne incognita* use volatile and non-volatile chemical signals to locate the roots of the solanaceous plant, *Capsicum spp*. The interactions between the root-knot nematode, *M. incognita,* and three pepper cultivars (California Wonder, Yolo Wonder and Long Red Cayenne) and one accession (AVDRC accession number: PP0237) were studied. Dual choice olfactometer assays to test chemotactic responses of J2s to root odors of the pepper plants were used. In addition, the responses of J2s when in contact with root exudates of the three pepper cultivars and the AVDRC accession were studied by observing the number of stylet thrusts per minute. Using a modified dual choice set up, the chemotactic responses of J2s to root exudates of the three pepper cultivars and the AVDRC accession were tested. Root volatiles were trapped on Super Q adsorbent, analyzed by Gas Chromatography linked Mass Spectrometry (GC/MS), and quantified using authentic standards. Root exudates were collected on XAD-4 amberlite adsorbent and analyzed using Liquid Chromatography coupled to Quadrupole Time of Flight Mass Spectrometry (LC-QToF-MS) to tentatively identify the components. The results indicated that the J2s preferred root odors (70-82%) of the three pepper cultivars than the AVDRC accession (60%) over sand controls. In stylet thrusting assays, the three pepper cultivars were observed to elicit thrusts five times more than the control, while the AVDRC accession elicited two times fewer thrusts compared to the three cultivars. Based on the chemotaxis assays with the pepper root exudates, nematodes were found to prefer (70-82%) the three pepper cultivars than the AVDRC accession (77%) over the control. GC/MS analysis of the root volatiles showed that the three pepper cultivars and the AVDRC accession shared six common components, of which five were confirmed using synthetic standards as α-pinene (**1**), limonene (3), 2-methoxy-3-(1-methylpropyl)-pyrazine (**8**), methyl salicylate (**10**) and tridecane (**12**). On the other hand, thymol (**11**) was identified as a component specific to the root odors emitted by the AVDRC accession. In olfactometer assays, J2s chose (90%) the arm permeated with different doses of a 5-component synthetic blend, but preferred less (74-93%) the arm permeated with thymol alone, and thymol combined with either the preferred natural plant root odors or the 5-component synthetic blend. These results provide new insights into the host finding and differential selection behavior of J2s to different cultivars of pepper plants, showing that the composition of root chemical signals determine J2 host choices. These results open up opportunities for the management of root-knot nematodes using semiochemical-based tools. Breeding programmes that exploit allomonal signals can also be explored to control RKNs.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Nematodes are unsegmented worm-like organisms that are microscopic in size classified in the phylum Nematoda. Some exist as free-living while others are parasites of animals and plants (Perry & Moens, 2006). Those that feed on plants are known as plant parasitic nematodes (PPNs). Various strategies have been used to categorize PPNs and they include: a) aerial parasites (those that feed on above-ground plant parts), b) root and tuber parasites (those that feed on below ground parts) (Coyne *et al.,* 2007). They can also be classified according to their feeding habits and motility. Ectoparasites, which mostly remain in the soil without entering the plant tissue and use their stylet to feed by puncturing root cells. Ectoparasites can exist as migratory or sedentary (Decraemer & Hunt, 2006). Migratory endoparasites which are mobile nematodes that feed inside the plant root tissue and sedentary endoparasites which once they have reached their feeding site inside the plant root tissue will cease to move and feed from a static location (Decraemer & Hunt, 2006; Coyne *et al*., 2007).

Economically, the most important PPNs include *Meloidogyne* species (root-knot nematodes), *Pratylenchus* species (lesion nematodes), *Heterodera* and *Globodera* species (cyst nematodes), *Ditylenchus* species (stem and bulb nematodes), *Tylenchulus* species (citrus nematodes) and *Rotylenchulus* species (reniform nematodes) (Chen *et al.*, 2004; Luc *et al.,* 2005; Perry & Moens, 2006; Onkendi *et al.,* 2014). Their infestation reduces product quality resulting in production losses. The first indication of nematode problems

is exhibited by a patch of poorly growing crop in an otherwise healthy crop. Their symptoms which are frequently misdiagnosed and their effects ascribed to other visible causes include: twisted leaves, discoloration of foliage, distorted shoots, and eventually galling on roots as in the case of root- knot nematodes (Khan, 1993; Nicol *et al.*, 2011).

Overall annual global losses caused by PPNs are estimated to be 11% for a total of USD 80 billion (Agrios, 2005) with crop production losses of 14.6% in tropical and subtropical regions compared to 8.8% in developed countries (Jones *et al.*, 2011). RKNs are reported to have caused an estimated annual loss of USD 157 billion globally (Abad *et al.*, 2008) and over 80% production losses in tomatoes in Kenya alone (Otipa *et al.*, 2004).

Root-Knot Nematodes (RKNs) are polyphagous, highly adapted obligate plant pathogens that parasitize nearly every species of higher plants including vegetable crops and are distributed worldwide (Karssen & Moens, 2006). They belong to the genus *Meloidogyne* containing more than eighty species that differ in nature and morphology depending on their host and environment (Chen *et al.,* 2004; Perry & Moens, 2006). They are sedentary and endoparasitic as feeding and reproduction occurs within plant roots. The second-stage juvenile (J2) is the infective stage which locates and penetrates a root then migrate intracellularly between the cortical cells down towards the root tip and line up parallel to the long axis of the root. They become sedentary and establish a feeding site, usually within the pericycle and vascular tissues (Perry *et al.*, 2009; Jones *et al.*, 2011). To sustain the subsequent parasitic stages, each J2 induces redifferentiation of five to seven parenchymatic root cells. A gall is formed due to hypertrophy and hyperplasia of the root

cells (Karssen & Moens, 2006; Coyne *et al.,* 2007). Galls on sweet and chilli peppers are often small (Luc *et al.,* 2005).

Geographically, the distribution of different species of RKNs is dependent on temperature, soil type and cropping history (Khan, 1993; Karssen & Moens, 2006). The species of RKNs with major economic importance based on their wide distribution and broad host range are the southern RKN, *Meloidogyne incognita* Kofoid and White, *Meloidogyne javanica* Chitiwood*,* the peanut RKN, *Meloidogyne arenaria* Chitwood that are found in the tropics (Taylor & Sasser, 1978; Luc *et al.,* 2005; Jones *et al.,* 2011). The northern RKN, *Meloidogyne halpa* Chitwood is a major species found in temperate regions (Luc *et al.*, 2005). Minor pest species include the rice RKN, *Meloidogyne graminicola* Golden & Birchfield (Karssen & Moens, 2006)*, Meloidogyne minor* Karsen, Bolk, van Aelst, van den Beld, Kox, Korthals, Molendijik, Zijlastra, van Hoof & Cook, found in sports fields and golf courses (Wesemael *et al.*, 2014) and *M. chitwoodi* (Luc *et al.*, 2005; Nicol *et al.*, 2011). Twenty two out of hundred species of *Meloidogyne* species have been reported in Africa, Australia and southern Asia (Taylor & Sasser, 1978; Onkendi *et al.,* 2014).

In Kenya, *Meloidogyne* species have been reported in all agro-ecological zones including high-, mid- and low- altitude zones. In Central Kenya, *M. incognita, M. javanica* and *M. arenaria* were reported in tomato (IITA, 1981; Birithia *et al.*, 2012) and French bean (Ogumo, 2014). In Nyanza and Western Kenya, RKNs have been reported in African leafy vegetables (Nchore, 2012; Mbogoh *et al.*, 2013). Recently, *Meloidogyne enterolobii*

Yang and Eisenback was reported in African nightshades in Eastern Kenya (Chitambo *et al.*, 2016)

Vegetables are an essential component of our daily diets as well as high value cash crops for small and large scale growers. Major vegetable producers in tropical and sub-tropical countries are Asia, Africa, South and Central America which are also areas highly infected by RKNs, an extremely important limiting factor in vegetable production (Luc *et al.,* 2005). The RKN host range includes, but not limited to, pepper, tomatoes, spinach, cabbage and pumpkin (Taylor & Sasser, 1978; Luc *et al*., 2005). Pepper is a high value vegetable crop grown in Kenya as a source of income by small and large scale growers and for domestic consumption. Crop yield and quality is affected by pests and diseases and RKN infestation is a major cause of production constraint especially for small holder farmers who cannot afford the expensive nematicides. Crop rotation is the most common method used for the management of RKNs by small scale farmers.

Nematodes are commonly controlled by rotating crops with plants that are not hosts to PPNs, using resistant cultivars, and application of nematicides (Hooks *et al.,* 2007). Synthetic nematicides are very effective against *Meloidogyne* species with good economic return on high value crops (Karssen & Moens, 2006). The use of nematicides as the principal method of controlling nematodes is very effective but also has negative effects on non-target organisms (Taylor & Sasser, 1978; Mitkowski & Abawi, 2003) and build-up of resistance by the nematodes (Onkendi *et al*., 2014). Environmental concerns have therefore caused reappraisal of synthetic nematicides (Bakker, 1993; Chitwood,

2002; Vos *et al.,* 2012). In addition, they are also expensive and unaffordable to small holder farmers in developing countries.

The crop production losses associated with RKNs are huge and pose a risk to food security globally. Conventional management strategies employed have been unable to fully control these polyphagous plant parasites. It is therefore paramount that alternate strategies be sought to improve management of RKNs. Understanding the chemical communication in plant - pest interactions has shown resounding success in developing cropping systems such as the 'push-pull' technology for striga management (Cook *et al.,* 2007). Similarly, understanding chemical communication in plant - RKN interactions can provide information towards developing alternate strategies for interrupting the life cycle of RKNs and the eventual management. To develop such strategies, knowledge of RKN response to semiochemicals is needed. This study aimed to investigate the chemical cues associated with the roots of peppers that mediate the host finding behavior of root-knot nematodes, *Meloidogyne* species.

1.2 Statement of the Problem

Root-knot nematodes pose a substantial threat to crop production globally due to the losses they cause in a wide range of agricultural crops posing a risk to food security (Abad *et al.*, 2008). The damages caused can be direct or indirect resulting in delayed maturity, toppling, reduced yields and quality of crops. Moreover, high costs of management and control leads to significant loss of income for the famers (Karssen & Moens, 2006). Nematicidal control of these pests used to be efficient and fast acting, but

development of resistance has rendered existing pest management programs ineffective. In addition, they are being reappraised due to the bioaccumulation of the nematicides in the environment (Schneider *et al*., 2003; Schneider *et al.,* 2006; Schneider & Hanson, 2009). Furthermore, these control agents are harmful to non-target species and also expensive for small holder farmers (Onkendi *et al*., 2014). Conventional chemical control, cultural and biological methods are unable to fully control the parasites and they are costly and labor intensive. This necessitates alternative ecofriendly strategies for the control of RKNs. One of such approaches would be to use chemical cues involved in the host plant-RKN interaction.

1.3 Justification of the study

Chemical ecology involves studying the origin, structure and function of naturally occurring compounds that mediate interactions between living organisms. Understanding the chemical communication in plant- RKN interactions is critical to elucidating the mechanism of RKN host location and providing tools that can be used for better management of these polyphagous pests. This can provide an alternative control strategy by interrupting the life cycle of RKNs during the infective second larval stage. To develop such strategies, knowledge of RKN attraction by semiochemicals is needed. However, little is known about such mechanisms. Both olfactory (Dillman *et al.*, 2012) as well as non-olfactory (Rasmann *et al.*, 2012) signals are considered to be important in the host seeking process. However, little investigation has been done on the semiochemical basis of RKN host location. This study therefore aimed to investigate the chemical cues

that mediate host seeking behavior of RKNs using the roots of different pepper cultivars to elucidate the underlying mechanism of RKN host location.

1.4 Hypotheses

i. RKNs are attracted from some distance by pepper root volatiles

ii. At close range, specific non-volatile compounds guide RKN to the host root

iii. Specific volatile organic compounds present in roots mediate plant-RKN interactions

1.5 Objectives

1.5.1 General Objective

To investigate the role of volatile and non-volatile compounds in the roots of different pepper cultivars that mediate behavioral responses of RKNs

1.5.2 Specific Objectives

i. To determine behavioral responses of RKN to root volatiles and exudates of different pepper cultivars.

ii. To identify chemical components of root volatiles and exudates of different pepper cultivars.

iii. To evaluate behavioral responses of RKN to blends of and specific identified root compounds.

1.6 Significance of the Study

The study was designed to characterize specific semiochemicals and blends that mediate RKN host location to preferred host or avoidance of non-hosts. The study was intended to lay down some groundwork for the development of novel semiochemical-based tactics to manage RKNs.

1.7 Scope and limitation of the study

This research only investigated the host seeking behavior of *Meloidogyne incognita* using three pepper cultivars and one accession. In addition crude root exudates were used for testing the semiochemical basis of RKN-host location.

CHAPTER TWO

REVIEW OF LITERATURE

2.1 Pepper production

Pepper, *Capsicum* spp., is one of the key vegetables produced globally with total production of green pepper estimated at >31.1 million tonnes and >3.1 million tonnes for dry pepper. In 2013, Africa was second after Asia with 20.7% of the total global production (FAO, 2014). In Kenya, production yield for 2013 were estimated at 3,000 tonnes for green chilies and peppers (FAO, 2014). It is widely grown in the Central region in open field and green houses, with favorable temperature range of 18-25^{o}C (Ashilenje, 2013). Peppers are used fresh for vegetable salads and can be cooked, for flavorings in food products, pharmaceuticals and cosmetics (Bosland & Votava, 2012). The bell pepper can be yellow, orange, red or green in color, with all ripening from a green color. Hot peppers are mostly red and green. Antioxidant vitamins A, C and E are present in high amounts in different pepper cultivars and pepper is also a focus for anticancer properties (Bosland & Votava, 2012).

The major production constraints reported in Kenya include diseases (bacterial wilt, fusarium wilt, downy mildew, late blight, leaf spot, pepper mild mottle and powdery mildew), arthropod pests (red spider mites, aphids, cutworms, African bollworm, thrips, leaf miners and whiteflies) and nematodes that lead to high economic losses (Ashilenje, 2013). Root-knot nematodes (RKN) are one of the major pathogens of *Capsicum spp*. worldwide. They cause root dysfunction by generally reducing the rooting volume,

nutrient and water intake efficiency. Eventually they limit fruit production and yield of the crop (Luc *et al.,* 2005).

Control of RKN in pepper has been done using resistant cultivars such as the Charlestone belle and Carolina wonder (Thies *et al*., 2008) conferred by the Me1 and Me3 resistant genes (Djian-Caporalino *et al.,* 2007). In Ghana, the drumstick tree, *Moringa oleifera* Lam. leaf powder was found to reduce nematode population, galling index and increased fruit per plant and number of leaves when applied in pepper plots (Sowley *et al.,* 2013).

2.2 Root Knot Nematodes (RKNs)

2.2.1 Morphology of RKNs

The body wall of RKNs has three major layers; the cuticle, the hypodermis and the somatic muscles (Figure 2.1). In females, the body wall protects her from the external environment but in second stage juveniles (J2s) and males, it enables them to move through the soil (Perry *et al.,* 2009). The cuticle which is transversely annulated, encloses the body of the root knot nematode, beneath it is a hypodermis (Luc *et al.,* 2005). The central cavity, pseudocoelom, contains a viscous fluid acting as a hydrostatic skeleton. The three major organ systems – digestive, reproductive and excretory are suspended within the fluid (Karssen & Moens, 2006).

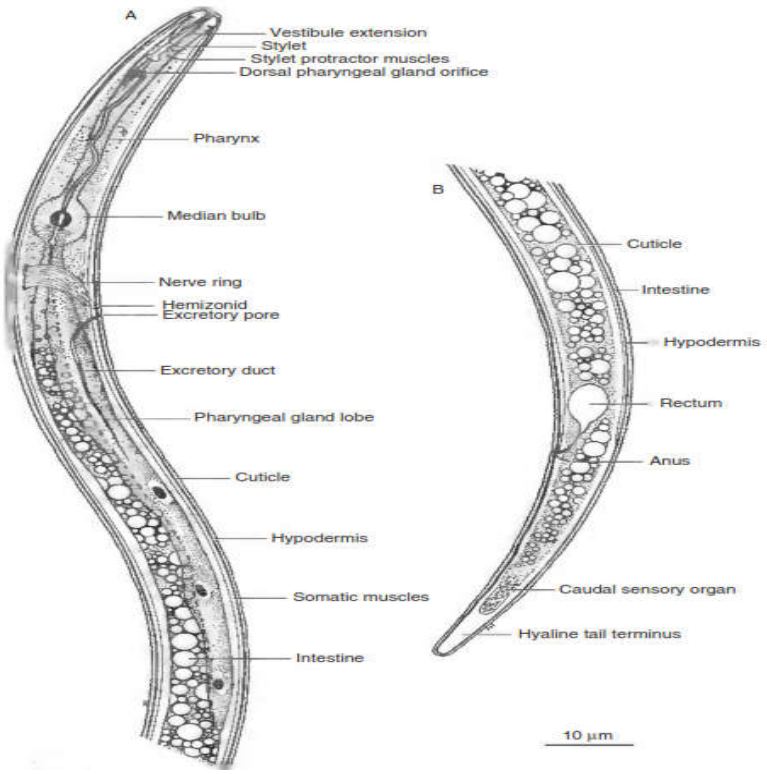


Figure 2.1: Second stage juvenile of a root knot nematode (A: anterior region; B: posterior region). (Source: Eisenback (1985); courtesy of N.C. State University Graphics)

The digestive system is responsible for nutrient uptake by the nematode from a source in order to support normal metabolic activities including growth and development, movement and reproduction (Perry *et al.*, 2009). The digestive system comprises a stoma, pharynx, intestine and rectum. The stoma is equipped with a hollow, retractable, hypodermic-needle-like stylet (Figure 2.1) that serves as an interface between the nematode and the plant (Jones *et al.*, 2011). The pharynx has three specialized gland cells responsible for some functions in the host-parasite relationship, and a metacorpus that pumps substances from the gland cells into the plant and from the plant into the intestine. The intestine serves as a storage organ and in females, six large rectal gland cells open

through this orifice, where they secrete a voluminous gelatinous matrix that serves to protect the eggs as they are deposited from the egg sac (Perry *et al.*, 2009).

Sedentary adult RKN female are pear shaped and white in color with a protruding, sometimes bending neck (Figure 2.2a). Their length ranges from 350 µm to 3 mm and in maximum width from 300 to 700 µm (Perry & Moens, 2006). Males are migratory and vermiform (Figure 2.2b), clearly annulated and ranges in length from 600 to 2500 µm. The infective second-stage juveniles (J2S) are vermiform (Figure 2.2c), annulated and 250 to 650 µm long. The delicate straight stylet is about 9–16 µm long. The third-stage juvenile (J3) and J4 stages are sedentary inside the root and swollen; they have no stylet and develop within the J2S cuticle (Figure 2.3) (Karssen & Moens, 2006).

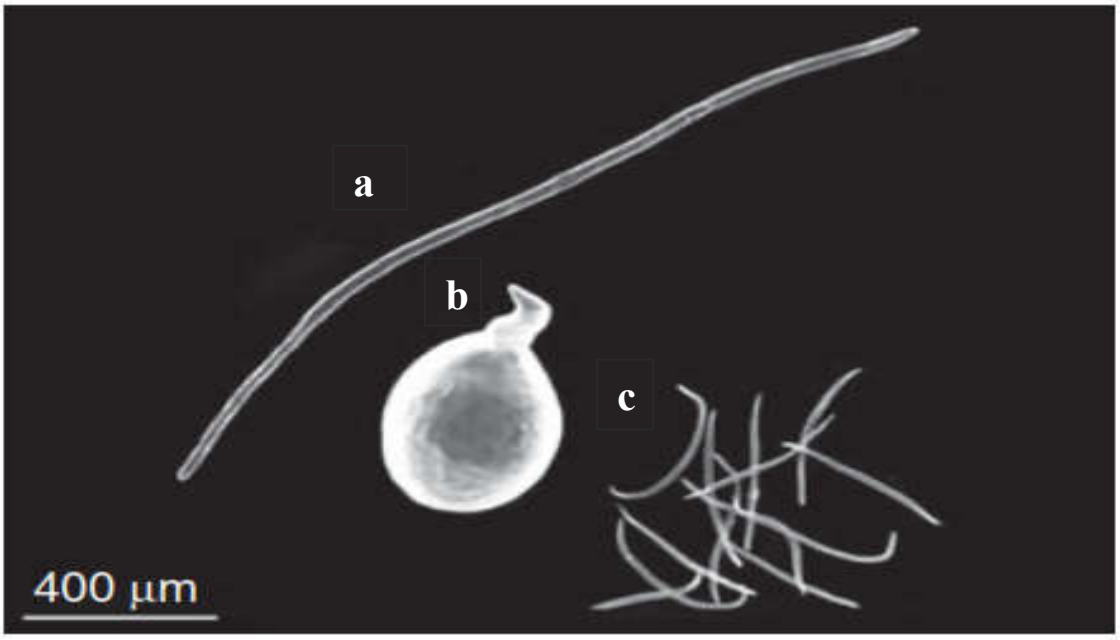


Figure 2.2: Scanning electron micrograph of root knot nematode
(a) male (left), (b) female (centre) and (c) 13 second stage juveniles (right). (Source: Eisenback and Triantaphyllou (1991). Courtesy of Marcel Dekker, Inc.)

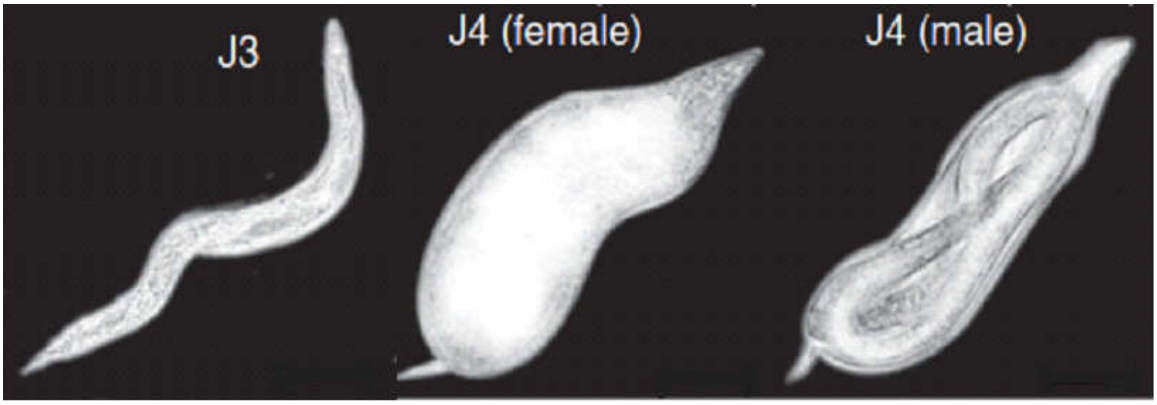


Figure 2.3: *Meloidogyne incognita* third and fourth stage juvenile, 400µm.
(Source: Perry *et al.*, (2009))

2.2.2 Reproduction and Life cycle of RKNs

Reproduction in most RKN species occurs by mitotic parthenogenesis, an asexual or clonal form in which the original diploid or polyploid chromosome number is retained (Jones *et al.,* 2011). The life cycle of the RKNs is divided into six stages; the egg, four juvenile stages and the adult (Figure 2.4). The eggs are usually enclosed in gelatinous sacs that are deposited on the surface of galled roots or within the galled tissue. Usually several hundred eggs are produced by each female (Perry & Moens, 2006). The first moult occurs within the egg following embryogenesis, giving rise to the second stage juveniles. Hatching of the eggs is temperature reliant and doesn't require stimulus from plant roots (Karssen & Moens, 2006). Under unfavorable conditions or in some temperate species such as *Meloidogyne naasi* Franklin, there can be a period of diapause before hatching occurs (Jones *et al.*, 2011).

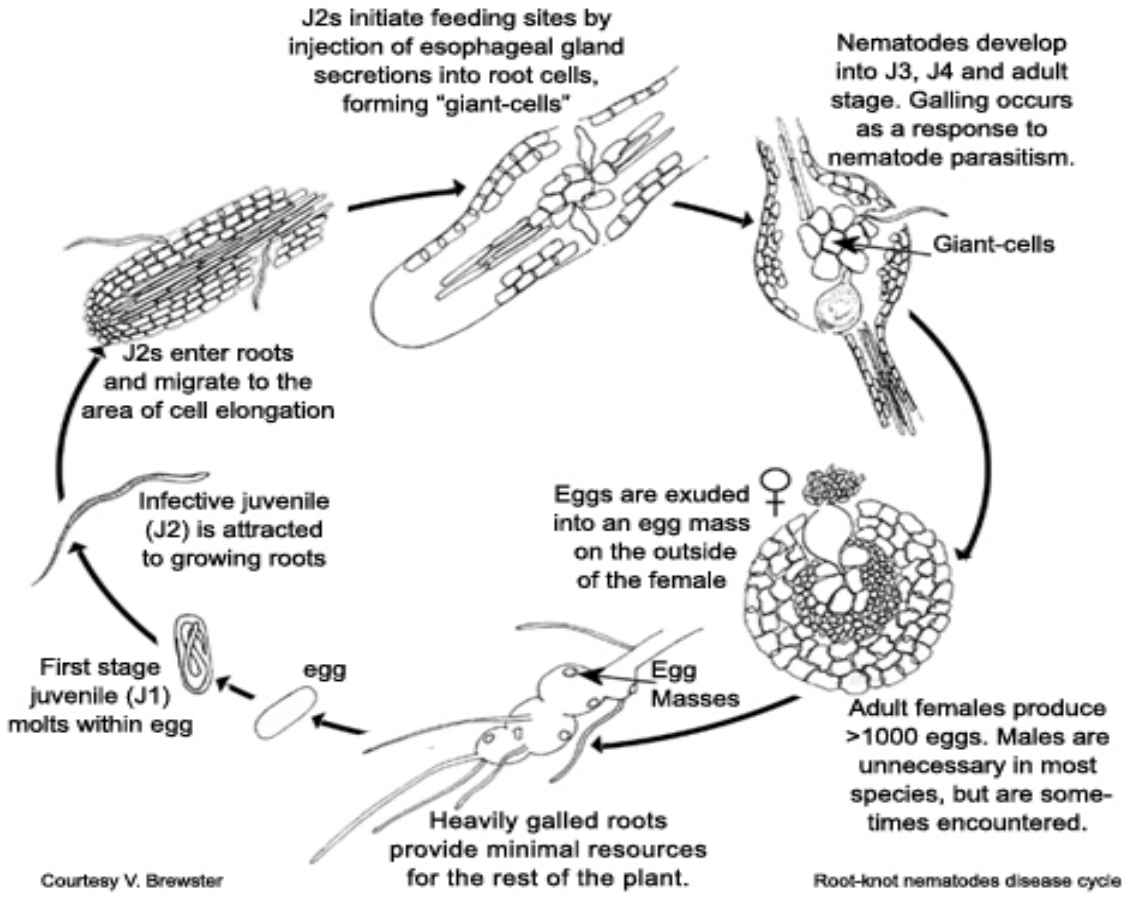


Figure 2.4: Life cycle of root-knot nematode (*Meloidogyne* spp.)
(Source: Mitowski & Abawi, 2003; courtesy of V. Brewster)

The J2s hatch from the egg and then find their food source by sensing substances being exuded from the roots. Infective juveniles have extraordinary capacity to move even a kilometer to locate host plant (Khan, 1993). Migration occurs in water films around soil particles or on root surfaces. The nematode loses its worm-like shape over a period of 20-30 days, and molts twice through further juvenile stages (J3 and J4) to become an adult. With suitable environment and adequate food sources, most of the adults are spherical females about 1 mm in diameter. However, males may also be produced when food supply diminishes or conditions are unfavorable for reproduction (Khan, 1993; Karssen & Moens, 2006).

2.2.3 Host Seeking Behavior of RKNs

The infective second-stage juveniles (J2s) rely on stored lipid reserves to provide the energy for their movement and do not feed during their migration in soil and roots. Plant signals are critical for nematodes to locate hosts and feeding sites before these reserves are overly depleted. The infective juveniles with >60% of their lipid reserves depleted are no longer capable of directed movement (Robinson *et al.,* 1987). Nematodes have the capacity to chemo-orientate using a combination of head and tail chemosensory organs to compare, simultaneously, the intensities of a stimulus at each end of their bodies (Curtis *et al.*, 2009). The plant cell wall is principally made of carbohydrate polymers, such as cellulose, hemicellulose and pectin, wall proteins and possibly phenolic compounds. Plant-parasitic nematodes, like bacteria and fungi, have developed enzyme systems for degradation of plant cell walls.

Molecular studies have reported the expression of genes encoding enzymes such as β-1,4-endoglucanases, also cellusases, in RKNs and both cyst nematodes (Abad & Williamson, 2010). On reaching the tip of a suitable root, enzymes are secreted which soften plant cell walls and it wounds the root using the stylet to create an entry point and then migrates between cells to a permanent feeding site in a day or two at optimum temperatures (Curtis *et al.*, 2009). The J2s induce the plant to convert some of its root cells into metabolically active ‘giant cells’ that serve as permanent supply of nutrients and the nematode becomes sedentary (Karssen & Moens, 2006; Jones *et al.,* 2011).

Chemotaxis is the principal means by which nematodes locate host plants; it is the directed orientation towards or away from a source of stimulation such as plant chemical cues (Reynolds *et al.*, 2011). Plant chemicals in the rhizosphere emanating from root exudates or sites of prior nematode penetration can influence nematode behavior. A number of plant compounds, some present in root exudates, have been shown either to attract nematodes to the roots, or to result in repellence, motility inhibition, or even death (Rao *et al.*, 1996; Curtis, 2007a; Dutta *et al.*, 2012). Infective juveniles (J2s) of *Meloidogyne spp*. are attracted to the zone of elongation in growing root tips and display characteristic nematode exploratory behavior at the root surface, including stylet thrusting, release of secretions (Figure 2.5) in preparation for root penetration, aggregation and an increase in mobility (Von Mende, 1997; Karssen & Moens, 2006).

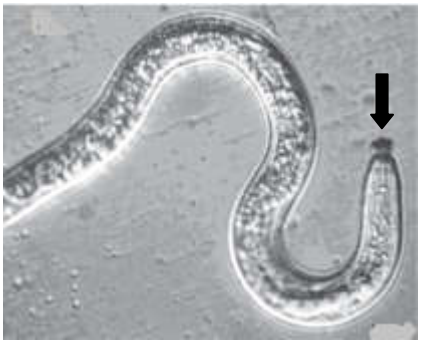


Figure 2.5: Stylet secretions (pointed by the arrow) of *Meloidogyne incognita* second stage juvenile visualized with Coomassie Brilliant Blue staining

Nematodes are attracted to plant roots by means of soluble and gaseous attractants produced by the root itself or by associated rhizosphere micro-organisms (Bird, 1959; Prot, 1980; Dusenbery, 1987; Fudali *et al.,* 2013). These attractants have been classified as long-distance, short-distance and local attractants (Perry, 1996; Curtis *et al.,* 2009). Long-distance cues are those that attract nematodes to the general root area and are

usually volatile organic compounds (VOCs) which belong to three chemical groups; terpenoids, phenylpropanoids and fatty acid derivatives (Baluska & Ninkovic, 2009; Wenke *et al.,* 2010). They tend to be lipophilic, low-molecular weight compounds (less than 300 Daltons) and have a high vapor pressure (0.01 kPa at 20^{o}C) (Wenke *et al.*, 2010). Short-distance attractants, which are typically water soluble compounds, enable the infective J2s to orientate to individual roots (Perry & Moens, 2006). Local attractants are those that enable endoparasitic nematodes, such as *Meloidogyne*, to orientate themselves to the preferred invasion site (Perry, 2005).

2.2.4 Damage caused by RKNs

Root knot nematodes cause minimal damage during infestation as the J2s migrates intracellularly through the cortex, becomes sedentary and secretes regulators as well as induces the plant to produce ethylene and auxin that causes hypertrophy and hyperplasia around its head forming giant cells (Perry *et al.*, 2009). The first plant reaction in response to attack by avirulent or virulent pathogens at the biochemical level is rapid production of reactive oxygen species (ROS), such as superoxide (O_2) and hydrogen peroxide (H_2O_2), the oxidative burst (Melillo *et al.*, 2006). Using molecular analysis to compare tomato-RKN interaction, it was found that for resistant plants, the initial nonspecific, weak and transient ROS production is followed by prolonged accumulation of ROS (Melillo *et al.*, 2006; Caillaud *et al.,* 2008). This could be detected two days post inoculation and was not present in compatible interactions (Abad & Williamson, 2010). The J2s continually secrete proteins with scavenger activity, from cuticle buildup of a surface coat that is likely to hide the nematode from host perception (Curtis,

2007b).These secretions are also thought to protect the parasite from the oxidative response of the host (Melillo *et al.*, 2006).

Successful establishment of RKN infestation has been associated with suppression of plant defense responses (Caillaud *et al.*, 2008). Galling interferes with vascular bundles in infected crops by inhibiting uptake of water and nutrients, causing loss in vigor, chlorosis, stunting, yield reduction and plant death during hot dry weather conditions (Agrios, 2005). RKNs attack on vegetable crops also increases severity of opportunistic infections such as the bacterial vascular pathogen *Ralstonia solanacearum* and vascular wilt pathogen, *Fusarium oxysporum* resulting in greater yield loss (Perry *et al.*, 2009). Losses in small scale production systems in Kenya have not been accounted for but can range between 30- 100 %.

2.2.5 Management and control of RKNs

2.2.5.1 Cultural Control

Cultural control for the management of RKNs employs measures such as crop and fallow rotation, trap crops, cover crops and soil amendments, solarization, and intercropping (Chen *et al.*, 2004; Perry *et al.*, 2009). Some crops have been used to control nematodes directly (trap crops) e.g. marigold (*Tagetes* species) which produce chemicals such as thiophenes that are toxic to nematodes (Krueger *et al.*, 2007; Hooks *et al,*, 2010; Faizi *et al*., 2011; Kalaiselvam & Devaraj, 2011). Other crops are used for suppressing nematode populations while providing other benefits such enhancing soil organic matter, reducing soil erosion or providing forage for grazing livestock (cover crops and green manures).

Crops such as the Brassicas e.g. broccoli, *Brassica oleraceae* L., (Roubtsova *et al.,*2007) when used as cover crops, produce glucosinolates which are natural biocides that are associated with reduction of nematode population.

Soil amendments, such as livestock or poultry manure and organic compost have nematicidal properties. They are applied in fields for intensive annual crop production, with the advantage that they are low cost and have the capacity to improve soil fertility (Riegel & Noe 2000; Perry *et al.*, 2009). The use of antagonistic plant parts, their extracts and products as organic amendments have also been used due to their nematicidal activity (Tsay *et al.*, 2004). Soil and organic amendments are environmentally friendly methods for nematode control but their limitation in large scale farming is that large quantities per unit are need rendering the strategy largely inapplicable (Mateille *et al.*, 2007).

2.2.5.2 Biological Control

Most common biological agents used for control of nematode are fungi and bacteria. Numerous nematophagous fungi exist, whereby the nematode is killed by trapping or endozoic parasitic mode (Chen *et al.,* 2004). Endozoic parasitic fungi from the genera *Trichoderma* and *Fusarium* (Lamovsek *et al.*, 2013) which infect the *Meloidogyne spp.* and other PPNs have spores which adhere to the nematode cuticle and germinate, forming tubes which penetrate into the body (Webster, 1972; Lamovseki *et al.,* 2013). *Pastueria penetrans and Bacillus spp.* are the major bacterial antagonists of nematodes, with endospores of *P. penetrans* attaching to the cuticle of juveniles, producing penetration structures that enter the nematodes causing death (Lamovseki *et al.,* 2013). Other fungi

parasitize the eggs and the RKN females such as *Pochonia chlamydosporia and Paecilomyces lilacinus* (Collange *et al.*, 2011; Qureshi *et al.*, 2012). In Kenya, *Trichoderma asperellum,* isolate TR900 an antagonistic fungi found in soil has been found effective against RKNs (RealIPM, 2016). The inability to economically generate huge amounts of biological material to be utilized over large areas is a significant drawback for effective use of biological control.

2.2.5.3 Chemical Control

Chemical control involves the use of nematicides; low-molecular weight fumigants and contact carbamates and organophosphates (Bakker, 1993; Whitehead, 1997). RKNs are difficult to manage as they are soil borne pathogens with a wide host range, thus chemical control would require large amounts of chemicals applied to the soil. Fumigants such as 1,3-dichloropropene, methyl bromide and dazomet are commonly applied as pre-plant treatments to reduce nematode numbers but they must penetrate large soil volumes to be effective (Mitkowski & Abawi, 2003). Some fumigants volatilize very quickly, a cover must be applied on treated soil so as to maintain it long enough. Methyl bromide which has been a very effective pre-plant treatment was phased out in 2005 due to environmental concerns of ozone depletion (Schneider *et al*., 2003; Schneider *et al.,* 2006; Schneider & Hanson, 2009).

Most nematicides have tended to be rather toxic or volatile, with poor target specificity and less-than-perfect human or environmental safety, such as groundwater contamination or atmospheric ozone depletion (Taylor & Sasser, 1978; Chitwood, 2002). Carbamates

and organophosphates such as aldicarb and nemacur (Luc *et al,*, 2005), are the current feasible methods for control of RKNs.

2.2.5.4 Plant resistance

In certain crops, nematodes are effectively controlled by resistant genes with actual economic benefits (Lilley *et al.,* 2011). Tomato cultivars harboring the Mi gene which was successfully obtained from the wild tomato, *Solanum peruvianum* L.*,* a wild relative to the common tomato confers resistance to three *Meloidogyne spp.* (Starr *et al*., 2002). However, the Mi gene breaks down at high temperatures (Luc *et al.*, 2005) which renders the crop unsuitable for RKN control in the tropics. The existence of resistance-breaking pathotypes is a major problem in breeding programmes in temperate crops (Luc *et al.,* 2005). Not many resistant cultivar traits are transferable to agricultural systems since, in some cases, the resistant trait may be linked to undesirable characteristics (Jones *et al.*, 2011).

2.3 Plant natural products involved in plant -nematode interactions

Plant roots produce natural products through rhizodeposition and rhizosecretion, containing low-molecular-weight organic compounds such as organic acids, amino acids, sugars and phenolic compounds. Some of these compounds, especially the phenolics, influence the growth and development of surrounding plants (Cseke, *et al.*, 2006). High molecular-weight compounds such as flavonoids, nucleotides, growth regulators, tannins, carbohydrates, steroids, alkaloids, triterpenoids, polyacetylenes and vitamins are released in large amounts (Baetz & Martinoia, 2014).

Root exudates constitute an important component of communication with rhizosphere-inhabiting microbes in plant-plant, plant-microbe, plant-nematode and plant-insect interactions (Narula *et al.*, 2009; Haichar *et al.*, 2014). A broad range of signaling molecules involved in these communications have been elucidated (Bais *et al.*, 2006; Narula *et al*., 2009; Rovira, 2015). They mediate both positive and negative interactions in the rhizosphere. The positive interactions comprise rhizobia, mycorrhizae and plant growth-promoting rhizobacteria (PGPR). Negative interactions include association with parasitic plants, pathogenic microbes and invertebrate herbivores (Bardi & Vivanco, 2009). Herbivore-induced volatile emissions in the subterranean environment benefit plant hosts by recruiting natural enemies of herbivorous insects, an important aspect for indirect plant defense. For example, the sesquiterpenes, (*E*)-β-caryophyllene (Figure 2.6) and giejerenes have been found to attract entomopathogenic nematodes (EPNs) *Heterorhabditis megidis* Poinar, Jackson & Klein and *Steinernema diaprepes* (Rhabditida: Steinernematidae) respectively (Rasmann *et al*., 2005; Ali *et al.*, 2010).

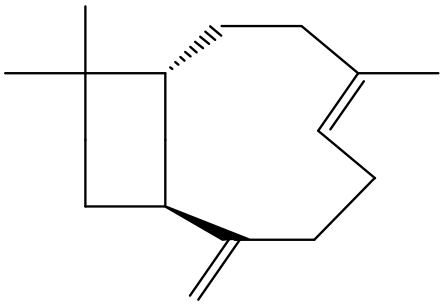


Figure 2.6: Chemical structure of (*E*)-β-Caryophyllene

Root-produced volatile signals may attract damaging pests (Wenke *et al.*, 2010), including plant parasitic nematodes (Hiltpold & Turlings, 2012). Carbon dioxide (CO_2) has previously been demonstrated to be a general signal attracting these nematodes (Pline & Dusenbery, 1987), although a recent study suggested that CO_2 may serve as a response

enhancer to more specific and reliable cues (Turlings *et al*., 2012). The monoterpene, beta myrcene (Figure 2.7) was found to attract the pine wood nematode, *Bursaphelenchus sylophillus* Steiner & Buhrer (Ishikawa *et al.,* 1986).

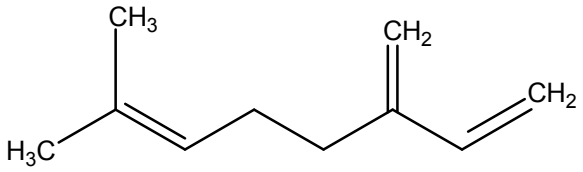


Figure 2.7: Chemical structure of β-myrcene

Water soluble root exudates mediate interactions in the subterranean environment. For instance, glycinoeclepins (Figure 2.8) A, B and C and solanoecelpin A (Figure 2.9) are triterpene compounds present in root exudates of kidney bean and potato respectively. Solanoeclepin A stimulates hatching of the cyst nematodes (Rasmann *et al.*, 2012). Some plants act as non-host trap crops using attract and kill strategy. For example, roots of French marigold *Tagetes patula and T. erecta* contain α-terthienyl (Figure 2.10) and other derivatives of bithienyl that inhibit populations of *Meloidogyne* and *Pratylenchus* (Hooks *et al.,* 2007) . Naturally occurring compounds, such as plant root semiochemicals, or their analogues can be potentially employed to realize interruption (Abad & Williamson, 2010).

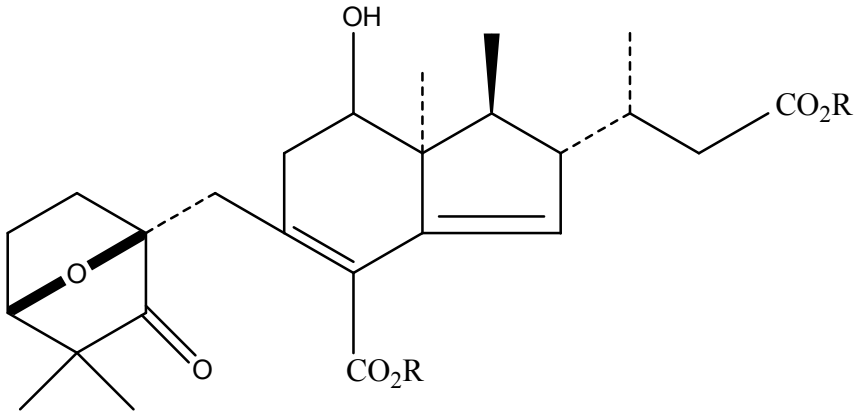


Figure 2.8: Chemical structure of Glycinoeclepins

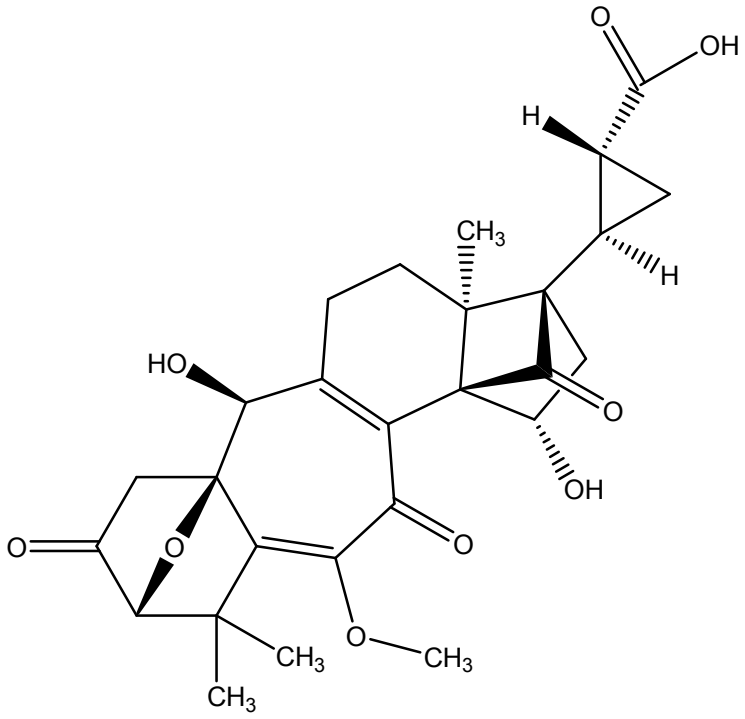


Figure 2.9: Chemical structure of Solanoeclepin A

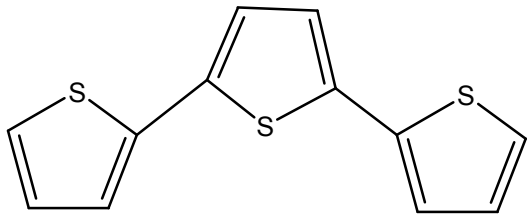


Figure 2.10: Chemical structure of α-terthienyl

CHAPTER THREE

MATERIALS AND METHODS

3.1 Plant materials

Two sweet pepper cultivars ('California Wonder' and 'Yolo Wonder'), and two hot peppers (Long red Cayenne and accession AVDRC PP0237) were used. Seeds of California Wonder and Long red Cayenne seeds were obtained from the Simlaw Seeds Company Limited, Nairobi, Kenya and Yolo Wonder from East Africa Seed Company, Nairobi, Kenya. Seeds of accession AVDRC PP0237 was obtained from the World Vegetable Center (courtesy of Dr. George Kariuki of Kenyatta University, Nairobi, Kenya).

All the pepper seeds were sown in rectangular basins (67 cm long, 40 cm wide and 5 cm deep) (Plate 3.1) containing sterilized (autoclaved (Astell MXN732) at 121^{o}C for 40 min) sand at the International Centre of Insect Physiology and Ecology (*icipe*) Duduville Campus, Nairobi, Kenya (1º 16' 60'' S; 36º 49' 0'' E). Seedlings were transplanted in 2 L plastic pots (17 cm top diameter, 13 cm base diameter and 15 cm deep) with sterilized sand two weeks after germination. Plants were watered daily each morning and maintained in a screenhouse at 22 ± 1^{o}C and 60-70% relative humidity (RH). Plants of 3-6 weeks old were used for the experiments.

Plate 3.1: Two weeks pepper seedlings in a nursery

3.2 Preparation of nutrient Solution

Nutrient solution to provide macro- and micro-nutrients prepared as described by Lambert *et al.* (1992) was used for watering the plants. The stock solution of Ca $(NO_3)_2.4H_2O$ (653g/L), $MgSO_4.7H_2O$ (399g/L), KNO_3 (184g/L) were autoclaved at 121^oC. Solutions of $NH_4H_2PO_4$ (108g/L), $FeSO_4.7H_2O$ 10g plus 72 ml of 500mM EDTA (pH 8.0) per liter and micronutrients (per liter; $MnCl_2.4H_2O$ (1.81g), $CuSO_4.5H_2O$ (0.1g), $ZnSO_4.5H_2O$ (0.22g), H_3BO_3 (2.86g), $H_2MoO_4.H_2O$ (0.02g) were filter-sterilized (Whatmann filter paper, Grade 1, 27 cm diameter). To formulate the amounts used for watering the plants, 50 L of distilled water was mixed with 25 ml of Ca $(NO_3)_2$, $MgSO_4$, $NH_4H_2PO_4$, Fe / EDTA, and micronutrients, and 75 ml of KNO_3, in a 50 L plastic container (Kenpoly Manufactures Limited, Nairobi, Kenya).

3.3 Nematode population

Meloidogyne incognita were obtained originally from tomato (*Lycopersicon ensculentum*) in Taita Taveta County, Kenya (3.3161° S, 38.4850° E) (courtesy of Dr. George Kariuki of Kenyatta University, Nairobi, Kenya) and molecular analysis was

carried out for identification in December 2014. Thereafter, they were maintained in pure cultures on Cal J tomato cultivar seedlings in pots containing sterilized sand in the screenhouse at *icipe* Duduville campus, Nairobi Kenya. Egg masses of *M. incognita* were detached from infected roots under a stereomicroscope (Leica M125, Leica microsystems, USA) and placed in distilled water to allow hatching, in the dark at 27 ± 2^{o}C for 2-5 days. First juvenile stage emerges within the egg after 1-2 days. The infective and motile second stage juveniles (J2s) that emerged were counted with a hand tally counter under the stereomicroscope and they were transferred into 15 ml falcon tubes using plastic Pasteur pipettes until use in bioassays.

3.4 Inoculation assays

This experiment was carried out to evaluate susceptibility or resistance of pepper to *M. incognita* using greenhouse screening technique as described by Holbrook *et al.,* (1983). Five plants were grown in 1L plastic pots (10 cm diameter x 15 cm height) filled with sterilized sand and each pot was inoculated with approximately 500 eggs. Approximately 45 days after inoculation, pepper plants were uprooted and washed clean of soil. Roots were placed in 500 ml beakers containing 300 ml 1.5% Phloxin B solution for 20 min (Coyne *et al.,* 2007) to stain the egg masses (Plate 3.2).

Plate 3.2: Egg mass of root-knot nematode-infected root stained with Phloxin B

Each plant was indexed for root galls and egg masses using the following scale: 0 = no galls or no egg masses; 1 = 1 to 2; 2 = 3 - 10; 3 = 11 to 30; 4 = 31 to 100, and 5= more than 100 galls or more than 100 egg masses per plant (Taylor & Sasser, 1978; Perry *et al*., 2009). The experiment was done in four replicates.

3.5 Dual choice soil olfactometer assays with intact plants

The response of second stage juveniles (J2s) to root produced volatiles was tested in a modified dual choice soil olfactometer (Sigma Scientific, Gainesville, Florida) (Rasmann *et al.* 2005). The olfactometer comprised of four components; the odor source chamber (A) and the control chamber (D) were both 85 mm diameter x 140 mm depth with a connector (15 mm diameter x 30 mm long) fitted with an ultra- fine mesh screen. The central release arm (B) (20 mm diameter x 60 mm length) linked to detachable connecting arms (C) (20 mm diameter x 70 mm length) that connected chambers A and B (Plate 3.3).

Plate 3.3: Dual choice olfactometer assays to test J2s responses to *Capsicum* root volatiles and synthetic blends
(A) Stimulus chamber, (B) Release arm (C) Connecting arm, (D) Control chamber

For the dual choice olfactometer assays, 30 plants were conditioned by placing in growth chambers containing 300 g of sterilized sand. They were watered with 20 ml of the nutrient solution daily for 3-5 days prior to the experiment in the laboratory and maintained at 25 ± 2^{o}C. In the control chamber, 300 g of sterilized sand was placed and 50 ml nutrient solution added. In the connection chamber, 30 g of sand was used and 20 ml nutrient solution added. Four replicates each comprising 500 juveniles were conducted.

After 4 hr (the optimal recovery time following preliminary studies testing response of J2s after 2, 6, 12, and 24 hr), the olfactometer was disassembled and the sand in each detachable section placed on a Baermann extractor (Coyne *et al.*, 2007) for 24 hr to recover the J2s. A 27 µm mesh standard test sieve was used to collect the J2s, which were stored in 50 ml falcon tubes. The number of J2s recovered from sections C (the connecting arms linking the odor and control chamber), A and D were counted using a hand tally counter under the stereo microscope at a magnification of 25x.

3.6 Collection of root exudates

A total of 1000 three weeks old seedlings were obtained from the nursery from the screenhouse at *icipe* duduville campus and the roots were cleaned gently with tap water and rinsed with distilled water to remove soil debris. Root exudates were collected for 48 hr using the dipping method (Gransee & Wittenmayer, 2000) in 2 litres of distilled water and thereafter filtered (Whatmann filter paper, Grade 1, 27cm diameter). Pre-conditioned XAD-4 amberlite (Sigma Aldrich, USA) (10g) adsorbent column maintained in a solvent

system 1:1 methanol: water v/v) was used to adsorb the organic components in the root exudates of pepper. Distilled water of 100 ml was passed through the column to clear any salts from the pepper root exudates. This was followed by 100 ml of methanol (Sigma-Aldrich, USA) which was used to extract the target compounds from the root exudates. The methanol was then concentrated to dryness in a rotary evaporator under vacuum at 40^{o}C. The residue was weighed and reconstituted at a concentration of 5 mg/ml in 100% distilled water for bioassays. A concentration of 5 mg/ml was prepared separately in 10% LC-MS grade methanol (Sigma-Aldrich, USA) for analysis.

3.7 Dual Choice soil assays for root exudates

Dual choice assays with root exudates were carried out by filling 5g autoclaved sand in a tube (14 mm diameter x 25 mm length) (Figure 3.1) after mixing with 1 ml, 5 mg/ml treatment on stimulus side and distilled water on control side respectively. Sand was added in the connecting tube and 200 J2s introduced at the release point. The experiment was done in four replicates. Baermann extraction was set up after 24 hours to recover the J2s.

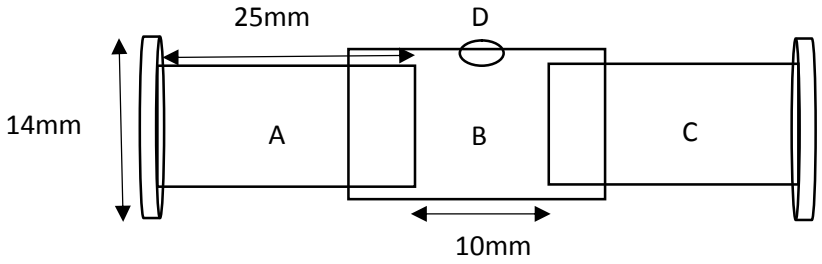


Figure 3.1: Schematic representation of the dual choice assay of root-knot nematodes to root exudates of pepper
(A) Stimulus tube, (B) connector, (C) Control tube (D) Release point

3.8 Stylet thrusting assays

Stylet thrusting experiments were carried out to investigate bioactivity when second stage juveniles are in contact with either the root exudates of pepper or distilled water (control). Exudate solution of 20 µl with a concentration of 5 mg/ml was transferred on to a glass slide and 20 µl nematode suspension containing about 50 J2s were introduced and covered with a cover slip. After 30 min when the nematodes had settled, activity of the J2s was observed using magnification of 200x on compound microscope (Leica DM 2500). Bioactivity was rated as the number of stylet thrusts per minute for each nematode. Data was taken for three separate minutes for each nematode and three replicates were done for each treatment (Dutta *et al.*, 2012).

3.9 Collection of root volatiles

Thirty pepper plants of either the varieties or accession were pre-conditioned for volatile sampling in glass chambers for 3-5 days. Volatiles were collected for 24 hr on a pre-cleaned (dichloromethane and nitrogen dried) Super Q (30 mg, Analytical Research System, Gainesville, Florida, USA) adsorbent. Each adsorbent was connected to a steel probe (17 cm long, 0.5 cm i.d, USDA/ARS-CMAVE, Gainesville Florida, USA) inserted in the plant sand root zone in the glass chamber (Figure 3.2).

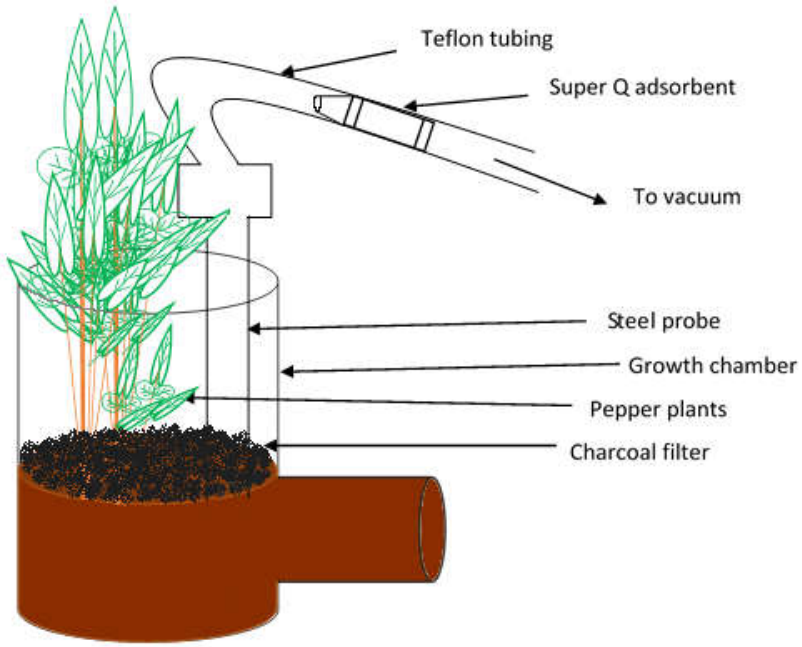


Figure 3.2: A schematic representation of the volatile collection set-up in the laboratory

The probe was connected to a vacuum pump that extracted volatiles from the soil at 170 ml/min. Cleaned charcoal filters (activated charcoal) were used to cover the sand to prevent adsorption of other odors from the surrounding air. The Super Q filters were eluted with 200 µl of GC-grade dichloromethane (Sigma Aldrich, St. Louis, Missouri, USA) and concentrated to 50 µl under a stream of nitrogen for use in GC/MS analysis. The experiment was carried out in triplicates to quantify the amounts of identified components in the root volatiles of pepper plants. For the control, volatiles were collected similarly from 300 g of pre-conditioned sand.

3.10 Analyses of root volatiles

Eluates of the root volatiles were analyzed using coupled gas chromatography-mass spectrometry (GC/MS) on an Agilent Technologies 7890B GC linked to a 5977 MS, equipped with a non-polar HP-5 MS ultra-inert column (30 m × 0.25 mm i.d., 0.25 µm)

(J&W, Folsom, CA, USA). The temperature program was 5 min at 35^{o}C, then 10^{o}C/min to 280^{o}C. An aliquot (1 µl) of each extract of the volatiles was analyzed in the splitless mode, using helium as a carrier gas at a flow rate of 1.2 ml/min. Spectra were recorded at 70 eV in the electron impact (EI) ionization mode (Plate 3.4).

Plate 3.4: Gas chromatography-mass spectrometer used for the analysis of root volatiles

Identification of compounds was done by comparison of mass spectral data with library data [Adams2 (Adams, 1995) and NIST08 (National Institutes of Standards and Technology, 2008)]. Unambiguous structure assignments were based on co-injection with commercially available authentic standards. Quantification was based on calibration curves (peak area vs. concentration) generated from authentic standards of the identified compounds (Njihia *et al.*, 2014; Wanjiku *et al.*, 2014; Murungi *et al.*, 2016).

3.11 Standards

Authentic standards of (R)-(+) *α*-pinene (purity 99%), 2-methoxy-3-(1-methylpropyl)-pyrazine (purity 99%), thymol (99%), and tridecane (purity 99%) were purchased from

Sigma Aldrich, St. Louis, MO, USA. Methyl salicylate (purity 97%) and (R)-(+)-limonene (purity 97%) were purchased from Sigma Aldrich, Steinhelm, Germany.

3.12 Bioassays with synthetic compounds

To determine the role of the identified root VOCs in the host-seeking behavior of RKNs, the responses of J2s were assessed in six different treatments (Table 3.1) in dual choice olfactometer assays.

Table 3.1: Synthetic compounds and blends used for testing response of J2s

	Treatments
1	5-Component blend versus sand
2	Thymol versus sand
3	5-Component blend versus thymol
4	California Wonder versus thymol
5	5-Component blend + thymol versus sand
6	California Wonder + thymol versus sand

Treatments were tested in dose response assays consisting of: a) a 5-component synthetic blend comprised of the shared compounds identified in the four pepper cultivars (mean of the naturally occurring proportions in pg/plant/hr: α-pinene, 40 pg/plant/hr; limonene, 92 pg/plant/hr; 2-methoxy-3-(1-methylpropyl)-pyrazine, 22 pg/plant/hr; methyl salicylate, 59 pg/plant/hr and tridecane, 126 pg/plant/hr), (the sixth component, 4,5-diepi-aristolochene, was identified based on comparison of mass spectral data with library data only and was not available commercially hence was not used for bioassays), b) thymol, 48 pg/plant/hr, identified as specific to the roots of the accession AVDRC PP0237. Three different concentrations of 48 pg/plant/hr (the natural amount of thymol detected in the

root volatiles of the resistant AVDRC PP0237, hence standardized across for comparison purposes), 96 pg/plant/hr and 192 pg/plant/hr (obtained by doubling the preceding concentration), each prepared in hexane.

These were applied by dispensing aliquots of 2 ml into the stimulus chamber containing 300 g of sterilized sand. The control consisted of 2 ml solvent (hexane) dispensed in 300 g sterilized sand. Each dose was tested against a control and carried out in four replicates. The optimal doses were used for further bioassays; thymol (96 pg/plant/hr) [see results section 4.5 responses of *Meloidogyne incognita* to selected volatile compounds and blends] was tested against cv. California Wonder (the most preferred plant in dual choice olfactometer assays with plants; see results section 4.2 response of *M. incognita* to pepper root volatiles relative to a control (sand)) and also paired with the optimal dose of the 5-component synthetic blend at a concentration of 192 pg/plant/hr. Another experiment tested the effect of spiking the 5-component synthetic blend (192 pg/plant/hr) as well as cv. California Wonder with thymol (96 pg/plant/hr) tested against a control (see results section 4.6 effect of thymol on natural plant odors and the preferred 5-component blend).

3.13 Analyses of root exudates

The chromatographic separation was achieved on a Waters ACQUITY UPLC (ultra-performance liquid chromatography) I-class system (Waters Corporation, Milford, MA, USA) (Plate 3.5). The UPLC was fitted to a Waters ACQUITY UPLC BEH C18 column (2.1mm × 50 mm, 1.7 µm particle size; Waters Corporation, Dublin, Ireland) heated to 40°C and an auto sampler tray cooled to 15°C. Mobile phases used were made up of

0.01% formic acid in water (A) and acetonitrile (B). The following gradient was used: 0 min, 5% B; 0–5 min, 5–50% B; 5–5.6 min, 50–100% B; 5.6–6.4 min, 100% B; 6.4–7 min, 100-5% B; 7–8 min, 5% B. The flow rate was held constant at 0.2 ml min^{-1} and the injection volume was 1 µL.

Plate 3.5: Liquid chromatography-quadrouple time of flight-mass spectrometer for analysis of pepper root exudates

The UPLC system was interfaced with electrospray ionization (ESI) to a Waters Xevo QToF-MS operated in full scan MS^E in positive mode. Data were acquired in resolution mode over the *m/z* range 100–1200 with a scan time of 1 s using a capillary voltage of 0.5 kV, sampling cone voltage of 40 V, source temperature 100°C and desolvation temperature of 350°C. The nitrogen desolvation flow rate was 500 L/h. For the high-

energy scan function, a collision energy ramp of 25–45 eV was applied in the T-wave collision cell using ultrahigh purity argon (≥99.999%) as the collision gas. A continuous lock spray reference compound (leucine enkephalin; $[M+H]^+ = 556.2766$) was sampled at 10 s intervals for centroid data mass correction. The mass spectrometer was calibrated across the 50 – 1,200 Da mass range using a 0. 5mM sodium formate solution prepared in 90:10 2-propanol/water (v/v).

MassLynx version 4.1 SCN 712 (Waters Corporation, Maple Street, MA) was used for data acquisition and processing. The elemental composition was generated for every analyte. Potential assignments were calculated using mono-isotopic masses with a tolerance of 10 ppm deviation and both odd- and even-electron states possible. The number and types of expected atoms was set as follows: carbon ≤ 100; hydrogen ≤ 100; oxygen ≤ 50; nitrogen ≤ 6. The empirical formula generated was used to predict structures which were proposed based on the online database, fragmentation pattern and literature (Wamalwa *et al.*, 2015; Murungi *et al.*, 2016; Musundire *et al.*, 2016).

3.14 Statistical Analyses

The number of galls and egg masses for scoring galling and egg mass indices were analyzed using analysis of variance and means were separated using Duncan's multiple range test. The number of responding nematodes obtained from the dual choice assays recorded as mean number of second stage juveniles that responded to the different treatments were expressed as percent response [(n/N) x 100]. N corresponds to the total mean of responding J2s while n is the mean number of J2s corresponding to a given

treatment. Non respondents were not included in the analysis. The data was analyzed by Chi-square goodness of fit to assess (a) *M. incognita* odor discrimination to plant root volatiles compared to a control (sand) and (b) attraction or avoidance of *M. incognita* to different doses of thymol and blends against their respective controls. The number of stylet thrusts obtained from the stylet thrusting bioassays (means of fifteen J2s per replicate for each treatment) were subjected to a generalized linear model assuming a quasi-poisson distribution error (to account for over dispersion) and logarithmic link function to examine the effect of the treatments. Multiple range tests were performed to compare means across all treatments. R version 2.15.1 software (R Core Team, 2015) was used to perform the statistical analysis and all tests were performed at 5% significance level.

CHAPTER FOUR

RESULTS AND DISCUSSIONS

4.1 *Meloidogyne incognita* infestation on pepper cultivars

The intensity of the root knot disease on the three pepper cultivars was generally high indicating successful RKN establishment. However, accession AVDRC PP0237 had the lowest galling index, with one gall per root system and no egg masses were observed (Table 4.1). Galling index is a measure for assessing root knot nematode infection on a plant by counting the number of galls per root system (Esfahani, 2009). Egg mass index assesses the reproduction of the nematodes and can be used as a measure for susceptibility or resistance of plants to RKNs (Taylor & Sasser, 1978; Perry *et al.*, 2009).

Table 4.1: Galling and egg-mass indices of California Wonder, Yolo Wonder, Long Red Cayenne and accession AVDRC PP0237

Pepper cultivar	No. of galls [Ŧ]	Galling index	Egg masses [Ŧ]	Egg mass index
California Wonder	44.75[a]	4.00[a]	36.50[a]	3.75[a]
Yolo Wonder	31.75[b]	3.75[ab]	34.75[a]	3.50[a]
Long Red Cayenne	21.5[b]	3.00[b]	24.00[a]	3.00[a]
AVDRC PP0237	0.75[c]	0.75[c]	0.00[b]	0.00[b]

[Ŧ] Mean number of galls and egg masses per root system. Means with the same letter are not significantly different (P<0.5, Duncan's Multiple Range test

These results are consistent with previous studies that showed Yolo Wonder and California Wonder were highly susceptible to RKNs (Thies & Fery, 2002; Djian-Caporalino *et al.,* 2007). On the other hand, AVDRC PP0237 did not support the growth and multiplication of *M. incognita.* It suggests that *M. incognita* uses some mechanisms for host selection and discrimination. We postulated that chemical cues may be involved during host location by the infective juveniles.

4.2 Responses of *Meloidogyne incognita* to pepper root volatiles relative to a control (sand)

Nematodes responded significantly to the root odors of the different pepper cultivars compared to the control (sand) (Figure 4.1). A greater ($P<0.0001$) number of J2s preferred root odors from cv. California wonder (82%, $\chi^2= 60.06$, df =1), Yolo Wonder (74%, $\chi^2= 28.14$, df =1), and Long Red Cayenne (71%, $\chi^2= 60.06$, df =1) over the control. However, the control was more preferred relative to AVDRC PP2037 (63%, $\chi^2= 5.94$, df =1, P = 0.01).

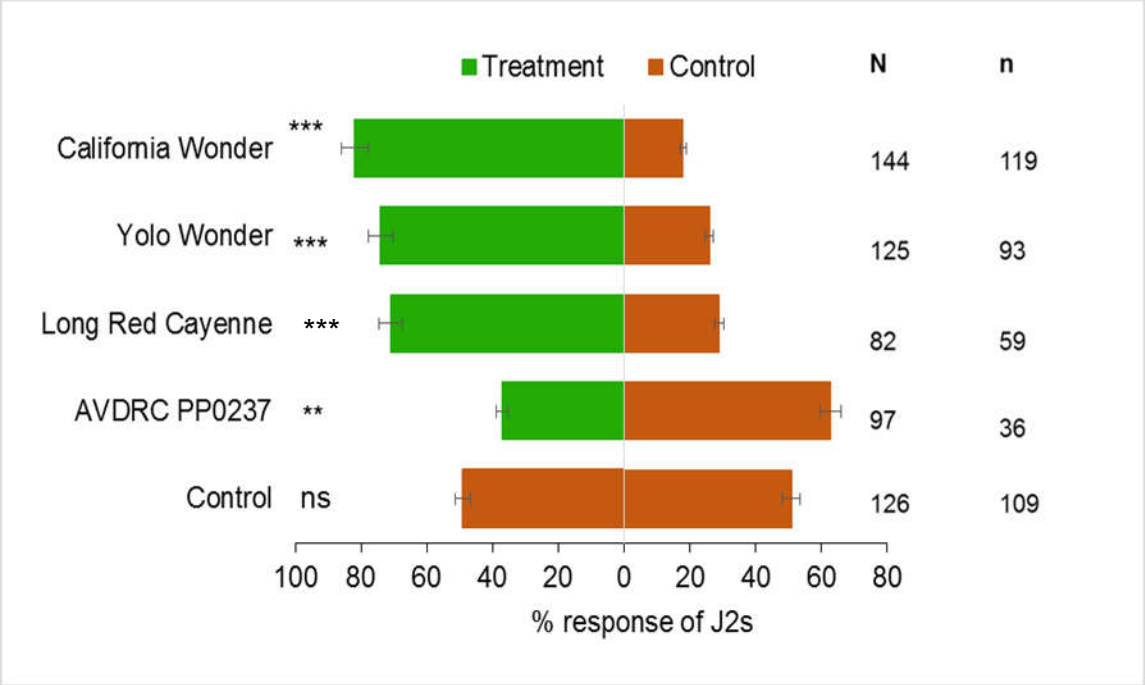


Figure 4.1: Attractive responses of *Meloidogyne incognita* infective juveniles (J2s) to root volatiles of four pepper cultivars compared to a (moist sand) control.
N is the total number of responding J2s while n is the number of responding J2s corresponding to a given treatment. The level of significance is indicated by: *** = P <0.0001; ** = P < 0.05; ns = not significant at P = 0.05

The experiments showed that *M. incognita* J2s responded differently to the root volatiles of the different pepper cultivars. The infective juveniles preferentially responded to the root odors of the three susceptible pepper cultivars California Wonder, Yolo Wonder and

Long red cayenne (Thies & Fery, 2002; Djian-Caporalino *et al.*, 2007), whereas they the control was more preferred compared to root odors from the RKN-resistant AVDRC PP0237. These results indicate that root chemical components play a key role in host attraction and discrimination by *M. incognita* J2s. These results support the earlier study by Prot (1980), which showed that host roots may attract or repel phytoparasitic nematodes, although the mediating host odor compounds were not identified in this specific study. The fact that J2s preferred less the root chemical cues from AVDRC PP0237 indicates that both the composition and quality of root semiochemicals play crucial roles in the host attraction behavior of RKNs. Evidenced by their ability to distinguish host signals even within species, it appears that *M. incognita* may have established a strong inclination for pepper plants that may best support their survival and multiplication.

4.3 Responses of *Meloidogyne incognita* to pepper root exudates relative to a control

Root exudates stimulated significantly ($F_{(4,670)} = 511.92$, $P < 0.0001$) more stylet thrusting in the J2s across all treatments when compared to the control (Figure 4.2). California Wonder ($t_{(4,670)} = 32.66$, $P<0.0001$), Yolo Wonder ($t_{(4,670)} = 31.43$, $P<0.0001$) and Long red cayenne ($t_{(4,670)} = 31.28$, $P<0.0001$) elicited thrusts five times more compared to the control. Accession AVDRC PP0237 ($t_{(4,670)} = 20.08$, $P<0.0001$) elicited thrusts three times more than the control ($t_{(4,670)}=47.17$, $P<0.0001$).

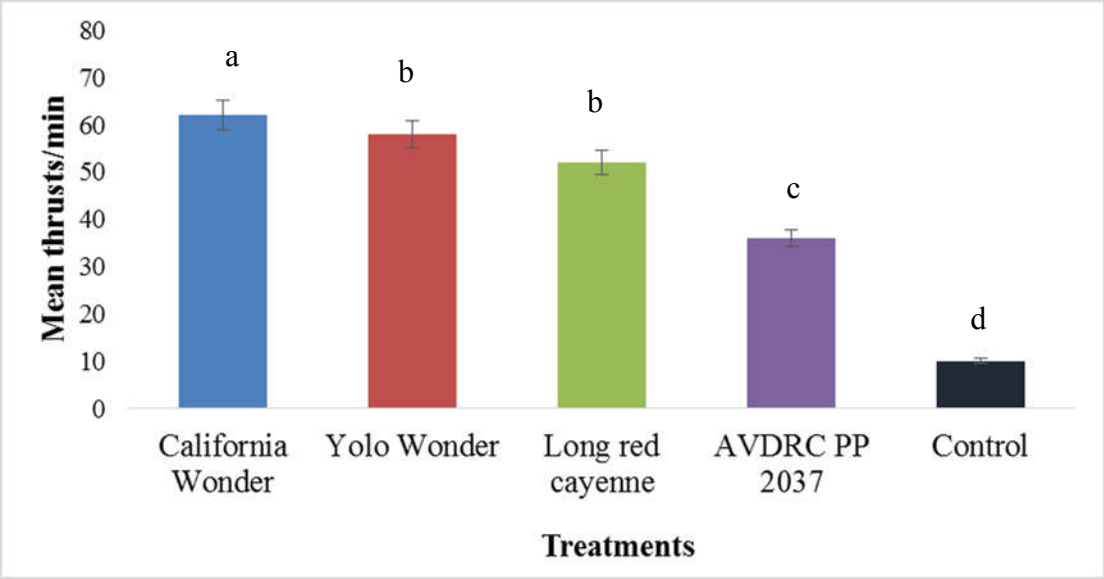


Figure 4.2: Stylet thrusting response of *Meloidogyne incognita* infective juveniles (J2s) to root exudates of four pepper cultivars.
Means with the same letters are not significantly different

Nematodes responded significantly to the root exudates of the different pepper cultivars compared to the control (sand) (Figure 4.3). J2s preferred root exudates from California wonder (χ^2= 49.54, df =1, P<0.0001), Yolo Wonder (χ^2= 17.12, df =1, P< 0.0001), and Long Red Cayenne (χ^2= 26.084, df =1, P<0.0001) over the control. However, the control was more preferred over the root exudates from AVDRC PP2037 (χ^2= 49.916, df =1, P < 0.0001).

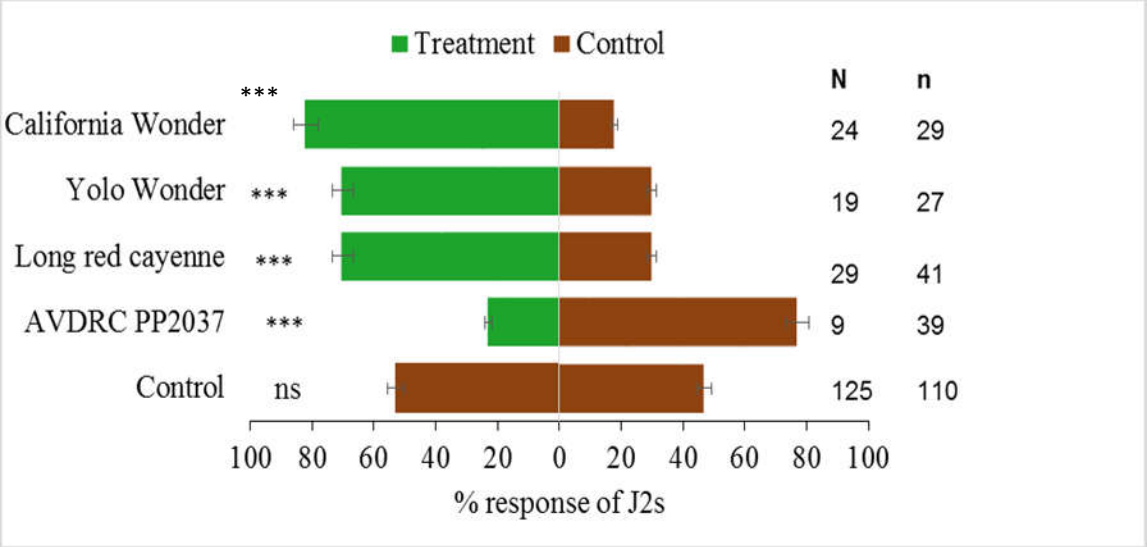


Figure 4.3: Responses of *Meloidogyne incognita* infective juveniles (J2s) to root exudates of four pepper cultivars compared to a (moist sand) control.
N is the total number of responding J2s while n is the number of responding J2s corresponding to a given treatment. The level of significance is indicated by: *** = P <0.0001 and ns = not significant at P = 0.05

Results from the stylet thrusting assays showed that J2s increased bioactivity when introduced into pepper exudates compared to the control. There was however reduced bioactivity when J2s were introduced in the RKN-resistant accession. Previous studies have shown that root exudates of potato stimulate aggregation and exploratory behavior of the cyst nematode *Heterodera schachtii* (Spiegel *et al.*, 2003). A previous study investigating the effect of neurotransmitters on J2s, showed that octopamine induced increased body movement but did not induce stylet thrusting. On the other hand resorcinol induced stylet thrusting and production of secretions but did not induce body movement (McClure & Von Mende, 1987). In another study, tomato and rice small lipophilic molecules, induced very little stylet thrusting and only minute quantities of stylet secretions after 16 hr of exposure (Dutta *et al.*, 2012).

In our study, the pepper root exudates from California Wonder, Yolo Wonder and Long Red Cayenne induced regular and more controlled stylet thrusting with a frequency of about one thrust s^{-1} which compared with that of resorcinol in the study of Dutta *et al.*, (2012). This may suggest that the pepper root exudates are perceived as a cue to a food source inducing the behavior that nematodes display when puncturing the roots of host plants.

The J2s produced more secretions in the presence of pepper root exudates (Figure 4.4) when visualized with Coomassie blue staining compared to J2s that were in the control. This suggests that the infective juveniles have the chemosensory ability to detect the presence of plant produced compounds that direct them to a host. It was observed that J2s moved more slowly or became inactive after 2 hr of exposure to pepper root exudates compared to those in the control. This may indicate that nematodes use up more lipid reserves when in contact with the pepper root exudates compared to the control (distilled water) which does not signal to a food source.

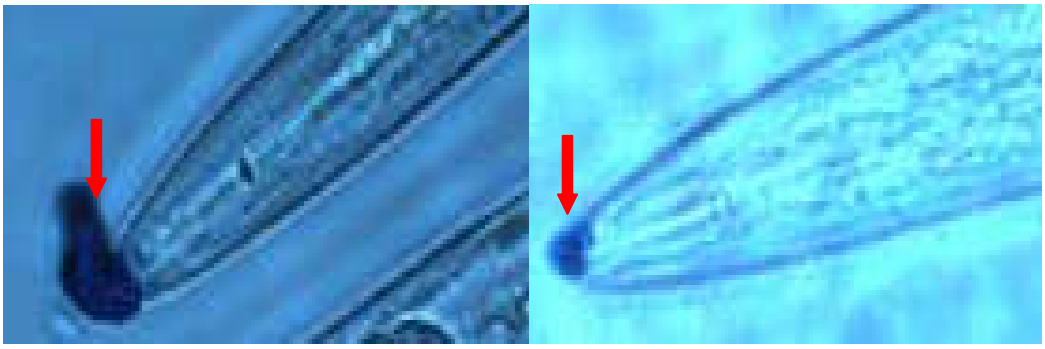


Figure 4.4: Stylet secretions (arrow) of *Meloidogyne incognita* second stage juvenile in California Wonder root exudates (left) and in control (right)

The dual choice experiments showed differential responses of *M. incognita* J2s to the root exudates of the different pepper cultivars. The root exudates of the three susceptible

pepper cultivars California Wonder, Yolo Wonder and Long red cayenne were preferred by the infective juveniles (Thies & Fery, 2002; Djian-Caporalino *et al.*, 2007) than the AVDRC accession compared to the control. This suggests that the pepper root exudates influence the directional orientation of the second stage juveniles towards the close vicinity of the roots.

4.4 Chemical analyses of pepper root volatiles

Gas Chromatography-Mass Spectroscopy analyses identified 18 components represented by the peaks 1, 3, 4, 5, 7 and 11 (monoterpenoids), 8 (methoxypyrazine) 2, 6, 9, 12 and 13 (alkanes), 10 (ester) and 14-18 (sesquiterpenes) in the root volatiles of the four pepper cultivars (Figure 4.5). The identities of compounds 1, 3, 4, and 6 to 12 were confirmed by comparison of their retention times and mass spectral fragmentation patterns with authentic standards. Compounds 2, 5, and 13 to 18 were tentatively identified based on mass spectral library data only. Of the emitted constituents, six (α-pinene, limonene, 2-methoxy-3-(1-methylpropyl)-pyrazine, methyl salicylate, tridecane, and 4,5-di-epi-aristolochene) were produced by the four cultivars but to varying relative concentrations (Table 4. 2).

Table 2: Mean amount of pepper root volatiles detected

				Mean amount detected pg/plant/hr ± SEM			
Peak No.	**RT (min)**	**Compound Name**	**Class of compound**	**California Wonder**	**Yolo Wonder**	**Long Red Cayenne**	**AVDRC PP2037**
1	9.76	α-Pinene[1]	Monoterpenoid	68.09 ± 34.10	47.72 ± 8.60	22.87 ± 4.56	21.90 ± 2.27
2	11.15	Decane	Alkane	25.41 ± 4.58	101.24 ± 9.87	_	_
3	11.7	D-limonene[1]	Monoterpenoid	61.24±8.33	173.91 ± 33.59	80.38 ± 13.93	52.81 ± 16.22
4	11.87	(Z)-β-ocimene	Monoterpenoid	_	87.75 ± 18.54	_	_
5	12.15	*p*-Cymene	Cyclic hydrocarbon	_	_	17.97 ± 4.66	24.29 ± 5.23
6	12.93	Undecane	Alkane	18.64 ± 5.03	76.67 ± 16.58	12.47 ± 4.22	_
7	13.74	Camphor	Monoterpenoid	_	103.36 ± 24.58	_	_
8	14.13	2-Methoxy-3-(1-methylpropyl)-pyrazine[1]	Pyrazine	13.92 ± 4.31	20.70 ± 2.79	39.97 ± 7.82	13.85 ± 1.04
9	14.43	Dodecane	Alkane	25.52±2.57	38.55 ± 3.97	_	48.42 ± 4.52
10	14.52	Methyl salicylate[1]	Ester	78.79 ± 7.91	57.86 ± 7.58	49.66 ± 4.72	48.29 ± 6.46
11	15.8	Thymol[2]	Monoterpenoid	_	_	_	48.43 ± 9.95
12	15.98	Tridecane[1]	Alkane	99.85 ± 5.18	172.45 ± 47.65	157.28 ± 13.73	75.56 ± 18.43
13	17.23	Tetradecane	Alkane	_	129.68 ± 30.77	284.71 ± 67.76	93.44 ± 36.68
14	18.13	γ - Himachalene	Sesquiterpene	68.37 ± 6.40	_	_	_
15	18.18	Allo-aromadendrene	Sesquiterpene	67.24±7.62	_	_	_
16	18.31	Alpha-Muurolene	Sesquiterpene	115.10 ± 12.01	_	_	_
17	18.58	4,5-Di-epi-aristolochene[1]	Sesquiterpene	145.61±31.89	231.18 ± 39.66	229.99 ± 39.33	234.44 ± 66.67
18	18.82	γ - Gurjunene	Sesquiterpene	553.83 ± 124.46	_	_	_

[1]Compounds common to the four pepper plants and [2]compound specific to AVDRC PP0237

The sweet pepper cultivars differed in their volatile root chemistry, with more sesquiterpenes dominating the odors of cv. California Wonder than cv. Yolo Wonder, while camphor and (*Z*)-β-ocimene were exclusively detected in cv. Yolo Wonder. Additionally, the two hot pepper cultivars, Long Red Cayenne and AVDRC PP 2037, also showed similarities and differences, with thymol being specific to the AVDRC PP0237 cultivar. Another compound 4, 5-di-epi-aristolochene was produced in greatest relative quantity in the accession AVDRC PP2037 and cv. Yolo Wonder was 4, 5-di-epi-aristolochene, and tetradecane in cv. Long Red Cayenne, and γ-gurjunene in cv. California Wonder. Structures of the tested synthetic compounds are given in figure 4.6.

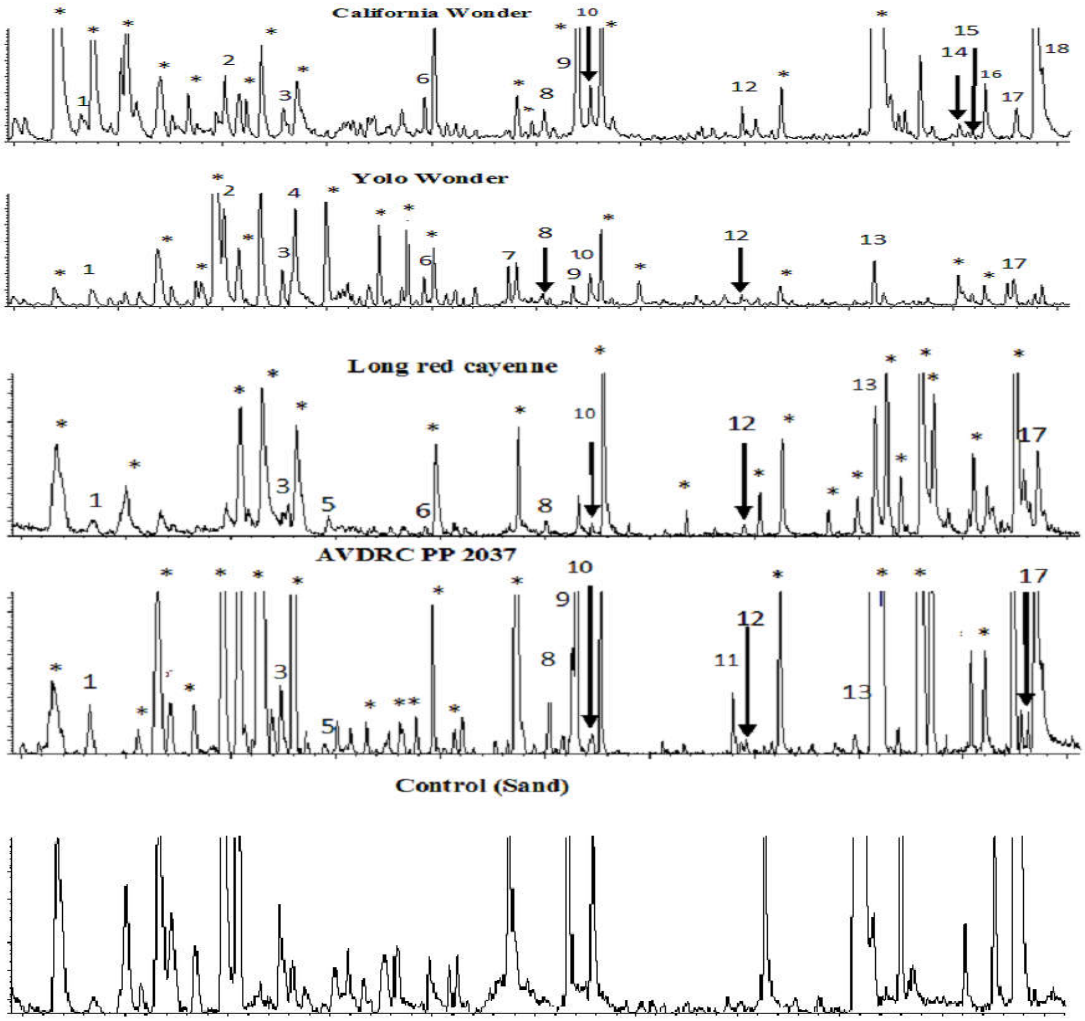
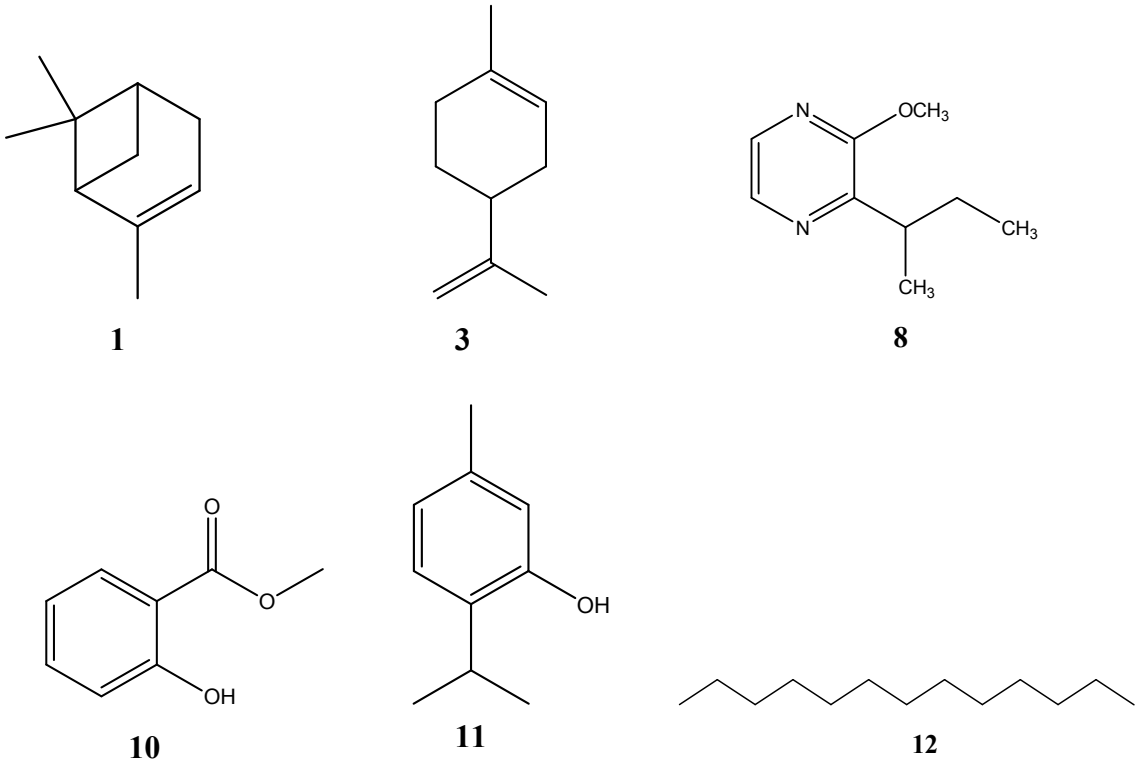


Figure 4.5: Gas chromatography-mass spectrometry chromatograms of root volatiles of *Capsicum annum*.
Numbers correspond to the following compounds (1) α-pinene, (2) Decane, (3) Limonene, (4) (*Z*)-β-ocimene, (5) *p*-cymene, (6) Undecane, (7) Camphor, (8) 2-methoxy-3-(1-methylpropyl)-pyrazine, (9) Dodecane, (10) Methyl salicylate, (11) Thymol, (12) Tridecane, (13) Tetradecane, (14) γ–himachalene, (15) Allo-aromadendrene, (16) α-muurolene, (17) 4,5-di-epi-aristolochene, and (18) γ–gurjunene (see also Table 1). Asterisk (*) indicates matrix interferences present in the control and impurities.



1 **3** **8**

10 **11** **12**

Figure 4.6: Chemical structures of the tested synthetic compounds
α-pinene (**1**), limonene (**3**), 2-methoxy-3-(1-methylpropyl)-pyrazine (**8**), methyl salicylate (**10**), thymol (**11**), and tridecane (**12**)

Although it has been determined that plant chemical cues are important for phytoparasitic nematodes to locate host roots (Perry & Aumann, 1998), the specific olfactory cues that trigger chemotactic host-finding behavior remain largely unknown for RKNs. We report for the first time the chemical signals involved in RKN-host interaction. Previously, most of the work undertaken on nematode-plant interactions has focused on herbivore-induced volatiles that attract entomopathogenic nematodes (EPNs). For example, the terpenoid (*E*)-β-caryophyllene has been shown to serve as a specific recruitment signal released by maize (*Zea* mays) roots damaged by the Western corn rootworms (*Diabrotica virgifera virgifera*) for the EPN *Heterorhabditis megidis* (Rasmann *et al*., 2005). Similarly, pregeijerene is released by citrus (*Citrus paradisi*

Macf. x *Poncirus trifoliata* L. Raf.) root stocks infested by larvae of the weevil *Diaprepes abbreviates* (L.) and attracts a variety of EPN species (Ali et al., 2010; Ali *et al.,*, 2012). Other than recruiting EPNs, plant produced volatiles also modulate inter-specific social behavioral plasticity, learning, and memory (Willet *et al.,* 2015).

Notably, for RKNs, previous work has shown that CO_2 serves as a general signal that attracts these nematodes (Dusenbery, 1987; Pline & Dusenbery, 1987). In the current study, the analytical methods used to capture volatiles excluded highly volatile components, such as CO_2. Nonetheless, the chemical analysis clearly demonstrated that pepper roots release a complex mixture of volatile organic compounds that modulated J2s responses.

Volatile organic compounds (VOCs) are also important for above-ground interactions. Some of the compounds identified in the current study have been reported to mediate various above-ground plant-herbivore interactions in other solanaceous crops. For instance, α-pinene, limonene, γ–gurjunene and α–muurolene were present in a complex mixture of headspace volatiles that act as host location and oviposition cues in the interaction between tomato and the tomato leaf miner, *Tuta absoluta* (Lepidoptera: Gelechiidae) (Proffit *et al.*, 2011; Caparros Megido *et al*., 2014; Bawin *et al.,* 2015).

A monoterpene blend which included camphor, α-pinene and limonene in essential oils of the African nightshade, *Solanum sarrachoides* Sendtner contributed to oviposition deterrence against the tomato red spider mite, *Tetranychus evansi* Baker and Pritchard

(Murungi *et al.*, 2013). Methyl salicylate is produced by *Nicotiana attenuatta* (Solanaceae) following attack by larvae of *Manduca quinquemaculata* (Lepidoptera, Sphingidae) (Kessler & Baldwin, 2001), as well as in tomato and *Datura wrightii* (Solanaceae) when damaged by *Manduca sexta* (Sphingidae) (Reisenman *et al.,* 2013). Additionally, methyl salicylate, alone or combined with linalool, was found to elicit attraction by *Phytosieolus longipes* Evans, a predator of *T. evansi* (Azandeme-Hounmalon *et al.,* 2016). This may suggest shared biosynthetic pathways for some of these compounds in the roots and leaves of solanaceous crops.

From a biosynthetic perspective, thymol is a phenolic monoterpene, a derivative of cymene. It has been suggested that γ-terpinene goes through aromatization to form *p*-cymene that is hydroxylated to thymol or its isomer carvacrol (Mikio & Taeko, 1962; Thompson *et al.*, 2003). In the current study, γ-terpinene was not detected in the pepper root volatiles. However, *p*-cymene was detected in the hot pepper cultivars, Long red cayenne and AVDRC PP0237, but it was absent in the sweet peppers. This may indicate that genetic variation for biosynthetic pathways exist in plants of the same species. It would be informative to investigate the genetic comparisons in the four pepper plants to determine if molecular tools can be deployed for some of these pathways.

4.5 Responses of *Meloidogyne incognita* to selected volatile compounds and blends

Nematodes responded significantly to the three doses of the 5-component synthetic blend compared to a control (sand) (Figure 4.7). The responses were dose-dependent 71% at 48 pg/plant/hr (χ^2= 5.04, P= 0.025); 81% at 96 pg/plant/hr (χ^2= 13.83, P<0.001); and 88% at

192 pg/plant/hr (χ^2= 21.95, P<0.0001) over the control. The dose of 192 pg/plant/hr was then used in further experiments.

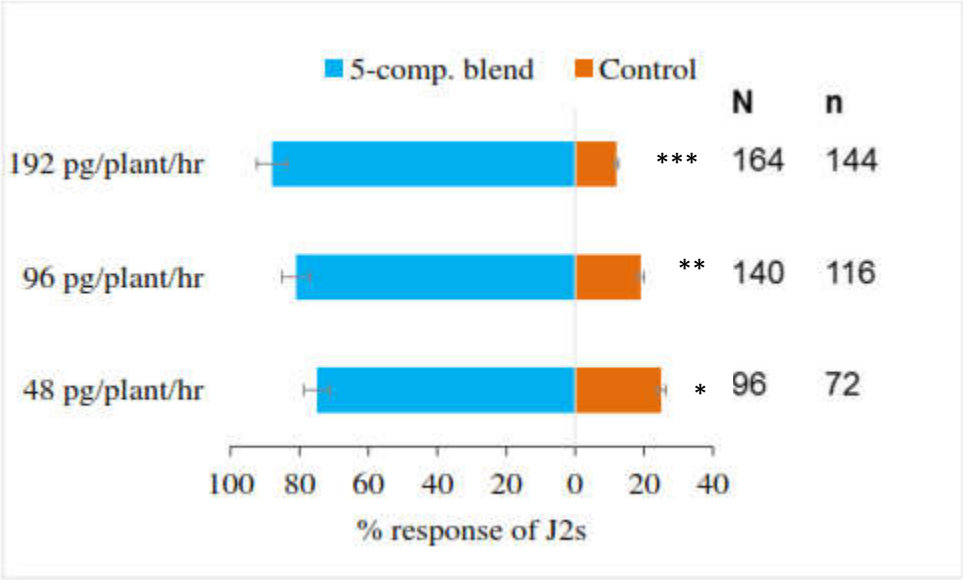


Figure 4.7: Response of *Meloidogyne incognita* infective juveniles (J2s) to different doses of the 5-component blend.
5-component blend includes α-pinene, limonene, 2-methoxy-3-(1-methylpropyl)-pyrazine, methyl salicylate and tridecane. N is the total number of responding J2 while n is the number of responding J2s corresponding to a given treatment. The asterisks indicate the significant levels with *** = significant at P <0.0001, ** = significant at P < 0.001 and * =P<0.05

Nematodes responded differentially to thymol at the three doses (Figure 4.8). At release rate corresponding to the natural emission (48 pg/plant/hr), 60% of J2s preferred less the treated part (χ^2= 4.99, df =1, P = 0.025); doubling the release rate (96 pg/plant/hr) further reduced nematode responses to 84% (χ^2= 27.11, df =1, P <0.0001). However, the highest dose, 192 pg/plant/hr, did not significantly 75% (χ^2= 3.06, df =1, P =0.08) affect the directional orientation of nematodes as this dose appeared to have a nematicidal effect since 905 of recovered J2s even at release arm were observed to be dead. Optimal activity was observed at a concentration of 96 pg/plant/hr based on statistical analysis; hence, this dose was used in further experiments.

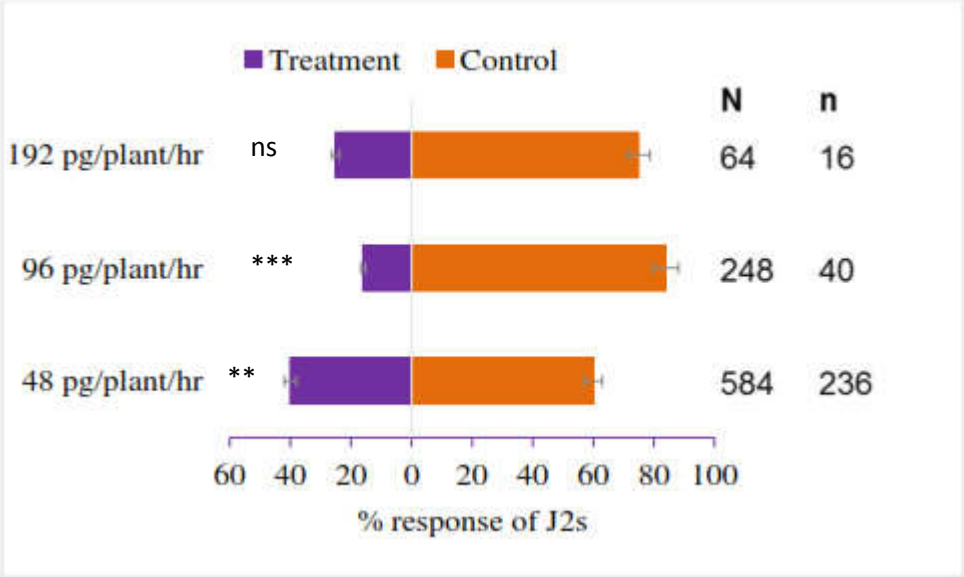


Figure 4.8: Response of *Meloidogyne incognita* infective juveniles (J2s) to different doses of thymol.
N is the total number of responding J2 while n is the number of responding J2s corresponding to a given treatment. The asterisks indicate the significant levels with *** = significant at P <0.0001, ** = significant at P < 0.05, and ns= not significant at P=0.05

4.6 Effect of thymol on natural plant odors and the preferred 5-component blend

Olfactometer assays testing the response of nematodes when thymol was paired with cv. California Wonder and the 5-component synthetic blend indicated significant preference for cv. California Wonder (χ^2= 41.39, df=1, P <0.0001) and the 5-component synthetic blend (χ^2= 34.24, df=1, P<0.0001) over thymol (Figure 4.9). *Meloidogyne incognita* J2s significantly avoided cv. California Wonder root odors spiked with 96 pg/plant/hr of thymol (χ^2= 11.03, df =1, P <0.001) and 192 pg/plant/hr of 5-component blend spiked with 96 pg/plant/hr of thymol (χ^2= 10.87, df=1, P<0.001) when compared to a control (Figure 4.9).

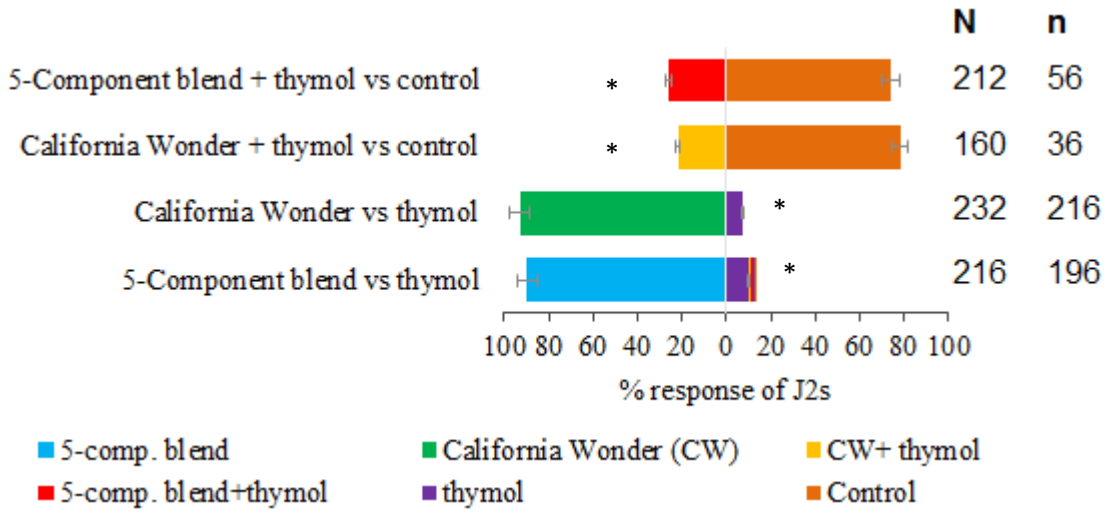


Figure 4.9: Effect of thymol on response of *Meloidogyne incognita* infective juveniles (J2s) to the five component blend and California Wonder (CW).
N is the total number of responding J2s while n is the number of responding J2s corresponding to a given treatment. The level of significance is indicated by * = significant at P <0.001

The presence of thymol, whether alone or combined with the natural root odors of the RKN-susceptible cv. California Wonder or the 5-component synthetic blend reduced the responses of nematodes. These results confirm our previous results, which showed that the J2s preferred less the root odors of AVDRC PP0237. Chemoreception can be disrupted by obstructing the chemoreceptors or creating chemical barriers by blocking the chemotactic signals emanating from the host roots as it is critical for host location and movement to feeding sites (Perry, 2005; Abad & Williamson, 2010). Our results support this phenomenon by demonstrating that although the four pepper cultivars shared similar chemical cues that contribute to J2s attraction, the presence of an antagonist, thymol, in AVDRC PP0237 was imperative in modulating chemotactic RKN-host location. Additional studies with other RKN-resistant pepper cultivars need to be undertaken to determine if thymol is the antagonistic chemical component or other compounds are involved across cultivars.

Thymol is a naturally occurring biocide in plants such as *Thymus vulgaris* (Crocol, 2011) known for its antimicrobial activity (Nostro *et al.*, 2007; Kifer *et al.*, 2016). It has been reported to have antifungal (Ahmad *et al.*, 2010) and antibacterial activity (Xu *et al.*, 2008; Santurio *et al.*, 2014). Previous studies have shown thymol to have nematicidal activity against *Meloidogyne arenaria* (Neal), the soybean cyst nematode, *Heterodera glycines* Ichinohe the soil saprophytic nematode, *Caenorhabditis elegans* Maupas and the pinewood nematode, *Bursaphelenchus xylophilus* (Steiner and Buhrer) Nickle (Soler-Serratosa *et al.*, 1995; Tsao & Yu, 2000; Choi *et al.*, 2007). In our study, thymol elicited avoidance behavior in J2s when present in root odors of the RKN-resistant pepper and in the presence of attractive odors, demonstrating a potential role for use in the disruption of chemotactic host finding behavior of the motile and infective stage of RKNs.

4.7 Chemical analyses of pepper root exudates

Liquid Chromatography-Time of Flight Mass Spectrometry analysis of root exudates tentatively identified a steroidal (**a**) and phenolic glycoside (**b**), a lignan (**c**) and a withanolide (**d**) (Figure 4.10). Elemental composition generated from MassLynx software given for each analyte was used to search online databases and to compare mass fragmentation pattern with those reported in literature (Table 4.3).

Table 4.3: LC-QToF-MS of different components in root exudates of different pepper cultivars and their tentative identities

Peak No.	RT (min)	Compound	[M+H]	Fragment ions (m/z)
(a)	4.88[1]	(25S)-spirostanol-3β,5β,26-triol-5β-D-glucopyranoside (reinocarnoside B)	595.382	465.3192, 453.2404, 433.3300, 352.1443, 345.1206, 317.1681, 289.2155, 271.2046, 253.1945, 223.0633, 161.1320
(b)	5.28[2]	2-[(6-Deoxyhexopyranosyl)oxy]cyclohexyl 6-ammonio-3-O-[(1S)-1-carboxylato-2-phenylethyl]-6-deoxyhexopyranoside	572.268	554.2565, 526.2621, 508.2501, 490.2425, 433.2351, 387.2309, 369.2208, 355.1463, 225.1266,171.0792
(c)	5.87[1]	4-[(2S,3R)-2,3-Dimethyl-4-(3,4,5-trimethoxyphenyl)butyl]-2-methoxyphenol	375.214	317.2106, 299.1997, 253.1941, 203.1791, 161.0948
(d)	6.37[1]	5β, 6β: 16α, 17α-diepoxy-4β-hydroxy-1-oxo-witha-2,24-dienolide (tubocapsenolide A)	469.257	486.2835 (M+OH), 491.2384 (M+Na), 507.2119 (M+K), 452.2276, 423.2515, 405.2408, 387.2306, 369.2202, 315.1733, 225.1270, 193.0866, 937.5054(2M+H), 959.4875(2M+Na), 975.4622(2M+K)

[1]Names suggested on basis of online database, literature and fragmentation pattern
[2]Names suggested on basis of online database and fragmentation pattern

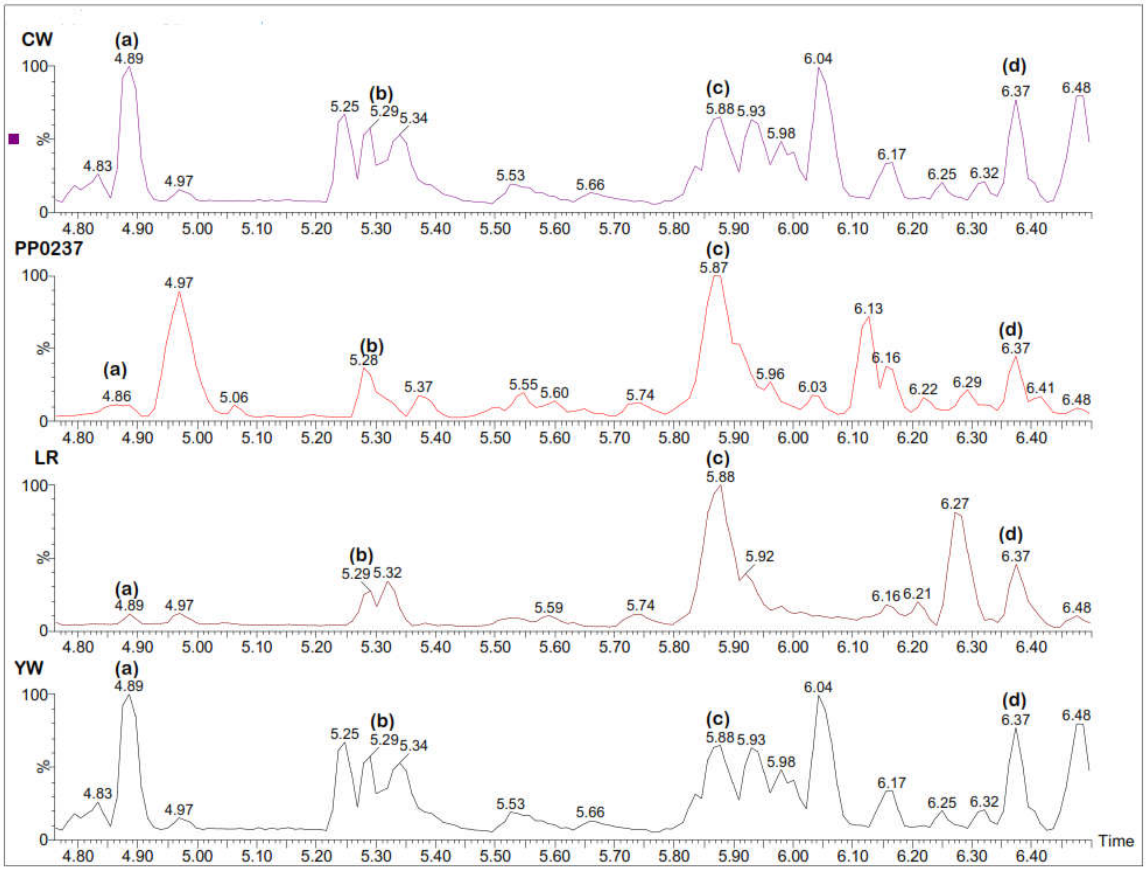


Figure 4.10: Liquid chromatography-time of flight mass spectrometry profile of root exudates of *Capsicum annum*
CW, PP0237, LR and YW represent California Wonder, AVDRC PP0237, Long Red Cayenne and Yolo Wonder pepper cultivars respectively.
(a) (25S)-spirostanol-3β,5β,26-triol-5β-D-glucopyranoside
(b) 2-[(6-deoxyhexopyranosyl)oxy]cyclohexyl-6-ammonio-3-O-[(1S)-1-carboxylato-2-phenylethyl]-6-deoxyhexopyranoside
(c) 4-[(2S,3R)-2,3-Dimethyl-4-(3,4,5-trimethoxyphenyl)butyl]-2-methoxyphenol
(d) 5β, 6β: 16α, 17α-diepoxy-4β-hydroxy-1-oxo-witha-2,24-dienolide

Compounds were tentatively identified as (**a**) (25S)-spirostanol-3β,5β,26-triol-5β-D-glucopyranoside (reinocarnoside B) (m/z 595, MF $C_{33}H_{54}O_9$) [Figure 4.11], (**b**) 2-[(6-deoxyhexopyranosyl)oxy]cyclohexyl-6-ammonio-3-O-[(1S)-1-carboxylato-2-phenylethyl]-6-deoxyhexopyranoside (m/z 572, MF $C_{27}H_{41}NO_{12}$) [Figure 4.12], (**c**) 4-[(2S,3R)-2,3-Dimethyl-4-(3,4,5-trimethoxyphenyl)butyl]-2-methoxyphenol (m/z 375, MF $C_{22}H_{29}O_5$)

[Figure 4.13], and (**d**) 5β, 6β: 16α, 17α-diepoxy-4β-hydroxy-1-oxo-witha-2,24-dienolide (tubocapsenolide A) (m/z 469, MF $C_{28}H_{36}O_6$) [Figure 4.14].

The lignan (**c**) was previously reported in ethanolic extract of *Machilus robusta* bark (Yanru *et al*., 2011). The steroidal glycoside (**a**) has been isolated from the roots of a medicinal herb, *Reineckia carnea* (Andr.) Kunth (Qian *et al.*, 2013). The withanolide (**d**) was previously extracted from roots of *Tubocapsicum anomalum* (Franch. & Sav.) and found to have cytotoxicity to cancer cells (Wu & Chang, 2009). Root exudates have previously been reported to mediate plant-nematode interactions (Hooks *et al.,* 2007). A complex terpene, solanoeclepin A present in potato, *Solanum tuberlosum,* stimulates egg hatching of the potato cyst nematode, *Globodera palllidipis*. Glycinoeclepins B and C are nortriterpenes present in the kidney bean that are active even at low concentrations of 0.01ng/ml (Schenk *et al.*, 1999; Rasmann *et al*., 2012;). In the current study, infective juveniles of *M. incognita* exposed to root exudates of the four pepper cultivars demonstrated different chemotaxis behavior. Notably, root exudates from the RKN-resistant accession elicited avoidance response to the infective juveniles while the susceptible cultivars were highly preferred and elicited more stylet thrusting. This demonstrates that root-released compounds influence host selection by the infective juveniles.

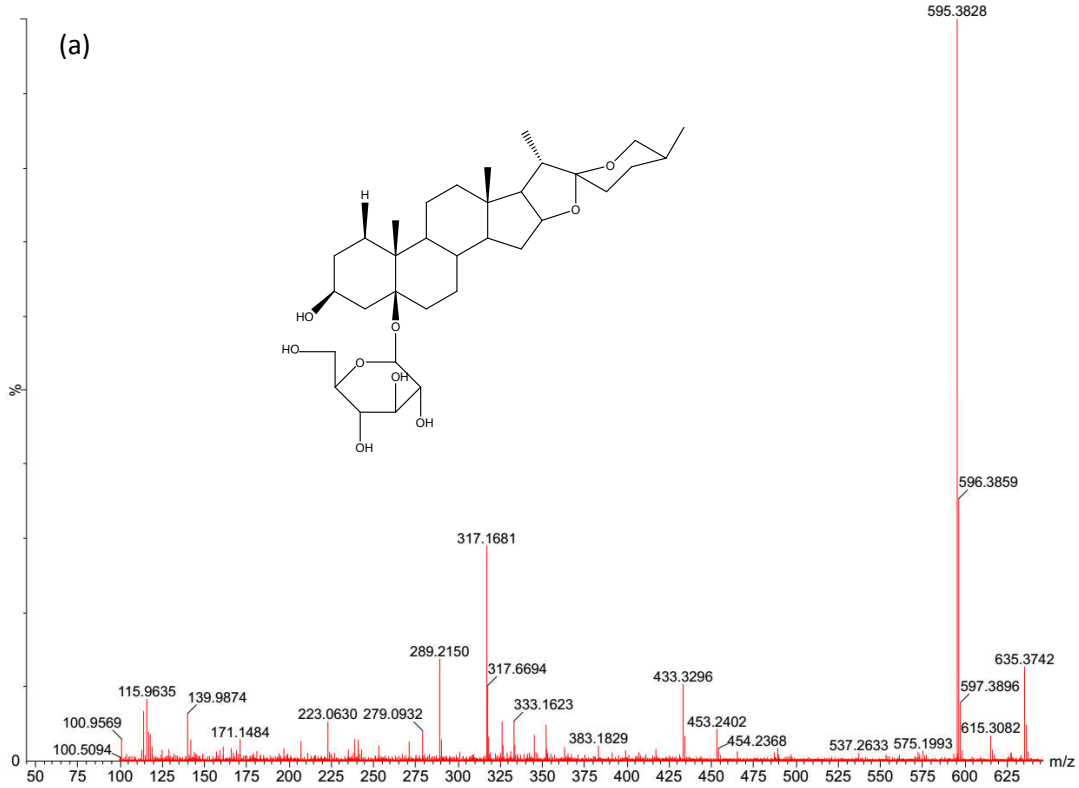


Figure 4.11: QToF-MS fragmentation pattern of (25S)-spirostanol-3β,5β,26-triol-5β-D-glucopyranoside (reinocarnoside B) ($C_{33}H_{54}O_9$)

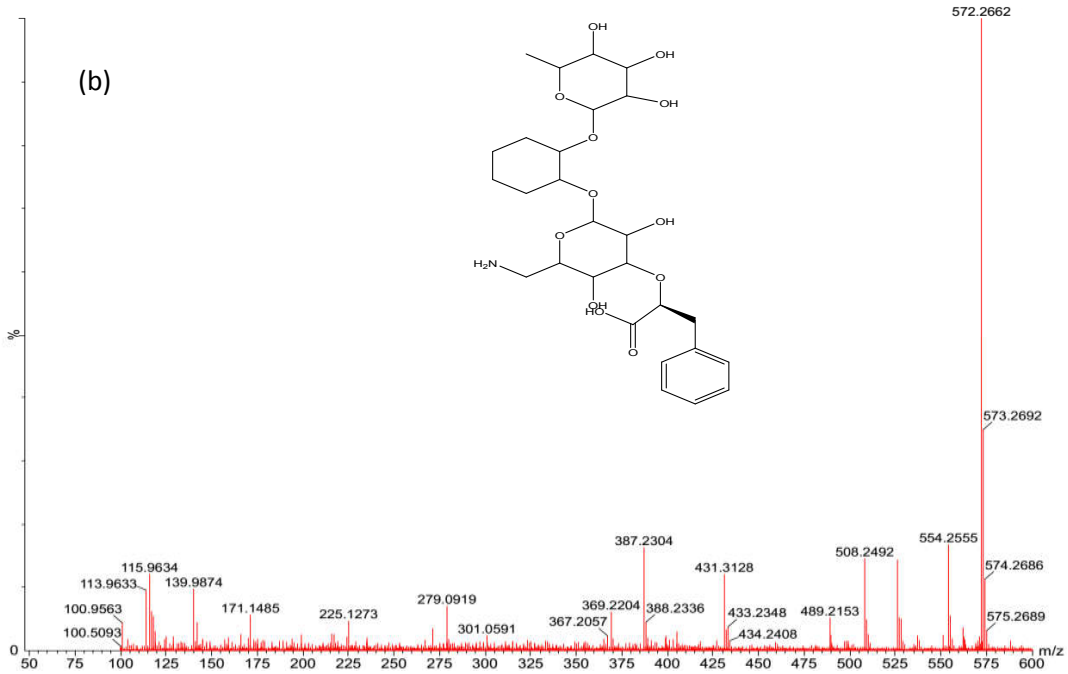


Figure 4.12: QToF –MS fragmentation of 2-[(6-deoxyhexopyranosyl)oxy]cyclohexyl 6-ammonio-3-O-[(1S)-1-carboxylato-2-phenylethyl]-6-deoxyhexopyranoside ($C_{27}H_{41}NO_{12}$)

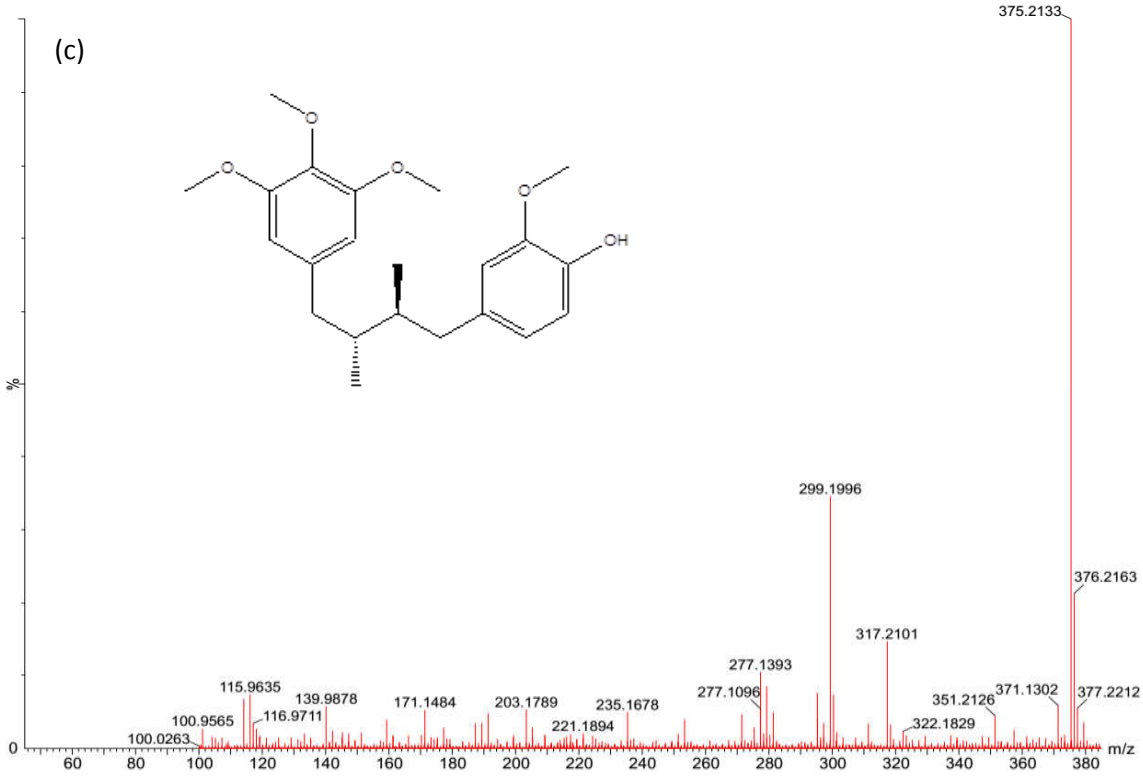


Figure 4.13: QToF-MS fragmentation of 4-[(2S,3R)-2,3-Dimethyl-4-(3,4,5-trimethoxyphenyl)butyl]-2-methoxyphenol

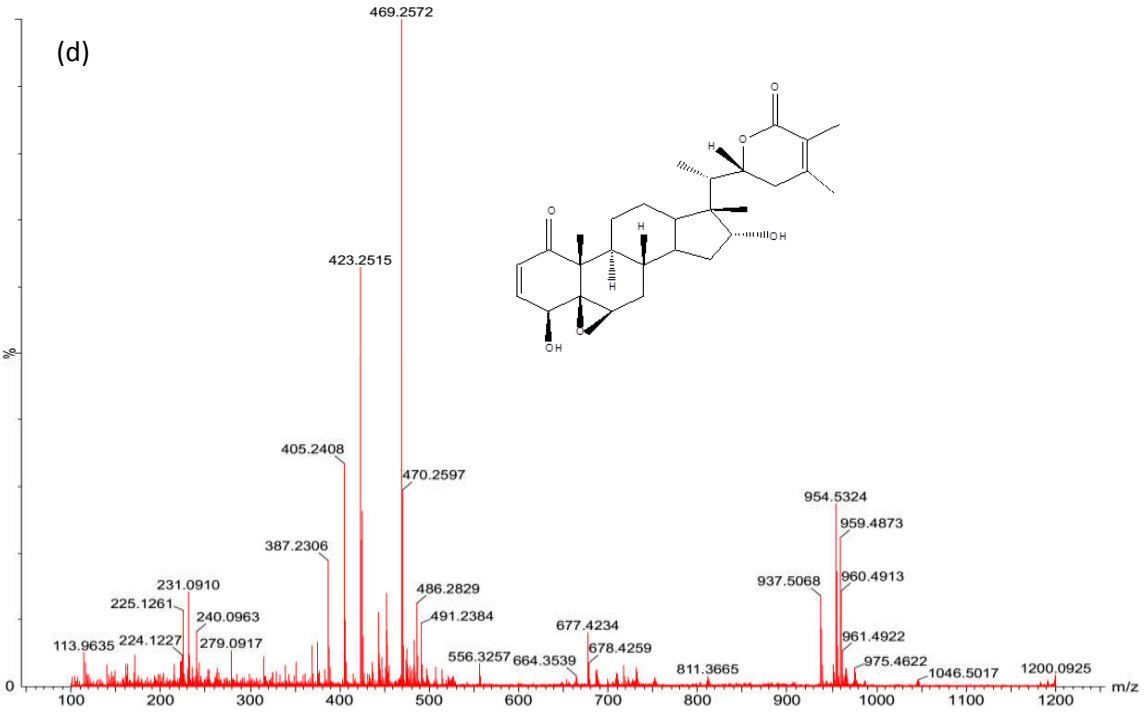


Figure 4.14: QToF-MS fragmentation of 5β, 6β: 16α, 17α-diepoxy-4β-hydroxy-1-oxo-witha-2, 24-dienolide (tubocapsenolide A) ($C_{28}H_{36}O_6$)

CHAPTER FIVE

CONCLUSIONS AND RECOMENDATIONS

In summary, our results indicate that plant root chemical cues are important for *M. incognita* J2s to locate their preferred host, and open a promising possibility for the use of semiochemicals in the management of RKNs. The efficacy of semiochemical based tools has been demonstrated in disease vectors (Heuskin *et al.*, 2011; Logan *et al.*, 2013) and in integrated pest management strategies of insect pests and the parasitic weed, striga (Agelopoulos *et al.*, 1999; Cook *et al*., 2007; Hassanali *et al.*, 2008; Soroker *et al.*, 2015). The present findings open up the possibility of deployment of molecular tools for plant breeders to incorporate genes responsible for the production of thymol to protect pepper from RKN infection. Development of seeds with resistant traits is promising approach by genetic modification of secondary metabolite pathways that produce insecticidal compounds (Birkett & Pickett, 2014). Other studies have identified resistant pepper cultivars, such as the Charlestone Belle and Carolina Wonder whose resistance is conferred by the Me1 and Me3 resistant genes (Djian-Caporalino *et al.,* 2007). This was associated with disease incidence where resistant cultivars recorded low galling index and egg production (Thies *et al*., 2008). Our study provides new insights towards linking molecular methods with biochemical processes for plant protection against these phytoparasitic nematodes.

5.1 CONCLUSIONS

1. Root volatiles and exudates of the four pepper cultivars influenced the host seeking behavior of *Meloidogyne incognita* J2s. There was a correlation between

responses of J2s to volatiles and exudates showing either attraction or avoidance to the different pepper cultivars.

2. Root chemistry of the four pepper cultivars showed similarities and differences in volatile and non-volatile chemical components with thymol being specific to the RKN-resistant cultivar.
3. Synthetic volatile compounds tested influenced chemotaxis in *M. incognita* J2s with shared components contributing to attraction of J2s. However, thymol which was specific to RKN-resistant cultivar elicited avoidance behavior when tested alone and when combined with preferred plant natural odors. These findings support the hypothesis that plant chemical signals play a role in pepper-RKN interaction and open new opportunities for use of semiochemical based tools in RKN management.

5.2 RECOMMENDATIONS

i. Root volatiles are produced based on the different biosynthetic pathways in plants. Molecular comparison between the susceptible and resistant pepper cultivars can be carried out to identify specific genes that can be deployed in crop improvement for management of RKNs

ii. These findings can provide further research to elucidate the full identities and bioactivity of the specific non-volatile compounds mediating host seeking behavior and parasitism in the RKN-pepper interactions.

iii. Volatile organic compounds can be tested in semi-field and field applications as slow release compounds to assess the performance as a management strategy for RKNs.

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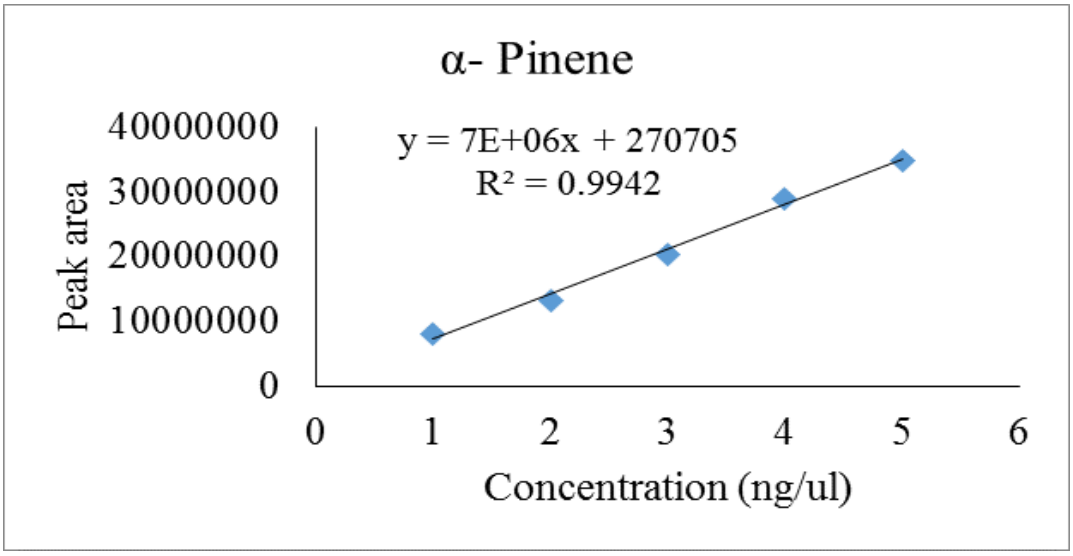
APPENDICES

APPENDIX I: CALIBRATION CURVE OF α-PINENE USED FOR QUANTIFICATION OF PEPPER ROOT VOLATILES



APPENDIX II: CALIBRATION CURVE OF HUMULENE USED FOR QUANTIFICATION OF PEPPER ROOT VOLATILES