BEHAVIORAL RESPONSE OF THE ROOT-KNOT NEMATODE, *MELOIDOGYNE INCOGNITA*, TO TOMATO, *SOLANUM LYCOPERSICON*, ROOT EXUDATES

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2020

Behavioral response of root-knot nematode, *Meloidogyne incognita,* to tomato, *Solanum lycopersicon*, root exudates

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A Thesis Submitted in Fulfillment for the Degree of a Master of Science in Plant Health Science and Management in the Department of Horticulture and Food Security of Jomo Kenyatta University of Agriculture and Technology

2020

DECLARATION

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

Signature..... Date.....

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This thesis has been submitted with our approval as supervisors.

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DEDICATION

I dedicate this work to my late Mum Julia Chemeli and my wife, Naumy, my son Ryan and daughter Ariana, for their unwavering support both spiritual and emotional during my endeavor for this degree.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my supervisors; Prof. Baldwyn Torto and Dr. Lucy K. Murungi for their valuable and unrelenting advice, encouragement, and their great mentorship throughout my research.

I am also grateful to the Department of Horticulture and Food Security at Jomo Kenyatta University of Agriculture and Technology for allowing me to pursue my study. Special appreciation to the Behavioral and Chemical Ecology Unit (BCEU), *icipe*, for the scholarship.

I am also indebted to my *icipe* colleagues; Xavier Cheseto, Ruth Kihika and Onesmus Wanyama for their moral support and technical assistance. Last but not the least; I would like to thank my family for the support they accorded me throughout my studies and my life in general.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AVRDC	Asian Vegetable Research and Development Center (The World Vegetable Center)
DEGO	Dorsal Esophageal Gland Opening
EST	Esterase
IPM	Integrated Pest Management
UPLC-QToF-MS	Ultra-Performance Liquid Chromatography-Quadrupole Time of Flight-Mass spectrometry
LC-MS	Liquid Chromatography coupled to Mass Spectrometry
PPNs	Plant Parasitic Nematodes
RKN	Root Knot Nematode

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Root-Knot Nematodes (RKNs) are major economically important group of plant parasitic nematodes distributed worldwide. RKNs cause over $100 billion yield losses annually on major crops. The damage caused to crops is due to plant root invasion by motile second stage juveniles (J2s). The J2s induce redifferentiation of the plant root cells into specialized feeding cells essential for nematode development. The hyperplasia and hypertrophy of cells surrounding the feeding cells lead to formation of typical root galls affecting transport of photosynthates from source to sink, and uptake of water and nutrients. Upon hatching from the eggs in the soil, J2s must successfully locate a host plant to invade and infect for them to survive and complete their life cycle. However, there is limited knowledge on what directs the J2s to their hosts. It is demonstrated that olfaction plays an important role in the nematode host-seeking process by following a chemical trail toward host-associated odors**.** The known attractants include volatile and non-volatile (water-soluble) compounds released by plant roots into the rhizosphere. The former mediates long distance attraction while the latter is involved in short distance attraction. The major source of these chemicals is root exudates from which several stimuli responses that include attraction, repulsion and aggregation have been postulated. In this study, the responses of the motile stage second stage juveniles (J2s) of *Meloidogyne incognita* to tomato root exudates and the non-volatile components identified in the root exudates were investigated. Using stylet thrusting, chemotaxis assays, and chemical analysis, specific metabolites in the tomato root exudates that either attract or repel J2s were identified. Ultra-Performance Liquid chromatography quadrupole time-of-flight mass spectrometry (UPLC-QToF-MS) analysis of bioactive fractions obtained from the root exudate revealed a high diversity of compounds. Five of these were identified as the phytohormone zeatin (cytokinin), the flavonoids quercetin and luteolin, and the alkaloids solasodine and tomatidine. In stylet thrusting and chemotaxis assays, the five compounds elicited concentration-dependent responses in J2s relative to 2% dimethyl sulfoxide (negative control) and methyl salicylate (positive control). Zeatin being attractive to J2s in all the concentrations and quercetin being attractive at lower concentrations and deterrence at high concentrations tested. These results indicate that J2 parasitism is influenced by root exudate chemistry and concentrations of specific compounds. Identification of zeatin and quercetin as an attractant of RKNs J2s improves our understanding of root knot nematodes chemical ecology, and that they could be used as a potential component in the IPM of root knot nematodes.

CHAPTER ONE

INTRODUCTION

1.1 Background

Plant parasitic nematodes (PPNs) are of considerable economic importance in agriculture worldwide (Bird *et al*., 2009). They are a major threat to a wide range of agricultural crops influencing their health, quality and yield (Singh *et al.*, 2015). Collectively, PPNs are estimated to cause $100-157 billion in annual crop losses amounting to 8.8- 14.6% of the total crop production globally (Bird *et al*., 2008; Singh *et al*., 2013). PPNs are small organisms, usually 0.25 mm to 3 mm long and cylindrical, tapering towards head and tail (Singh & Phulera, 2015). Females of RKN lose their worm shape as they mature, becoming pear-, lemon- or kidney- shaped (Lambert & Bekal, 2002). PPNs are classified by their mode of feeding and host-parasite relationship as either ecto-parasites or endo-parasites and as migratory or sedentary (Luc *et al*., 2005). Most of PPNs have similar life cycle stages, an egg, four juvenile stages and the adult. The first stage juvenile (J1) molts within the egg and emerges as a motile second stage juvenile (J2), which then molts into the third and fourth juvenile stages before molting into an adult stage (Moens *et al*., 2009). Among the PPNs, root knot nematodes (RKNs) are the most damaging and they estimated to cause yield losses amounting to $100 billion per year worldwide (Mukhtar *et al*., 2014; Muturi *et al*., 2003).

Root knot nematodes (*Meloidogyne sp.*) are group of PPNs distributed globally in the tropics, subtropics, and temperate regions (Castagnone-Sereno *et al.*, 2013). The survival success of most of these *Meloidogyne sp*. is attributed to wide host ranges, short generation times and high reproductive rates (Singh *et al.*, 2013) and as such, they are able to parasitize majority of cultivated plants species (Trudgill & Blok, 2001). RKNs are characterized by how they establish an intimate relationship with their host plants inducing the root cells to differentiate into specialized feeding cells (giant cells), that constitute an exclusive source of nutrients for their development (Caillaud *et al*., 2008).

The formation of these specialized feeding structures disrupts the normal functioning of the host plant root systems thus hampering the uptake of water and nutrients, and the flow of the photosynthates (Teillet *et al*., 2013). Although more than 100 species of the RKN, *Meloidogyne sp.* have been identified, four species have been categorized as the major ones worldwide, whereas the others are minor or emerging (Onkendi *et al.*, 2014). The four major species *Meloidogyne arenaria, Meloidogyne hapla, Meloidogyne incognita* and *Meloidogyne javanica* comprise 98% of the worldwide population of *Meloidogyne sp.* (Elling, 2013; Esbenshade & Triantaphyllou, 1987). They cause most of the damage reported on agricultural crops as they have a wide range of host plants (Khan *et al*., 2014). *Meloidogyne arenaria, M. incognita* and *M. javanica* are the three major species found in the tropics, subtropics, and glasshouses in temperate regions while *M. hapla* is the major species found in the temperate regions (Moens *et al*., 2011). The minor/emerging species of *Meloidogyne sp.* include; *Meloidogyne enterolobii, Meloidogyne exigua* and *Meloidogyne graminicola* found in tropical climatic regions, *Meloidogyne chitwood, Meloidogyne naasi* and *Meloidogyne fallax* are some species found in temperate climates (Moens *et al*., 2011; Singh *et al*., 2013). Some of the crops affected by these *Meloidogyne sp.* include groundnuts, lettuce, potatoes, barley, carrots, coffee, rice, maize, and tomatoes (Moens *et al*., 2009). Almost all the major species and some of the minor species mostly affect tomato plants. *Meloidogyne incognita* has been reported to cause suppression in yields of tomatoes as high as 85% (Ros Ibáñez *et al*., 2014), *M. hapla* and *M. enterolobii* found in tropical climatic regions cause up to 50% and 65% respectively (Singh *et al*., 2013).

Tomato (*Solanum lycopersicon*) is one of the most important vegetable crops grown in most regions of the world (Seid *et al*., 2015). In Africa, tomato production is approximately 20M tonnes and Kenya is ranks 6th with a total production of approximately 400K tonnes (FAO, 2014). In Kenya, tomato production is mainly carried out by smallholder farmers who produce for home consumption and for domestic market (Sigei *et al*., 2014). Also, tomato is the second leading vegetable in terms of production after potatoes (FAO, 2014). The production of tomato is divided into two

categories; the open field, which accounts for 95% and high tunnels 5% of production (Sigei *et al*., 2014). Tomato varieties grown in Kenya include: Money maker, Eden, Onyx, Monyalla, Tanzanite, 'Cal-J', Shanty F1, Galilea F1, and Nyota F1 for open field and marglobe, super marmande, caltana F1, Anna F1, Kilele F1, Tylka F1, Pink Red, Montelle and Celebrity among others are mostly for high tunnel (Sigei *et al*., 2014). Tomato production is faced with a myriad of challenges such as pests and diseases, high cost of pesticides and environmental factors (Ngundo *et al*., 2012). RKNs are responsible for yield losses ranging from 40-46% in Kenya (Ngundo *et al*., 2012)

Olfaction is believed to play a crucial role for RKNs to successfully locate the host plant and be able to invade and infect (Dillman *et al*., 2012). However, the cues that play the role in host seeking are least investigated (Fudali *et al*., 2013). Given that RKNs have a wide host range, it is suggested that plant chemical cues in the rhizosphere influence host location (Fudali e*t al*., 2013). These attractants include volatile and non-volatile (water soluble) compounds released by plant roots into the rhizosphere (Curtis, 2008). The former mediates long distance attraction while the latter is responsible for short distance attraction. The major source of these chemicals are root exudates from which several stimuli responses that include attraction, repulsion and aggregation have been postulated but no compounds have been identified so far (Spiegel *et al.*, 2003). As such, this study sought to test the response of RKNs to the tomato root exudates and identify the chemical constituents by mass spectrometry that are responsible for the short distance signaling

1.2 Statement of Problem

Root Knot Nematodes cause huge losses in crops worldwide and mostly crops that are of high agricultural importance (Mitsumasu *et al*., S2015). Losses attributed to RKNs, principally *M. incognita,* are between 5-43% in the tropics and subtropics (Surendra *et al*., 2014). Majority of small-scale farmers in Africa are often unaware of the RKNs or the damasge they cause but recognize reducing yields (Jones *et al*., 2013). As such, mitigation strategies to control RKNs and improve crop yields are of great importance.

However, management strategies such as crop rotation, biological control, resistant varieties and nematicides that were used are faced with many challenges. Of these existing methods, fumigants nematicides such as methyl bromide were highly promising in the management of *M. incognita* and other soil borne pathogens. But due to their adverse effects on marine ecosystems, humans and animals health, and ozone depletion, their use have been completely phased out (Moens *et al.*, 2009; Stoll *et al.*, 2003) necessitating alternatives ecofriendly control strategies. One such alternatives method is the use of semiochemicals involved in the RKNs-host plants interactions.

1.3 Justification

Agriculture is the backbone of the Kenyan economy contributing about 30% of gross domestic product and accounts for over 80% of employment (HCD, 2014). The Kenyan government has put emphasis into agriculture as highlighted through Kenya Vison 2030, the Medium-Term Plan III and most recently the Big Four agenda for 2017-2022, that seeks to have 100% food and nutrition security (Republic of Kenya, 2018). For the government to achieve its target of sustainable food and nutrition security, mitigating challenges of production (pests and diseases) of high value vegetables crops such as tomato are required. Vegetables contributed 36% of the domestic value of horticulture estimated at KES 70.9 billion of these, tomato production accounted for 19% of the total value coming second after potatoes which accounted for 43% of value (HCD, 2014). Tomato production is faced with many challenges such as pest and diseases. Among the pests, root knot nematodes are the leading below-ground pest that attack the plant root system hampering it from getting nutrients and water for healthy growth. To control root knot nematodes, chemicals nematicides have been used for management, but they have many adverse effects on the environment and water systems thus need for alternatives control measures (Stoll *et al.*, 2003).

Advances in the understanding of plant-pest interactions has resulted in alternative pest management approaches that are highly selective, effective, and environmentally friendly not only alleviating the problems posed by conventional pesticides but also increasing

yield and marketability of produce. Plant-pest interactions are an invaluable source of insight for crop protection strategies (Cook *et al.*, 2007). Research has shown that there is chemical communication between the nematodes and host plants suggesting plant roots produce chemical signals that guide the host seeking process (Fudali *et al*., 2013; Kihika *et al*., 2017; Murungi *et al*., 2018). Curtis (2008) proposed that nematodes are possibly attracted by volatiles and water-soluble compounds secreted by the roots or other organisms present in the rhizosphere. Small lipophilic molecules from tomato root exudates have been shown to have inhibitory impact on motility of *M. incognita* and *M. graminicola* (Dutta *et al.*, 2012). Spiegel (2003) also demonstrated that root exudates of tomatoes have attraction effect on *Ditylenchus dipsaci* that is also a plant parasitic nematode. These research outputs have unlocked more research into various mechanisms of insect-plant interactions below-ground. In this study, identification and subsequent understanding of the functions of the chemical compounds in the root exudates of tomato plants would not only address the existing knowledge gap, but also offer hope for the development of a new approach or the improvement of existing RKN control strategies.

1.4 Hypotheses

1. There is no behavioral response of RKN to root exudates of host plants
2. There is no behavioral response of RKN to water-soluble fractions of host plant root exudates
3. There are no water-soluble secondary metabolites involved in host attraction

1.5 Objectives

1.5.1 General objective

To determine the mechanisms for short distance signaling of root knot nematode to the host plant roots focusing on naturally occurring behavior-modifying chemicals in the tomato root exudates.

1.5.2 Specific objectives

1. To evaluate the behavioral responses of root knot nematode, *Meloidogyne incognita*, to tomato root exudates
2. To screen fractions of tomato root exudates for their bioactivity against *Meloidogyne incognita*
3. To identify the chemical components in bioactive fractions of tomato root exudates and determine their effects on *Meloidogyne incognita* behavior

CHAPTER TWO

REVIEW OF LITERATURE

2.1 Tomato (Solanum lycopersicum L)

Tomato is one of the most widely cultivated crops in the world (Garcia *et al*., 2015). The crop is native to South and Central America and it belongs to the *Solanaceae* family of plants that includes other species such as spotato, tobacco, peppers, nightshade, and eggplant (Balemi & Negisho, 2012). The fruits are known for a source of lycopene, which is an antioxidant that is important for bone health and as a source of vitamins A and C as well as calcium and potassium (Srivastava & Kulshreshtha, 2013). Tomato is one of the most important cash crops for small holders and medium-scale commercial farmers in Africa (Onkendi *et al*., 2014). In Kenya, it is the second leading vegetable in terms of production and value after potato and is grown for consumption and as source of income (Sigei *et al*., 2014).

Tomato exists in two types of growth habit i.e. determinate and indeterminate types. The determinate varieties are bushy types and have a defined period of flowering and fruit development and are mostly grown in open fields. They include varieties like Eden, Onyx, Tanzanite, Monyalla and 'Cal-J' as they are high yielders (Musyoki *et al.*, 2005; Tembe *et al*., 2018). The indeterminate varieties are mostly heirloom garden varieties that produce flowers and fruits throughout the life of the plant. They grow mostly in controlled environments such as in greenhouses and glasshouses. The indeterminate varieties include: Kenom, Marglobe, Monset, Nemonneta, Anna F1, among others.

Tomato production is faced with many constraints that include pests and diseases (Ochilo *et al*., 2019). Of the diseases bacterial wilt, early and late blight, leaf curl, tomato spotted wilt virus, leaf spot and powdery mildew are the most problematic. Insect pests and other arthropods include spider mites, leaf miners, tomato leaf miner (*Tuta absoluta*), thrips, whiteflies, African bollworm and root knot nematodes leading to high economic losses

(Birithia *et al*., 2012). Root knot nematodes are the major below-ground pathogens of tomatoes worldwide that limits its production (Luc *et al*., 2005).Root knot nematodes, (*Meloidogyne sp*.)

Root knot nematodes (*Meloidogyne sp.)* are the top leading plant-parasitic nematodes based on scientific and economic importance (Jones *et al*., 2013). They were first noticed by Berkeley in 1855, when he observed galls on roots of greenhouse grown cucumber plants (Moens *et al*., 2009) and recognized them as belonging to the genus *Heteroidera*. In 1884, Muller classified RKNs as *Heteroidera radicicola* and later in 1887 he reclassified them into different *Meloidogyne sp.* giving general description of the genus *Meloidogyne* and differentiating it from the genus *Heteroidera* (Eisenback *et al*., 1981). The *Meloidgyne sp*. then were re-described by Chitwood basing on the examination of morphologicsal features and morphometrics from all the life stages of the species evaluated (Eisenback & Triantaphyllou, 1991). Root knot nematodes in the *Meloidogyne species* belong to Phylum Nemata, Order Tylenchida, suborder Tylenchina, superfamily Tylenchoidea, family Heteroderidae, subfamily Meloidogyninea, genus *Meloidogyne* (Abad *et al*., 2003).

2.2 Morphology

Use of morphological and morphometrics features have been the key for the preliminary identification of RKNs (*Meloidogyne sp*.). The features mostly used in females are body shape, stylet length, knob, and perennial pattern shape. The general morphology of *Meloidogyne* species is that females are pearly white in color with rounded to pear shaped body with a protruding and or bend neck. The female body length ranges from 350 - 3000 µm and width from 300 - 700 µm, the stylet length ranges from 10 - 25 µm. The males are motile vermiform and clearly annulated, with their body length ranging from 600 - 2500 µm, head composed of a head cap and head region, stylet length ranges from 13 - 33 µm. Their dorsal esophageal gland opening (DEGO) is located 2 - 13 µm behind the stylet knobs. In addition, the J2 are vermiform with annulated body and their length ranges from 250 - 600 µm, stylet length ranges from 9 - 26 µm and DEGO position is 2 -

12 µm behind the stylet knobs (Eisenback *et al.*, 1991; Onkendi *et al.*, 2014).

2.3 Reproduction

Despite having considerable conserved morphological features across the genus, *Meloidogyne species* exhibit a degree of reproductive plasticity (Bird *et al*., 2009; Castagnone-Sereno *et al*., 2013). Most species of economic importance are dioecious and gonochoristic (i.e. the males and females are morphologically distinct) e.g. *Meloidogyne hapla*, *Meloidogyne incognita* (kofoid and white) Chitwood that are highly damaging and polyphagous. Some species are amphimictic (i.e. sperm and egg from different individual parents), reproduce solely by outcrossing and are not significant agricultural pathogens e.g. *Meloidogyne carolinensis* (Bird *et al*., 2009). Many RKN species of agricultural importance reproduce by mitotic parthenogenesis, i.e development of an embryo from an unfertilized egg and have various degrees of polyploidy and aneuploidy (Liu *et al*., 2007) that include *M. arenaria, M. incognita* and *M. javanica* (Bird *et al*., 2009). However, *M. hapla* which is also widely distributed in temperate regions, reproduces by facultative meiotic parthenogenesis, where sexual reproduction occurs but also parthenogenetic offspring are also produced (Liu *et al*., 2007).

2.4 Life cycle of *Meloidogyne* spp.

The life cycle comprises of six stages *viz.* egg, four juvenile stages and adult. The embryonic development results into the first-stage juvenile (J1) that molts within the egg and emerges as a second-stage juvenile (J2), which is the infective stage. The J2 moves through the soil in search of a new host plant or nearby roots of the host plant. During this period, the J2 depends solely on the energy reserves stored in the intestine and their ability to invade the roots is reduced after long periods in the soil (Moens *et al.*, 2009). Infective juveniles enter the root tip of the plant through mechanical disruption of the root tissue by use of the stylet and during this process, they produce in their sub-ventral glands cell wall-degrading enzymes such as β-1,4- endoglucanases that aid in the penetration (Hussey, 1989; Rosso *et al*., 1999). The J2s then migrate through the intercellular space

within the undifferentiated root cells and move towards the elongation zone of the root where it establishes a permanent feeding site and becomes sedentary.

When the J2 has established a suitable feeding site, it pierces the cell walls with their stylets and the esophageal glands release secretions that are injected into the cells during feeding. These secretions induce formation of giant cells as a result of repeated nuclear divisions, without cytokinesis (cytokinesis is the process of cell division which divides the cytoplasm, organelles and cell membrane to form daughter cells), and cortical cells proliferation and hypertrophy resulting in formation of typical root galls (Cabello *et al*., 2014; Matthews *et al*., 2011). Following the initiation of the feeding site and the giant cell formation, the J2 becomes flask-shaped and once inside the root tissue they molt three times into the third (J3) and fourth stage (J4) and adult. The third (J3) and fourth stage (J4) are dormant stages as they do not have stylets and they do not feed (Pierre *et al*., 2003; Eisenback *et al*., 1991; Williamson & Hussey, 1996). During the J4 stage, the RKN differentiate into male and female having their reproductive organs developing into maturity. At the fourth and final molt, the adults nematodes are reveled having the three previous juveniles cuticles, the stylets reappears in both sexes, perennial pattern is observed in females and sperm production is initiated in males (Eisenback *et al*., 1991). The mature females deposit their eggs in a gelatinous matrix that hold them together outside the root surface. The matrix provides physical protection to the eggs and acts as a barrier to temperature fluctuations and water evaporation (Moens *et al*., 2009).

The length of the life cycle in RKN is greatly influenced by temperature (lower minimum, optimum, and maximum) and it takes approximately 25-30 days from eggs to adults. Earlier reports indicate that cool climate species such as *M. hapla* have different temperatures for different stages of development, hatching, mobility, invasion of roots, growth, reproduction and survival than those which occur in warmer regions such as *M. incognita, M. javanica* and *M. arenaria*. Optimum temperature ranges from 15 ^{o}C to 25 ^{o}C for cool climate species and 25 ^{o}C to 30 ^{o}C for the warmer climate species (Taylor, & Sasser, 1978). Under adverse environmental conditions, the proportion of males increases; as reproductive function implies a greater spend of energy, differentiation of

females is favored when food is available. Males are vermiform and migrate out of the roots; females are globose and remain sedentary, laying several eggs into a gelatinous matrix on the surface of a galled root or inside the galls (Maleita, 2011). In a susceptible host, the site serves as food source for the development and reproduction (Vovlas *et al*., 2005). If feeding site is not supportive, the nematodes die or leave the roots, because the access to water and nutrients is limited (Goverse *et al*., 2000).

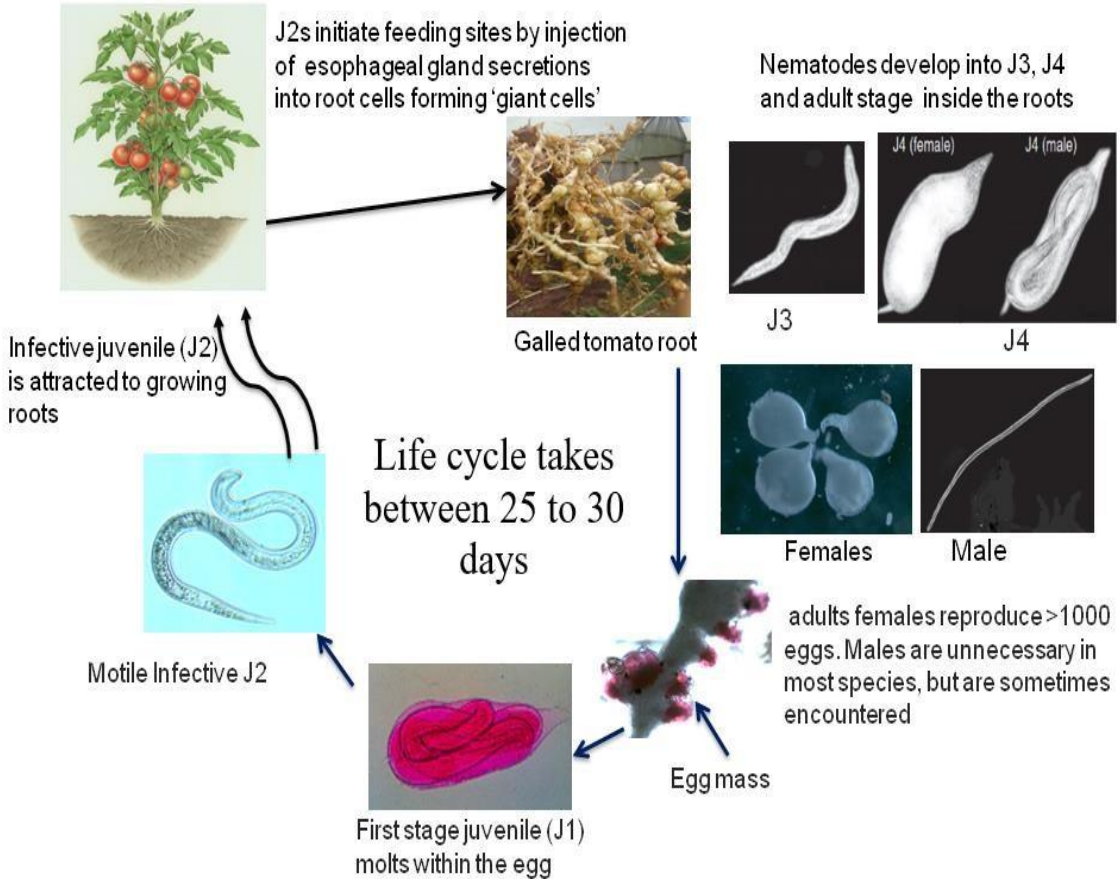

Figure 0.1: Basic life cycle of root-knot nematode (*Meloidogyne spp*.) (modified from: Mitowski and Abawi, 2011; courtesy of V. Brewster).

2.5 Distribution and host range of RKN

Root knot nematodes (*Meloidogyn*e species) are the most successful of all the plant parasitic nematodes species in the phylum Nemata and have high negative impact on

agricultural crops (Abd-Elgawad & Askary, 2015). These is attributed to their global distribution and their ability to parasitize a wide range of cultivated plant species thus affecting the production and quality of plants of economic importance (Pierre *et al*., 2003; Eisenback *et al*., 1991). The most common species that are encountered in most agricultural fields are, *M. incognita, M. javanica, M. arenaria and M. hapla* and these species represent about 95% of genus *Meloidogyne* (Hussey & Janssen, 2002). The rest of the species such as *M. chitwoodi, M. naasi, M. graminicola, M. fallax, and M. exigua* have restricted distribution and affect specific plant species (Moens *et al*., 2009). The three most common species of RKN, *M. incognita, M. javanica, and M. arenaria* are known worldwide and are restricted to the temperate regions to glasshouses. They also reproduce on many monocotyledonous and dicotyledonous plants. *M. hapla* is the other major species that has been found worldwide from temperate regions and in tropical and subtropical regions at higher altitudes (Moens *et al*., 2009). This species reproduces in many dicotyledonous host plants including food crops and ornamentals. In the presence of RKN in any field, the main functions of plant roots, water, and nutrients absorptions, are seriously hampered leading to decreased rate of photosynthesis in leaves. RKNs also manipulate plant processes such as translocation of photosynthates from shoots to roots to support nematode development and reproduction (Moens *et al*., 2009). This compromises the health of the plant increasing its susceptibility to attack by other pathogens such as fungi and bacteria.

2.6 Damage symptoms of RKNs

Root knot nematodes damage to plants may not be recognized from above ground symptoms since the parasites inhabit the rhizosphere. The damage they cause to plant is through penetration of root tissues and the subsequent movement in the roots. During this penetration process the RKN inflicts physical damage on the root surface and tissue by use of the stylet thrusting and release of cellulytic and pectolytic enzymes that breakdown the root tissues (Jones *et al*., 2013). Upon penetration into the root from the tip, the J2 migrates intercellularly to the cortex in the cell differentiation region and move into cell elongation site thus causing damage of the cells. The impact of the damage is the

interruptions of water uptake and nutrients by the plants. This is characterized by both above- and below -ground symptoms that are produced on any infected plants having a damaged and a malfunctioned root system. The symptoms include suppressed shoot growth and deformed root, chlorosis on leaves due to nutrients deficiencies, wilting during mild water shortage and even when there is adequate availability of moisture (Jones *et al*., 2013; Khanzada *et al*., 2012). In addition to the direct damaged to plants, some of the nematodes transmit plant viruses (tobraviruses and nepoviruses) and/or renders the plants to be more vulnerable to attack by other pathogens such as bacteria and fungi (MacFarlane & Robinson, 2010). All these effects on the root systems and the development of the plants lead to reduction and even total yield losses in crop production.

2.7 Management of RKNs

Control of RKNs is aimed at reducing nematode populations in soil and consequently limit the damage to levels that are economically acceptable and increase crop yields (Coyne *et al*., 2014). In this regard, several management strategies are in place and others are still under development. These include chemical control, cultural controls, biological control, and integrated management that combine either all or some of the existing methods.

2.7.1 Chemical control

Nematicides for controlling plant parasitic nematodes dates to 1950s and were grouped as soil fumigants or non-fumigants and systemic. However, persistent use has raised concerns on their effects to human health, animal health and the environment (Danchin *et al*., 2013; Taylor *et al*., 1978). As a result, effective nematicides such as DBCP (dibromochloropane), EDB (ethylene dibromide), which have been used as fumigants, have been withdrawn from the markets due to their possible effects on humans and the environment (Oka *et al*., 2000). Methyl bromide that was most effective and widely used for nematodes and other soil borne diseases and weeds has been banned from being used

and was completely withdrawn from the market in 2005. Following the ban on methyl bromide other non-fumigants nematicides such as aldicarb have been put under sharp focus after being detected in ground water (Oka *et al*., 2000). Nevertheless, nematicides continue to be part of integrated nematode management programs necessitating introduction of new strategies that are more efficient, ecologically sound, and safe (Adegbite & Adesiyan, 2005).

2.7.2 Cultural control

Cultural methods remain the most successful RKN control approach, as they are environmentally sustainable and friendly. The methods that are used in the fields include crop rotation where non-host crops are rotated with the host crops. For example, *M. hapla* infested vegetable field can be planted non host crop such as corn (Mitkowski *et al*., 2011). Use of cover crops is yet another strategy, where crops grow outside the agricultural season some of which are antagonistic to nematodes. Besides reducing nematode population, cover crops also have additional benefit as they stabilize the soil and improve its quality (Chitwood, 2002; Mitkowski *et al*., 2011). Other techniques that are used for nematodes control includes solarization and flooding of fields. Despite the obvious value in these approaches, the downside is that they require extensive planning and economic investment before successful implementation can be achieved which is not always the case. Moreover, RKNs are polyphagous thus complicating crop rotation as a management strategy.

2.7.3 Biological control

Biological control methods that have been put in place for control of nematodes includes use of pathogenic fungi that infect eggs, rhizobacteria, endophytic fungi and obligate parasitic bacteria (Lamovšek *et al*., 2013). Most of these microorganisms could control *Meloidogyne spp.* and *Heteroidera avenae* by exerting antagonistic actions through various mechanisms. Non- pathogenic bacteria control nematodes by inducing plant resistance, degrading signaling compounds to which nematodes are attracted to or by

colonizing the roots thus blocking the penetration of the J2s (Lamovšek *et al*., 2013). Bacteria, *Telluria chitinolytica* and *Bacillus cereus* have potential as biocontrol agents of *M. javanica*. The bacteria, *T. chitinolytica* colonize root surfaces and these feature enables the bacteria to interrupt the host recognition process of nematodes and to decompose the nematodes eggs (Oka *et al*., 2000). *B. cereus* culture filtrate has been shown to have nematicidal effect when incorporated with proteinaceous compounds such as ammonia and nitrite in the soil (Oka *et al*., 2000).

Fungal biocontrol agents such as *Trichoderma herzianum* have been used in screen houses for soil treatment in peat-bran formulation and effect reduced root galling caused by *M. javanica* (Sharon *et al*., 2001). Other funguses that have been used to control nematodes are *Pasteura penetrans* and *Verticillium clamidosporum*. Other types of biological control agents are the rhizospheric and endophytic fungi and bacteria, which may protect plants directly or indirectly rather than through parasitism of the nematodes by inducing resistance or inhabiting nematodes recognition sites. Although many bio-control agents of nematodes have been tested against nematodes, they have not led to the development of commercial products. But still they are part of an integrated nematode management program.

2.8 Host Plants - Root Knot Nematodes interactions

Generally, plant parasitic nematodes must locate a host plant before energy reserves are exhausted. For the parasites to successfully locate and penetrate the host, they rely on behavioral strategies facilitated by their elaborate nervous system and on special structures such as stylets for efficient root location, penetration and establishment of feeding site (Curtis, 2007). Root knot nematodes are no exception, as J2s must locate the hosts efficiently. Previous reports have shown that RKNs locate their host plants by chemotaxis where plant roots or microorganisms in the rhizosphere produce water soluble and gaseous attractants (Reynolds *et al*., 2011). The latter comprise highly volatile molecules that diffuse very fast in the soil and therefore mediate long-distance attraction that brings the nematodes to the general root area. The water soluble attractants on the

other hand, facilitate short distance attraction that allows the nematode to zero in on the actual root and with the help of other local cues it orients itself to the preferred site of invasion (Farnier *et al*., 2012; Reynolds *et al*., 2011; Spence *et al*., 2008). Subsequently, the J2s aggregate at the regions of apical meristem, cell elongation and near points of lateral roots. They enter roots behind the root cap and penetrations involves mechanical action of the stylet as well as enzymatic action through esophageal glands secretions (Caillaud *et al*., 2008; Sijmons *et al*., 1994). After penetration, the J2 migrate intercellularly to the region of cell elongation where they become sedentary and establish a mutual nutritional relationship with their host through inducement of feeding sites. In severe RKN infestation, plant above ground appear patchy, have stunted growth, lack of vigor, and develop chlorosis or other coloration on the foliage. The foliage also thins, leaves roll, and temporal wilting occurs in periods of water stress and high temperatures. When fertilizer and water is applied, the plants fail to respond normally and become vulnerable to diseases because of the RKN infections (Coyne *et al*., 2014).

2.9 Plant root exudates

The main function of plant roots is to provide anchorage to plants and a medium through which plants get nutrients, water and minerals for its growth and development. However, it has been noted that the root secretes thousands of different compounds into the rhizosphere whose functions are very diverse (Jaffuel *et al*., 2015). Root sexudations are part of rhizodeposition that is a major source of soil organic carbon compounds released by the plants. The amounts of the root exudates released into the soil depends on the age and species of the plants and the external factors such as biotic and abiotic stresses (Badri & Vivanco, 2009). The exudates contain ions, inorganic acids, oxygen, water, and mainly carbon-based compounds (Bais *et al*., 2006). The organic compounds are divided into two categories; the low molecular weight compounds which includes amino acids, organic acids, sugars and phenolics and high molecular weight compounds such as proteins, enzymes, sterols, lignin, glucosinolates and flavanols (Hage-Ahmed *et al*., 2013).

Root exudates mediate both positive and negative interactions in the rhizosphere. The

positive interactions include symbiotic associations with beneficial microbes such as mycorrhizae, rhizobia, plant growth promoting rhizobacteria (PGPR). The negative interactions include associations with pathogenic microbes, parasitic plants, and invertebrates' herbivores (Bais *et al*., 2006). Apart from supporting beneficial symbiotic associations, root exudates are also involved in an array of ecological functions that include changing the soil chemistry, increasing nutrient uptake, and even protecting the plants against metal toxicity (Bertin *et al*., 2003; Ogbemudia, and Thompson, 2014).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Plants materials

Two tomato cultivars ('Cal-J' and Moneymaker) widely cultivated in Kenya and three accession cultivars (AVRDC: VIO43619, VIO45764 and AVT01210) from The World Vegetable Center (courtesy of the late Dr. George Kariuki of Kenyatta University Nairobi Kenya) were used. 'Cal-J' and Moneymaker cultivars are among the high yielding cultivars in Kenya and mostly grown in the open field. 'Cal-J' tomato is grown mostly as for a processing while Moneymaker is for the fresh market.

Seeds of tomato 'Cal-J' and Moneymaker were purchased from Simlaw Seeds Company Nairobi, Kenya. The accession cultivars AVRDC: VIO43619, VIO45764 and AVT01210 were obtained from AVRDC - The World Vegetable Center. Seeds were sown in a rectangular plastic basin (67 cm × 40 cm × 5cm) (Kenpoly Manufactures Limited, Nairobi, Kenya), containing sterilized sand (autoclaved at 121 $^{\circ}$C for 40 min). They were placed in a screen house at the International Centre of Insect Physiology and Ecology (*icipe*) Duduville Campus, Nairobi, Kenya (1° 13' 18.96"S, 36° 53' 47.94"E) maintained at 23-± 2 $^{\circ}$C temperature, 60-70% relative humidity (RH) with 12 h:12 h light: dark photoperiod. Seedlings were transplanted into 2 L plastic pots (17 cm top diameter ×13 cm base diameter ×15 cm depth) containing a mixture of sterilized sand and loamy soil (2:1) and watered daily with nutrient solution as detailed below. Plants used for the experiments were 4-5 weeks old.

3.2 Preparation of nutrient solution.

A nutrient solution was prepared by mixing micro- and macro-nutrients (Lambert, 1992; Kihika *et al*., 2017). The stock solution contained autoclaved (121 °C) $Ca(NO_3)_2.4H_2O$, 653 g/L; $MgSO_4.7H_2O$, 399 g/L; KNO_3, 184 g/L and filter-sterilized (0.22 mm filters) $NH_4H_2PO_4$, 108 g/L; $FeSO_4.7H_2O$ 10 g plus 72 mL of 500 mM EDTA (pH 8.0) per liter

and micronutrients (per liter; $MnCl_2.4H_2O$, 1.81 g; $CuSO_4.5H_2O$, 0.1 g; $ZnSO_4.5H_2O$, 0.22 g; H_3BO_3, 2.86 g; $H_2MoO_4.H_2O$, 0.02 g). For watering plants, $Ca(NO_3)_2$, 25 mL; $MgSO_4$, 25 mL; KNO_3, 75 mL; $NH_4H_2PO_4$, 25 mL; Fe/EDTA, 25 mL and 25 mL, each of the micronutrients were mixed with distilled water and made to a final volume of 50 liters in a 50 L plastic container (Kenpoly Manufactures Limited).

3.3 Root knot nematode culture

The inoculum of *M. incognita* was obtained from tomato (*S. lycopersicum*) collected from Taita Taveta County (3.3161° S, 38.4850° E), Kenya, and maintained in pure cultures on tomato cultivar 'Cal-J' seedlings in pots containing sterilized sand under similar conditions described in section 3.1. Egg masses were extracted under a stereomicroscope at 20x magnification (Leica M125, Leica microsystems, USA) from galled tomato roots that were initially stained with Phloxine B dye (15 mg/L) for 20 minutes. Egg masses were subsequently placed in 6-well culture plates filled with 2 ml of distilled water each and placed in the dark at 27 ± 2 °C in the laboratory for 2 - 5 days to allow hatching and emergence of J2s. The freshly emerged J2s were picked with a plastic pipette dropper and transferred into a counting dish and the numbers determined under the stereomicroscope using a hand tally before being transferred into sterile 15 mL falcon tubes for use in the behavioral assays.

3.4 Collection of root exudates.

4-5-week-old tomato plants of each cultivar were brought to the laboratory from the screenhouse for collection of exudates. The plants were carefully uprooted from the soil and washed under running tap water, to remove the sand and soil debris, followed by rinsing twice with distilled water. To collect root exudates, 500 plants of each cultivar with cleaned roots were placed into 4 L rectangular containers (21 cm×14 cm×15 cm) filled with 1.5 L of distilled water. The containers were covered with aluminum foil up to the stems of the tomato plants to avoid contamination of the exudates from the leaves and photodegradation. Each tomato cultivar exudates were collected in three batches of

500 plants. Each batch treated as a replicate. Plants were maintained in the 4 L rectangular container at 23 ± 2°C for 48 hr., and the distilled water was replenished every 24 hr. Root exudates, extracted in the distilled water, were filtered using a Whatman No. 1 filter paper to remove particulate matter. Filtrates were freeze-dried in a benchtop freeze drier (VirTis AdVantage 2.0, SP scientific, U.S.A), weighed then divided into two equal portions for use in either bioassays or chemical analysis. Distilled water was used as negative control and Methyl salicylate (MeSA) used as a positive control. Methyl salicylate was chosen as positive control because it has been identified to be an root knot nematode attractive compound (Kihika *et al*., 2017; Murungi *et al*., 2018).

3.5 Bioactivity of root exudates.

To determine the effect of root exudate on RKNs, a stock solution of 5 µg/µL of the freeze-dried root exudate for each of the cultivar was prepared in distilled water and serially diluted to obtain three concentrations of 1.25, 2.5 and 5 µg/µL. Distilled water and methyl salicylate (MeSA) (100 ng/µL) (Kihika *et al*., 2017) served as the negative and positive controls, respectively. The bioactivity of the respective exudates and the controls were tested on freshly emerged J2s in two different experiments.

3.5.1 Stylet Thrusting Bioassay

Stylet is a hardened hollow protractible spear-like structure at the head of nematode (Jones *et al*., 2013). The nematode uses it to puncture plant cells and suck food and nutrients from the plant cells. Nematodes use stylet to eject secretions from its salivary glands into and around plant cells. The secretions could be enzymes and or metabolites that assists the nematode in plant root invasion and parasitism (Singh *et al*., 2015)

To determine the response of J2s to the root exudates, the number of stylet thrusts/min were recorded when juveniles were in contact with different concentrations of the exudates and the positive and negative controls under the stereomicroscope. Prior to measuring stylet thrusting response, 20 µL of each exudates solution were pipetted into a

previously formed ring of 30 µL of 23% Pluronic gel (Williamson *et al.*, 2009) (Sigma-Aldrich, St Louis, MO) on a microscope glass slide (Figure 3.1). A 20 µL suspension that contained approximately 50 J2s were added into the ring (Dutta *et al*., 2012) A cover slip was placed to cover the ring and then slight pressure was applied to the cover slip to ensure an airtight fit and to aid the spread of the nematodes to contact the Pluronic gel. The set up was left for the nematodes to settle for 15 min and J2 behavior, the stylet thrusting, were observed and the number of thrusts/min recorded. The stylet thrusting response was observed on 15 J2s, chosen singly per slide (replicate) under a compound microscope (Leica DM 2500, Leica microsystems, IL) at 200x magnification. Three replicates per treatment were carried out each using fresh root exudate.

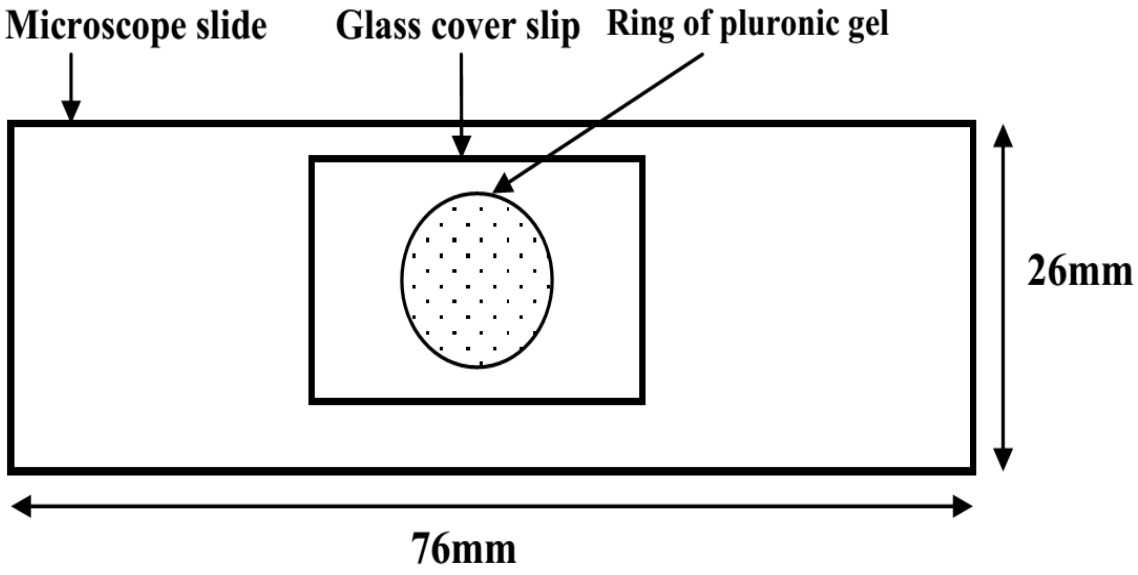

Figure 0.1: Schematic representation of a stylet thrust assay set up

3.5.2 Chemotaxis Bioassay

Nematode preference was tested on the different tomato cultivar root exudates in a dual choice sand assay (60 mm length x 14 mm diameter) divided into three sections (Figure 3.2). The respective sections (treatment and control) were filled with sterilized clean moist sand (Figure 3.2 A and 3.2 B, 5 g and 3.2 C, 2 g) mixed with the treatments, either

exudate or the positive control on the stimulus side, and distilled water on the control side. The exudate was tested at three concentrations; 1.25, 2.5, and 5 µg/µL. J2s (200), were introduced into the release point (3.2C). After 24 h, a modified Baermann extraction (Coyne *et al*., 2014) was used to recover J2s from the respective tubes. The number of J2s in each arm of the tube was counted under a stereomicroscope. Three replicates each comprising 200 nematodes were carried out per treatment.

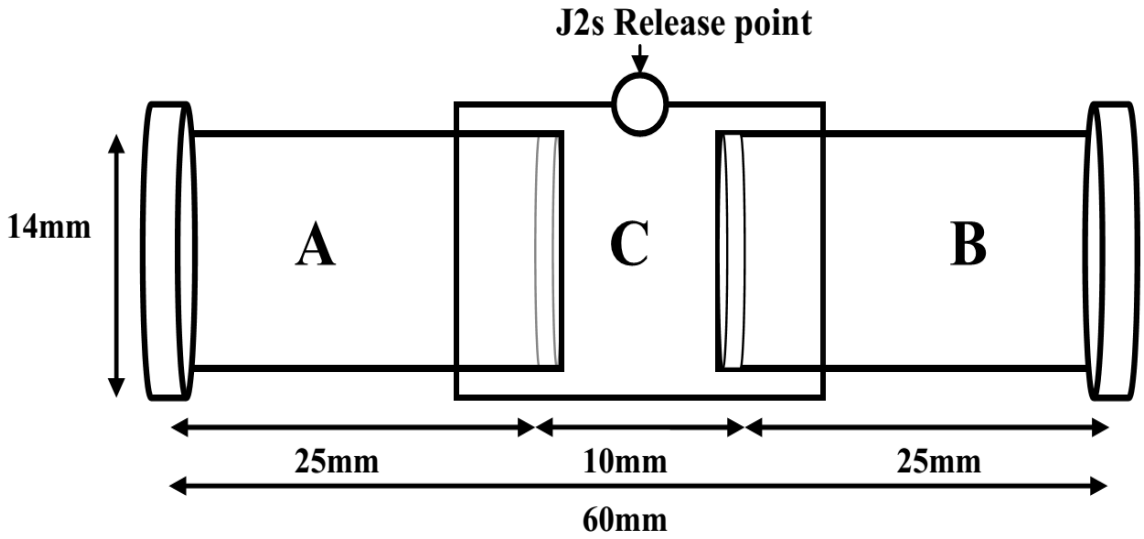

Figure 0.2: Schematic representation of a dual choice sand assay (A) stimulus tube, (B) control tube, and (C) connecting tube with hole for releasing second stage juveniles

3.6 Chemical analysis of root exudate

The freeze-dried crude exudate from each tomato cultivar, 1µg /µL was prepared in water: methanol (90:10, v/v), vortexed for 1 min, ultrasonicated for 5 min and centrifuged at 12,000 rpm for 5 min. The supernatant was then passed through Whatman 0.2 µm pore size syringe filters and analyzed on an analytical high-performance liquid chromatography (HPLC) system (Shimadzu Nexera X2 Series, Shimadzu, Kyoto, Japan) and ultra-performance liquid chromatography coupled with time-of-flight mass spectrometry (UPLC-QToF-MS). The UPLC was Waters ACQUITY I-class system (Waters Corp., Milford, MA, USA). The HPLC system was equipped with a prominence

SPD-M30A diode array detector (190-700 nm). The column oven was set at 30 °C with the following column parameters, 250 mm SPD-M3i.d., ACE 5 RP-18, column (Advance Chromatography Technologies, Aberdeen, Scotland). The mobile phase A (0.01% formic acid in water) and B (acetonitrile) used a low-pressure elution at a flow rate held constant at 1 ml/min and a total run time of 50 min. The following gradient program was employed at 0 min, 5% B; 0−10 min, 5−20% B; 10−15 min, 20% B; 15−23 min 20-70% B; 23-30 min, 70% B; 30−38 min 70-100% B; 38−45 min 100% B; 45− 48 min 100-5% B; 48-50 min 5% B with a total run time of 50 min.

The UPLC, ACQUITY I-class system (Waters Corp., Milford, MA, USA) fitted with ACQUITY UPLC BEH C_{18} column 2.1 x 100 mm, 1.7 µm particle size (Waters Corp., Wexford, Ireland), heated at 40 °C, and with an autosampler tray cooled at 5 °C, was used. The mobile phase included water (solvent A) and acetonitrile (solvent B) each containing 0.01% formic acid. The following gradient was used: 0-2.72 min, 5-20% B; 2.72-4.08 min, 20% B; 4.08-6.26 min, 20-70% B; 6.26-8.16 min, 70% B; 8.16 – 10.34 min, 70 - 100% B; 10.34 – 12.24 min, 100% B; 12.24 -13.06 min 100 - 5 % B; 13.06 – 13.88 min, 5% B. The flow rate was held constant at 0.3 ml min^{-1} for all the analyses. The injection volume was at 0.2 µl. The UPLC system was interfaced with electrospray ionization (ESI) to a Waters Synapt G2-Si QToF-MS operated in full scan MS^E in positive mode. Data was 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, sample 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 50-1,500 Da mass range using a 0.5 mM 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 20-ppm deviation and both odd- and even-electron states possible. The empirical formula generated was used to predict structures that were proposed based on online database (ChemSpider, Metlin), fragmentation pattern and literature. When authentic sample was available, co-injection was carried out to confirm identities of compounds (Cheseto *et al.*, 2017; Jared *et al*., 2015; Musundire *et al*., 2016).

Based on the results of bioactivity (section 4.1 and 4.2) and analysis of the exudates, 'Cal-J' root exudate was further fractionated, and the bioactivity of the fractions was tested. 'Cal- J' root exudates were fractionated into four fractions using HPLC method described above (fraction 1 (3-12 min), fraction 2 (12-23 min), fraction 3 (23-30 min) and fraction 4 (30-45 min). The fractions were concentrated in vacuo using a rotary evaporator to give 100 mg, 12 mg, 0.7 mg, and 0.5 mg of fractions 1, 2, 3 and 4, respectively. For each of the fractions, three concentrations were prepared and tested for their bioactivity on J2s following procedures described in section 3.5 on stylet thrusting and chemotaxis. The bioactive fractions were analyzed using methods described in section 3.6 (UPLC-QTOF-MS) and the identified compound tested for their bioactivity against nematodes.

3.7 Bioactivity of the identified compounds

Bioassays were carried out using five compounds identified after UPLC-QToF-MS analysis of the bioactive fractions and co-injections with authentic compounds (Figure 4.7). The compounds were a phytohormone zeatin, the flavonoids luteolin and quercetin and the alkaloids solasodine and tomatidine. The compounds were prepared in 2% Dimethyl sulfoxide (DMSO) to make stock solutions of 1000 ng/µL. The DMSO was used to enhance the solubility of the compounds in water. For the assays, solutions of the compounds were prepared by serially diluting four-fold the stock solution to yield the concentrations 250 ng/µL, 62.3 ng/µL, 15.6 ng/µL and 4 ng/µL. Bioactivity of each compound was tested on J2s in stylet thrusting and chemotaxis bioassays as described in section 3.5 in three replicates. Tomatidine was commercially obtained and tested as

tomatidine hydrochloride.

3.8 Chemicals

Analytical grade methanol (≥99.9%), acetonitrile (≥99.9%), formic acid (98-100%), water (LC-MS chromasolv). *trans*-zeatin, Luteolin (≥98%), Quercetin (≥95%), Solasodine (≥95%), Tomatidine hydrochloride (≥85%) Dimethyl sulfoxide (DMSO) (≥99.9%) and Methyl salicylate (MeSA) (≥99%), were sourced from Sigma-Aldrich (St Louis, MO, USA).

3.9 Statistical analysis

The number of stylet thrust per minute was log-transformed prior to analysis of variance to normalize the data and stabilize the variance. Mean separations were carried out using the Tukey's HSD test. The numbers of responding nematodes obtained from the chemotaxis assays were recorded as means of J2 that responded to the different treatments and expressed as percent response [(n/N) × 100]. N corresponds to the total number of responding J2, while n is the number of J2 corresponding to a given treatment. The data obtained from the chemotaxis assays was analyzed by Chi-square goodness of fit to assess attraction and/or avoidance of *M. incognita* to the different doses of tomato root exudates, fractions and the synthetic compounds tested individually compared to controls (distilled water and 2% DMSO) (Kihika e*t al.*, 2017) Non- respondents were not included in the analysis. All tests were performed at 5% significance level using R software version 3.2.3 (R Core Team, 2015).

CHAPTER FOUR

RESULTS

4.1 Bioactivity of root exudates of tomato cultivars

The results of the bioactivity of the root exudates are presented in two figures. Figure 4.1 showing response of RKNs J2s stylet to the 'Cal-J' and moneymaker tomato cultivar and AVRDC tomato accessions VI045764, VI043619 and AVT01210 root exudates. Figure 4.2 showing the behavioral response, chemotaxis, of RKNs J2s to 'Cal-J' and moneymaker tomato cultivar and AVRDC tomato accessions VI045764, VI043619 and AVT01210 root exudates.

The root exudate of the tomato cultivars induced stylet thrusts that were significantly different to the negative control (distilled water). 'Cal-J' ($F_{(4,220)}$ =508.3, P<0.001), Moneymaker ($F_{(4,220)}$ = 100.11, P<0.001), AVRDC_VI045764 ($F_{(4,220)}$ = 449.8, P<0.001), AVRDC_VI043619 ($F_{(4,220)}$ = 486, P<0.001), AVRDC_ATV01210 ($F_{(4,220)}$ = 772.7, P<0.001) (Figure 4.1). The rates of stylet thrusts elicited by the different concentrations within the cultivar root exudate were concentration dependent. The stylet thrusts induced by the cultivars at high concentrations (5.0µg/µl) were comparable to the positive control (MeSA) except that the stylet thrusts induced by 'Cal- J' root exudate at 2.5 and 5.0µg/µl were relatively higher than the positive control (Figure 4.1).

On the other hand, in chemotaxis assays, differential response of J2s of *M. incognita* to the concentrations of the tomato cultivars root exudates was observed. Root exudates of 'Cal-J' tomato was preferred in all the concentrations tested with significant preferences observed at 2.5 µg/µl, (77.2%, χ^2 = 26.1, df = 1, $P < 0.001$) and 5.0 µg/µl., (62.8%, χ^2 = 7.06, df = 1, $P < 0.01$) compared with the distilled water (Figure 4.2). Similarly, AVRDC_VI045764 had significant preference at 2.5 µg/µl, (67.5%, χ^2 = 5.9, df = 1, $P <$ 0.05). There was no significant preference for root exudates of moneymaker, AVRDC_VI043619 and AVRDC_ATV01210 for all the concentrations tested (Figure

4.2).

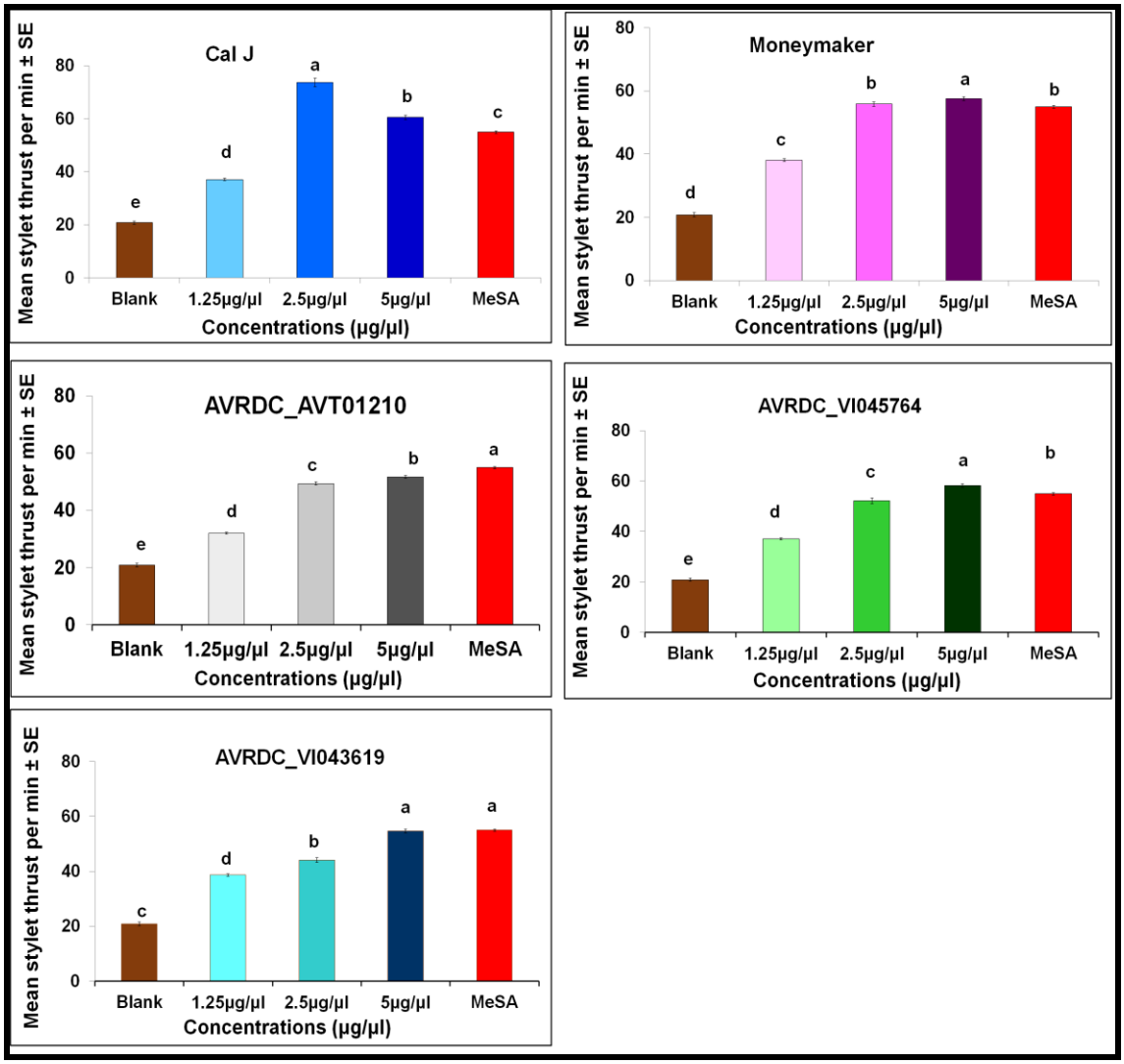

Figure 0.1: Response of *M. incognita* J2s to different concentrations of 'Cal-J' and Moneymaker tomato cultivars and AVRDC tomato accessions root exudates. The bars show the mean stylet thrusting/minute elicited by different concentrations of the exudate. Bars capped with different letters are significantly different between the root exudate concentrations (Tukey HSD, P < 0.05).

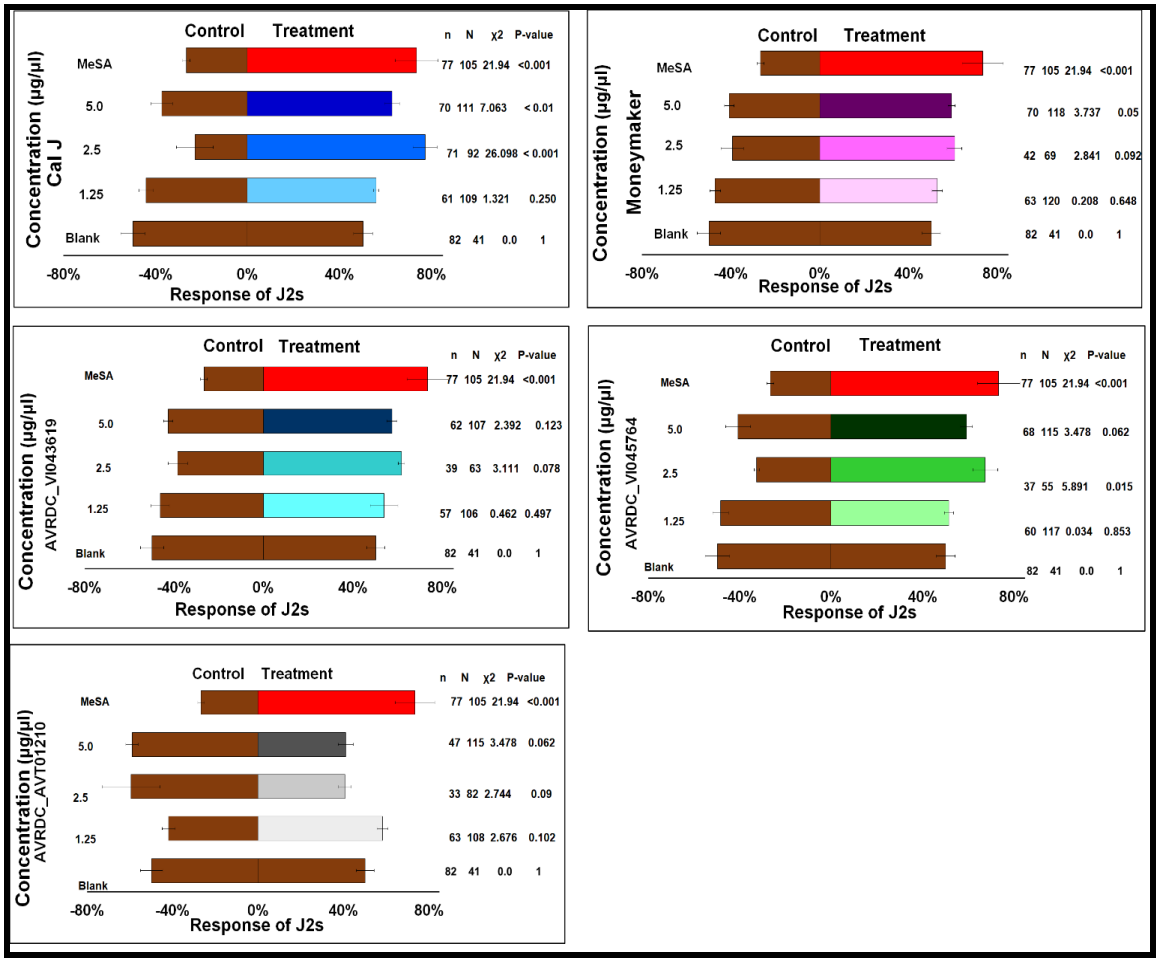

Figure 0.2: Response of J2s to different concentrations of 'Cal-J' and Moneymaker tomato cultivars and AVRDC tomato accessions root exudates compared to controls. N corresponds to the total mean of responding J2s while n is the number of J2s corresponding to a given treatment. Control = distilled water, MeSA = methyl salicylate.

4.2 Chemical profiling and analysis of the tomato root exudates

Profiling of the root exudates of the different tomato cultivars and the tomato accessions using UPLC-QTOF-MS, showed a similarity in their chemical composition (Figure 4.3).

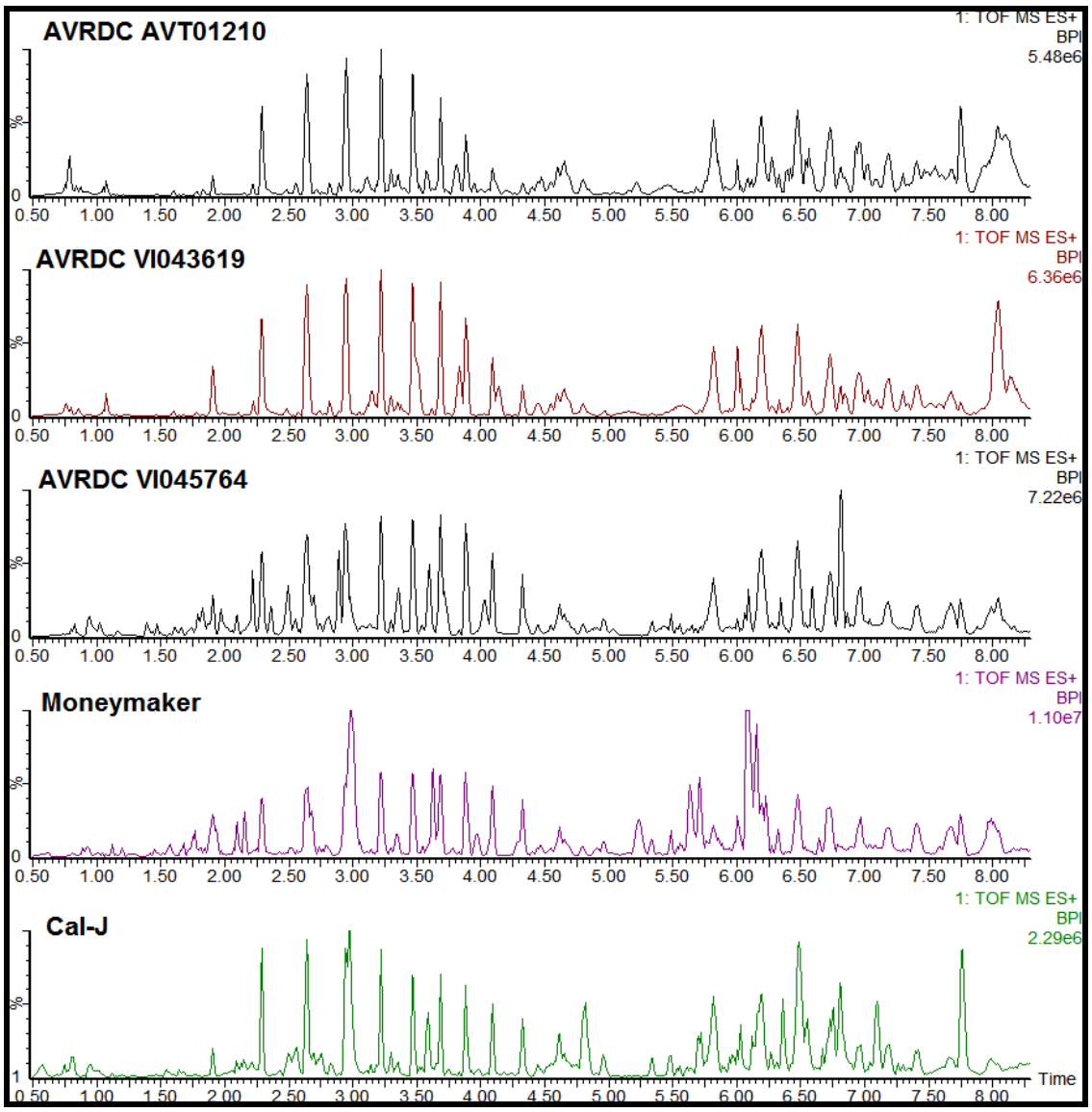

Figure 0.3: Liquid chromatography coupled with quadrupole time of flight mass spectrometry (UPLC-QToF-MS) overlay profile of different tomato cultivar root exudates.

Based on the behavioral assays using the crude root exudates and similarity in chemical profiles (Figure 4.1, 4.2 and 4.3), 'Cal-J' root exudates were further fractionated using HPLC and the bioactivity of the fractions tested (Figure 4.4).

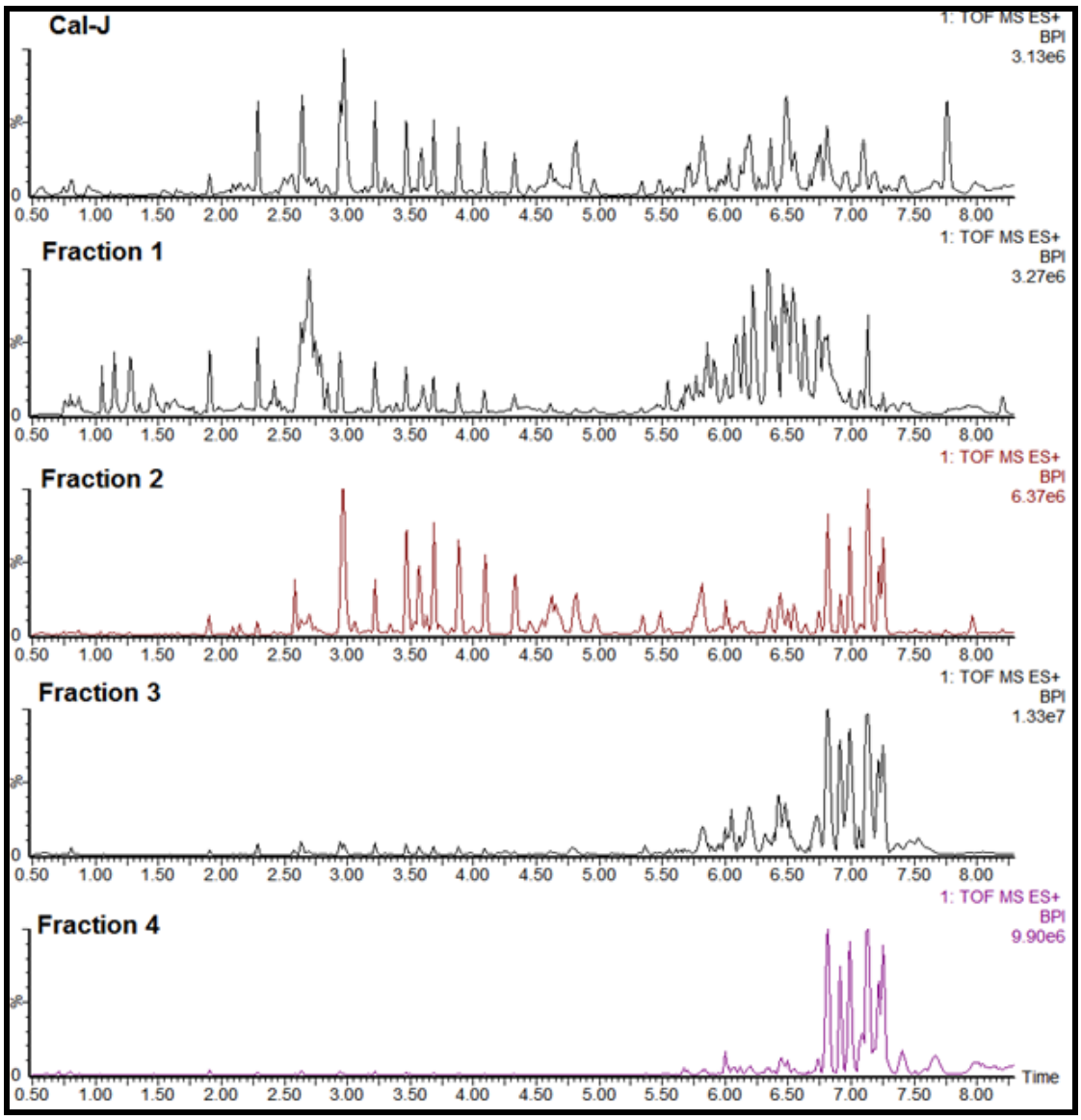

Figure 0.4: Liquid chromatography coupled with quadruple time-of-flight mass spectrometry (UPLC-QToF-MS) profile of 'Cal-J' tomato root exudate and fractions

4.3 Bioactivity of 'Cal-J' root exudate fractions

The fractions (Figure 4.4) obtained by chromatography of the root exudate elicited significantly higher and concentration-dependent stylet thrusting in J2s than the negative control (fraction 1: $F_{(4,\ 220)} = 783.6$, $P < 0.001$; fraction 2: $F_{(4,220)} = 637.2$, $P < 0.001$

fraction 3: $F_{(4,220)} = 512.6$, $P < 0.001$; fraction 4: $F_{(4,220)} = 698.3$, $P < 0.001$) (Figure 4.5). Whereas stylet thrusting responses elicited by fractions 1 and 2 compared favorably with the positive control, they were lower for fractions 3 and 4 (Figure 4.5). These results indicate that all the individual fractions contained compounds which may be necessary for J2 host detection; however, the more potent components appeared to be present in fractions 1 and 2. UPLC-QToF-MS analysis identified fractions 1 and 2 as the more polar fractions, suggesting J2 utilization of polar compounds for host seeking.

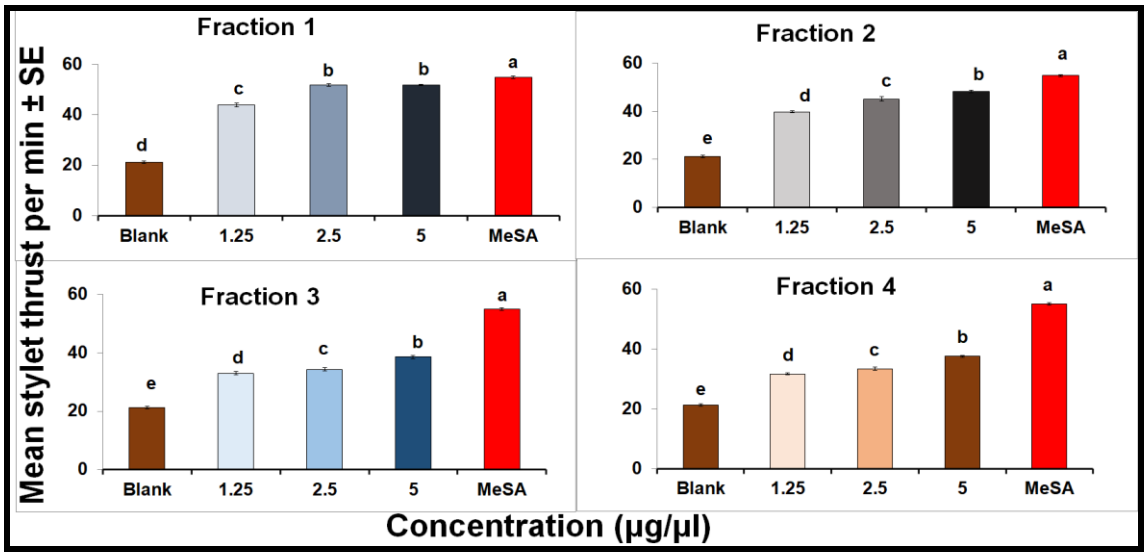

Figure 0.5: Response of *M. incognita* J2s to different concentrations of 'Cal-J' tomato root exudate fractions. The bars show the mean stylet thrusting/minute elicited by different concentrations of the exudate fractions. Bars capped with different letters are significantly different; those with similar letters are not significantly different between the concentrations of each fraction (Tukey HSD, P < 0.05)

The results of the chemotaxis assays confirmed this observation, whereby differential significant concentration-dependent responses were recorded for fraction 1 (2.5 µg/µL, 64.1%, $\chi^2 = 8.43$, df = 1, $P < 0.01$) and fraction 2 (5 µg/µL, 70.7, $\chi^2 = 17.12$, df = 1, $P < 0.001$). (Figure 4.6). Fractions 3 and 4 on the contrary elicited chemotaxis responses which were not significantly different from that elicited by the negative control, except

at the concentration of 1.25 µg/µL for fraction 4 (59.9%, $\chi^2 = 5.88$, df = 1, $P < 0.05$) (Figure 4.6).

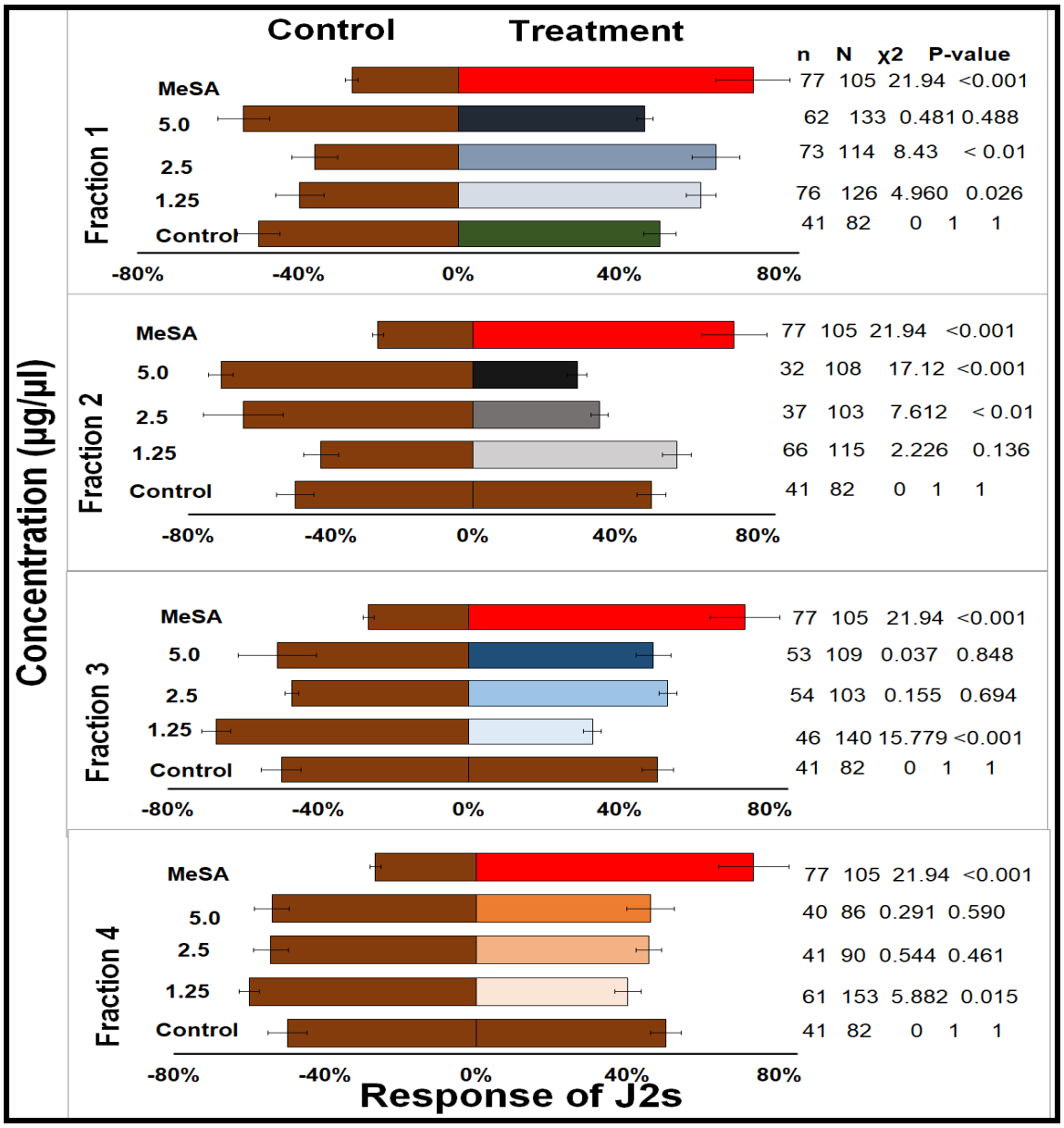

Figure 0.6: Response of *M. incognita* J2s to different concentrations of 'Cal J' tomato root exudate fractions compared to controls. N corresponds to the total mean of respondinJ2s while n is the number of J2s corresponding to a given treatment. Control = distilled water, MeSA = methyl salicylate.

4.4 UPLC-QToF-MS identification of compounds in bioactive fractions

Chemical analysis of the bioactive fractions using UPLC-QToF-MS identified a complex blend of polar compounds in fractions 1 and 2 (Figure 4.7). Among these, five compounds, including the phytohormone zeatin (cytokinin), flavonoids quercetin and luteolin and alkaloids tomatidine and solasodine present in fractions 1 and 2 (Table 4.1), were identified based on retention time (RT), mass fragmentation and, confirmed with authentic standards by co-injections (Figure 4.7). In addition, tomatine was tentatively identified based on mass fragmentation pattern and literature data. Zeatin (**1**) eluted at RT 1.97 min and had a molecular ion peak $[M+H]^+$ at *m/z* 220.1558 with two major fragment ions at *m/z* 202.1425 $[M - H_2O]^+$ and 136.0877, a characteristic adenine derivative ion $C_5H_5N_5^+$ (Imbault *et al*., 1993) (Figure 4.7). Tomatine (**2**) eluted at RT 5.54 min and had a molecular ion $[M+H]^+$ peak at *m/z* 1034.5531, with the aglycone tomatidine fragment at *m/z* 416.3516 and fragment ions at *m/z* 902.5118, 740.4571 and 578.4064 that could be due to consecutive losses of a xylose and two glucose moieties (Cataldi *et al*., 2005; Distl & Wink, 2009; Jared *et al*., 2015) (Figure 4.7). Luteolin (**3**) eluted at RT 6.02 min and was identified based on a molecular ion $[M+H]^+$ peak at *m/z* 287.1150, with key characteristic fragment ions at *m/z* 153.0175 and *m/z* 135.0436, in addition to *m/z* 269.0483, *m/z* 257.0455 and *m/z* 213.0543, corresponding to dehydration of product ion to $[M+H- H2O]^+$, followed by two sequential losses of CO: $[M+H-H_2O-CO]^+$ and $[M+H-H_2O- 2CO]^+$ respectively (Tsimogiannis *et al*., 2007) (Figure 4.7). Quercetin (**4**) eluted at RT 6.05 min and was identified baseds on a molecular ion $[M+H]^+$ peak at *m/z* 303.0529, with characteristic fragment ions at *m/z* 165.0177, *m/z* 153.0169 and *m/z* 137.0230 that were in tandem with authentic standard fragment ions (Figure 4.7). In addition, fragment ions corresponding to a loss of H_2O and 2CO were detected at *m/z* 285.0423 and *m/z* 229.0515 respectively (Tsimogiannis *et al*., 2007) (Table 4.1). Tomatidine (**5**) eluted at RT 6.32 min and was identified based on a molecular ion $[M+H]^+$ peak at *m/z* 416.4434, and a fragment ion $[M - H_2O]^+$ peak at *m/z* 398.4297 (Caprioli, Cahill, Logrippo, & James, 2015) (Figure 4.7) Solasodine (**6**) eluted at RT 6.33 min and was identified based on a molecular ion $[M+H]^+$ peak at *m/z*

414.4323, with a fragment ion $[M - H_2O]^+$ peak at *m/z* 396.4179 (Table 4.1) (Cahill et al., 2010). Additional compounds were tentatively identified based on mass spectra and diagnostic fragment ions: glucoside derivative of caffeic acid eluting at RT 1.57 min with a molecular ion $[M+H]^+$ peak at *m/z* 342.1393, and *m/z* 180.0872 corresponding to loss of a sugar moiety, and at *m/z* 163.0228, representing loss of H_2O; quercetin glucoside eluting at RT 2.75 min, with a molecular ion $[M+H]^+$ peak at *m/z* 465.1898, and *m/z* 303.1298 (quercetin aglycone) due to loss of a sugar moiety; and luteolin glucoside at RT 3.12 min, with a molecular ion $[M+H]^+$ peak at *m/z* 449.1610, and *m/z* 287.1051 (luteolin aglycone) due to loss of sugar moiety (Fridén & Sjöberg, 2014) (Figure 4.7).

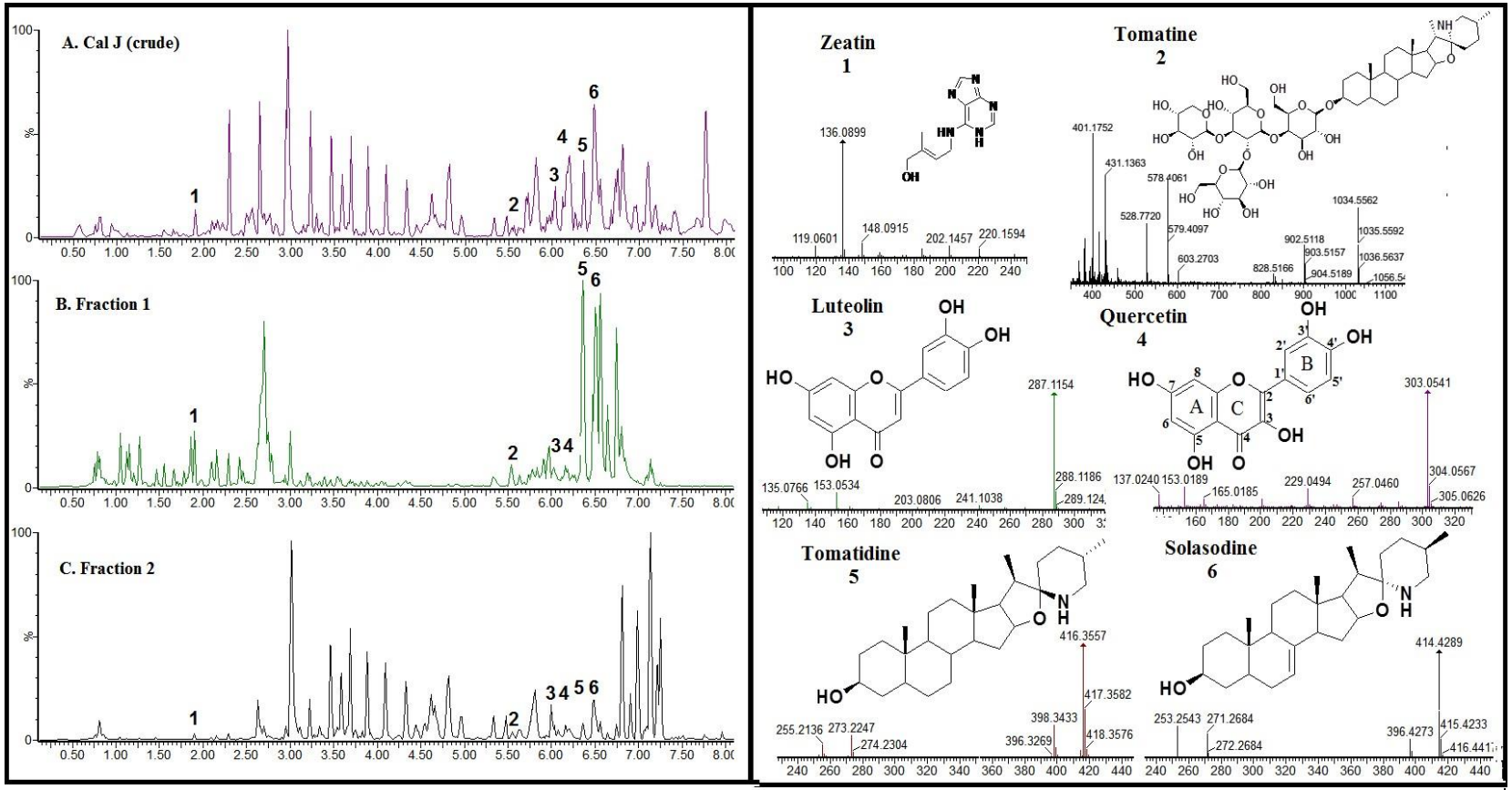

Figure 0.7: UPLC-QToF-MS chromatogram of (A) 'Cal-J' root exudate, (B) fraction 1 and (C) fraction 2 with identified compounds (1, 2, 3, 4, 5 and 6)

Table 0-1: Identified compounds in 'Cal J' tomato root exudate fractions

RT (min)	compound name	molecular formula	$[M+H]^+$	fragment ions (*m/z*)
1.57[b]	caffeic acid glycoside	$C_{15}H_{18}O_9$	342.1393	180.0872, 163.0228
1.97[a]	zeatin	$C_{10}H_{14}N_5O$	220.1558	202.1425, 136.0877
2.75[b]	Quercetin glycoside	$C_{21}H_{20}O_{12}$	465.1898	303.1298
3.12[b]	luteolin glycoside	$C_{21}H_{20}O_{11}$	449.1610	287.1051
5.54[b]	tomatine	$C_{50}H_{83}NO_{21}$	1034.5531	902.5118, 740.4575, 578.4064, 528.7714, 416.3516, 383.1640
6.02[a]	luteolin	$C_{15}H_{11}O_6$	287.1150	269.0483, 213.0543, 153.0175, 135.0436
6.05[a]	quercetin	$C_{15}H_{11}O_7$	303.0529	285.0423, 229.0515, 165.0177, 153.0169, 137.0230
6.32[a]	solasodine	$C_{27}H_{43}NO_2$	414.4289	396.4179,271.2716, 253.2582
6.33[a]	tomatidine	$C_{27}H_{45}NO_2$	416.3557	398.3433, 273.2247, 255.2136

[a]Compound identities confirmed with authentic standards. [b]Compounds tentatively identified.

4.5 Bioactivity of the synthetic compounds identified in fractions 1 and 2 of Cal J tomato root exudates

The five compounds identified and confirmed by co-injections with pure standards (Figure 4.7) elicited significantly higher stylet thrusting than the negative control (2% DMSO), with the rate increasing as the concentration of the compounds increased

(zeatin (**1**); $F_{(6, 308)} = 546.2$, $P < 0.001$, luteolin **(3)**; $F_{(6, 308)} = 585.2$, $P < 0.001$, quercetin (**4**); $F_{(6, 308)} = 633.2$, $P < 0.001$, tomatidine (**5**); $F_{(6, 308)} = 469.8$, $P < 0.001$) and solasodine (**6**); $F_{(6, 308)} = 446.4$, $P < 0.001$. However, at all the concentrations tested, none of the compounds elicited a stylet thrusting response that was higher than the positive control (Figure 4.8).

In chemotaxis assays, the phytohormone zeatin (**1**), was attractive to J2s at all the concentrations tested, with significant attraction observed at 62.5 ng/µL (71.9%, $\chi^2 = 16.84$, df = 1, $P < 0.001$) (Figure 4.9). The flavonoids luteolin (**3**) was less preferred by J2s, with significant deterrence at 1000 ng/µL (67.1%, $\chi^2 = 13.22$, df = 1, $P < 0.001$). Quercetin (**4**) on the other hand, was significantly attractive at 15.6 ng/µL (66.6%, $\chi^2 = 12.47$, df = 1, $P < 0.001$) and 62.5 ng/µL (65.4%, $\chi^2 = 11.22$, df = 1, $P < 0.001$), however, increasing its concentration to 1000 ng/µL, significantly deterred J2 responses (62.0%, $\chi^2 = 7.37$, df = 1, $P < 0.01$) (Figure 4.9). Although alkaloids, tomatidine (**5**) and solasodine (**6**) induced significant stylet thrusting responses compared to the negative control, they elicited no significant attraction in J2s of *M. incognita* at all the concentrations tested, except at the highest concentration, 1000 ng/µL, of tomatidine (**5**) where significant deterrence was observed (59.8%, $\chi^2 = 2.82$, df = 1, $P < 0.05$) (Figure 4.9).

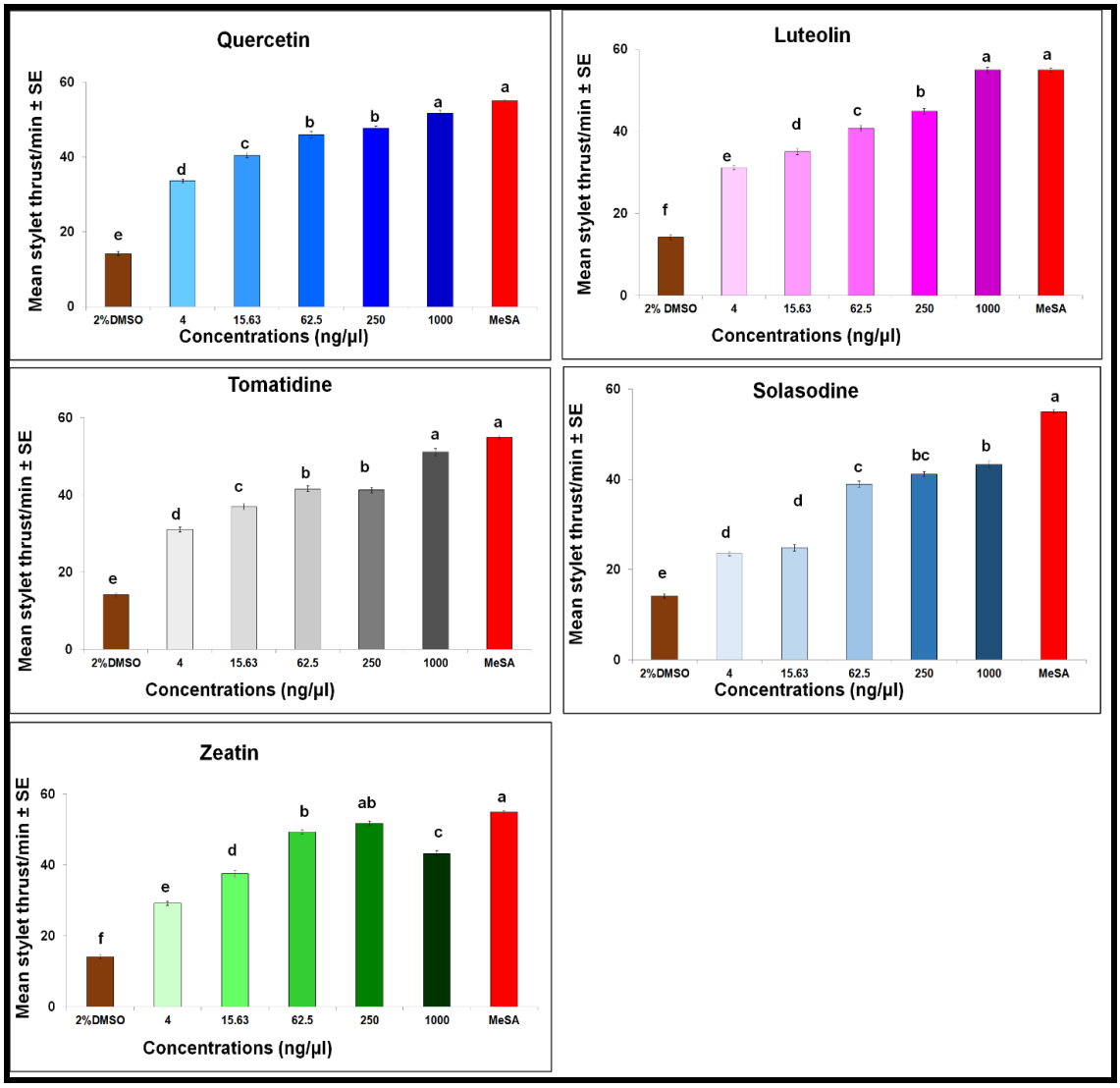

Figure 0.8: Response of *M. incognita* J2s to identified compounds; quercetin, luteolin, tomatidine, solasodine and zeatin. The bars show the mean stylet thrusting/minute elicited by different concentrations of the identified compounds. Bars capped with different letters are significantly different, those with similar letters are not significantly different between the concentrations (Tukey HSD, P < 0.05).

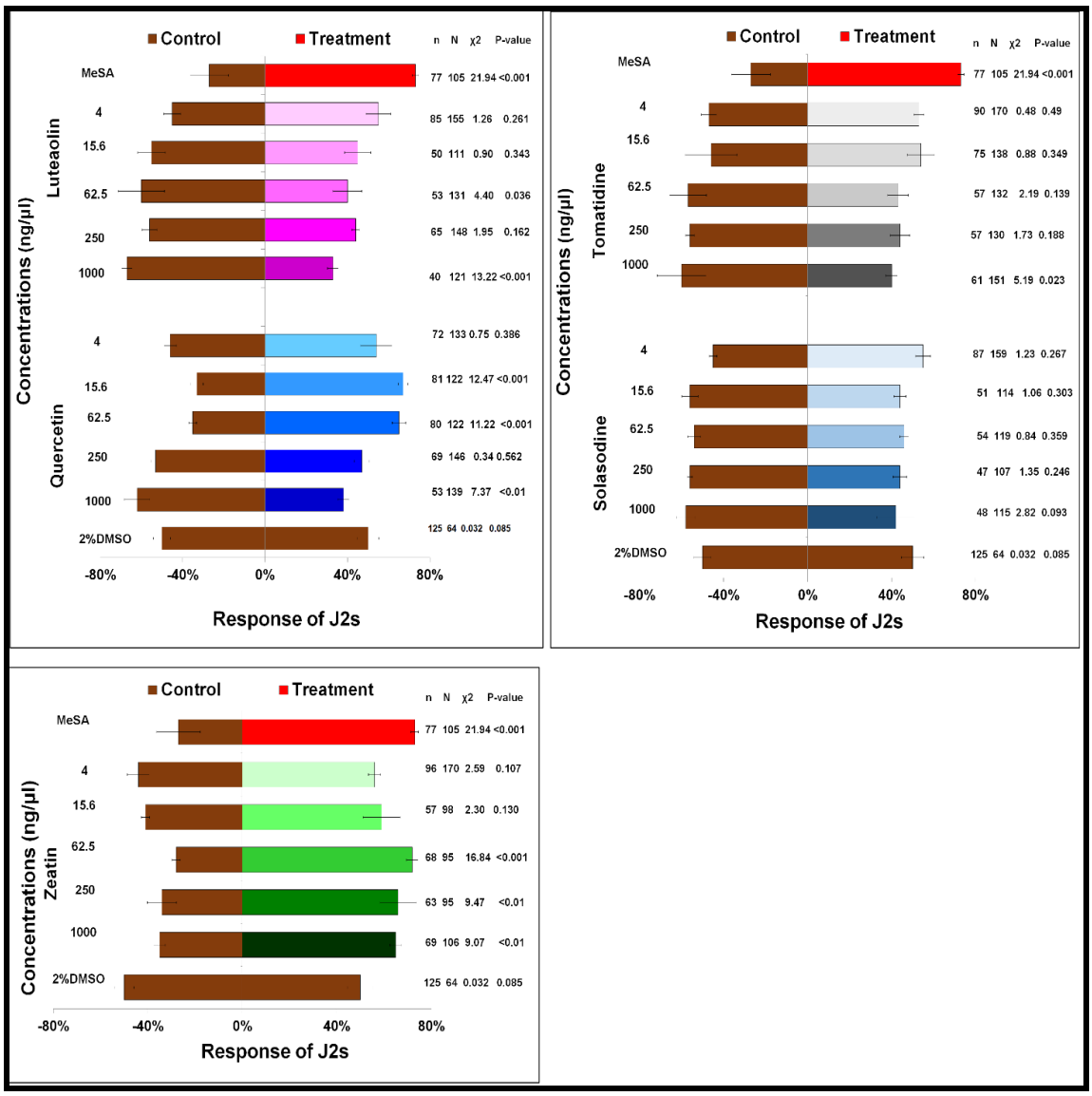

Figure 0.9: Response of M. incognita J2s to identified compounds; quercetin, luteolin, tomatidine, solasodine and zeatin compared to controls. N corresponds to the total mean of responding J2s while is the number of J2s corresponding to a given treatment. DMSO = dimethyl sulfoxide, MeSA = methyl salicylate.

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

Studies on root knot nematode-plant interactions shows that chemical components released by the roots of host plants have differential effects on nematodes. The chemicals may act as deterrents or attractants of nematodes. Some are egg hatching factors stimulants or hatching inhibitors. Others may have nematicidal effects thus effecting mobility of the nematodes (Jada *et al*., 2013; Wuyts *et al*., 2006). In this study, the effects of tomato root exudates on the behavior of RKNs is demonstrated. The different concentrations of the tomato root exudates elicited significant stylet thrusting of the *M. incognita*, compared to the negative control (distilled water). These results concur with a previous study which found that small lipophilic molecules in tomato and rice root exudates, and the water-soluble neurotransmitter, resorcinol, induced stylet thrusting in *M. graminicola* and *M. incognita* (Dutta *et al*., 2012). It also indicates that specific components or blends of these components in the root exudate influence *M. incognita* J2s.

On the other hand, chemotaxis assays showed that different tomato root exudates have different attraction ability to *M. incognita* J2s. Comparison of the response of J2s to the different tomato cultivars showed that 'Cal J' was significantly preferred suggesting that it could have components that make it attractive to nematodes. Linking both behavioral assays, it is observed that tomato root exudates might have similar components that elicit stylet thrusting on *M. incognita* J2s. Profiling the root exudates using UPLC-QToF/MS showed that tomato root exudates have similar chemical components, but the differences might be the ratios released by the individual cultivar. These results indicate that host finding in J2s is complex and may involve semiochemicals derived from different classes detected at specific concentrations or ratios. This may explain why the 'Cal-J'

root exudates elicited significant stylet thrusting and were highly preferred by the *M. incognita* J2s. On further analysis of the 'Cal-J' root exudate, fractionation was carried out and response of *M incognita* J2s to the fractions tested.

The response of *M incognita* J2s on the fractions indicated that, the fractions contained compounds, which elicited stylet thrusting as the crude ('Cal J' exudates) but at lower rates. In chemotaxis assay, *M. incognita* J2s showed significant preference to fraction 1 and fraction 2 and no significant difference in fraction 3 and 4. These results indicate that the exudate fractions contained components that may be necessary for J2 host detection; however, the more potent components appeared to be present in fractions 1 and 2 since they elicited stylet thrusting and also were preferred in the chemotaxis assay. During fractionation of the 'Cal-J' root exudate, fractions 1 and 2 were categorized as more polar components than fractions 3 and 4 suggesting that J2s may utilize polar components of the exudates for host plant detection and location. This result also suggests that semiochemicals derived from different classes of compounds detected at specific concentrations or ratios might be used as cues in host finding process by the J2s (Najar-Rodriguez *et al*., 2011).

Using UPLC-QTOF-MS, different classes of compounds were identified in the bioactive fractions, by co-injections with pure compounds. *Meloidogyne incognita* J2s were found to elicit differential responses to a phytohormone, flavonoids and alkaloids from the bioactive fractions. The phytohormone zeatin elicited stylet thrusting and was preferred by the J2s. These results indicate that zeatin (**1**) may play an important role as a host finding signal in *M. incognita*, and because it is a phytohormone, suggests its presence may serve as an indication of a healthy plant and availability of resources. Previous studies have shown that when zeatin (**1**) is exogenously supplied to resistant tomato plants it increased their susceptibility to *M. incognita* (Akhkha *et al*., 2002; Dropkin *et al*., 1969).

Additionally, it has been reported that phytohormones are not only responsible for plant

development processes, but that they also mediate plant-microbe interactions (Bais *et al*., 2006; Chanclud & Morel, 2016; Favery *et al*., 2016; Nahar *et al*., 2013). The cytokinin zeatin (**1**), has been identified in the secretions of *M. incognita* and the beet cyst nematode *Heterodera schachtii*, suggesting that nematodes not only biosynthesize certain phytohormones, but may use them to prime host plants to facilitate penetration and possibly avoid detection during root invasion. In addition, phytohormones could function as site induction molecules during the initial stages of J2 root invasion (De Meutter *et al*., 2003; Vanholme *et al*., 2004). Moreover, there is evidence that cytokinins play a role in cell cycle activation and formation of feeding cells of *M. incognita* and *H. schachtii* (Dowd *et al*., 2017).

Furthermore, the phytohormone auxin has been shown to work synergistically with kinetin, another cytokinin, and other phytohormones in stimulating J2 root penetration and subsequent development (Dropkin *et al*., 1969; Fridén & Sjöberg, 2014). The flavonoids identified and their bioactivity tested in the current study differentially influenced J2 responses in the chemotaxis assay. Although luteolin (**3**) and quercetin (**4**) are structurally similar, the presence of the additional hydroxyl group at the C-3 position on the C ring in quercetin could account for the differential attractiveness (Kumar & Pandey, 2013). The additional hydroxyl group increases the polarity of the molecule, which would make it more soluble in water and possibly facilitate binding to more receptor sites through hydrogen bonding with chemosensory receptors of J2s. These results suggest that host plants of RKNs that release high concentrations of luteolin (**3**) and quercetin (**4**) beyond a certain threshold concentration in their root exudates would be less preferred for host invasion by J2s. In a previous study, tests with the structurally similar flavonoids patuletin, which has a methoxy group at C-6, and quercetin (**4**) identified in extracts of *Tagetes patula,* demonstrated a higher nematicidal activity for patuletin than quercetin (**4**), which was attributed to the presence of a methoxy group in patuletin (Faizi *et al*., 2011). Other authors have shown that the presence of the hydroxyl group at position 3 in the C ring is important in flavonoids for antibacterial activity

(Johnson *et al*., 2011; Wu *et al*., 2013). Furthermore, a study testing the effect of apigenin and its derivatives on larval growth of *C. elegans*, found that positioning and number of hydroxyl groups in the molecule determine the effect of the compound on larval growth (Yoon *et al*., 2006). Conversely, salicylic acid, a polar compound from mint extract had nematicidal activity against *M. incognita.* Additionally, reactive carbonyl species in carvone and pulegone, non-polar compounds, could also have contributed to their nematicidal activity against *M. incognita* (Caboni *et al*., 2013). These findings suggest it would be interesting to investigate structure-activity relationships in root exudate metabolites that influence J2 chemosensory detection and host finding behavior.

Several flavonoids have been shown to play a role in the ecology of various organisms. Their allelopathic effect and influence in plant-microbe interactions in stimulating rhizobial nod gene expressions and chemo-attraction of rhizobia towards plant roots, inhibition of root pathogens, and promotion of mycorrhizal spore germination and hyphal branching is well documented (Hassan & Mathesius, 2012; Mierziak *et al*., 2014; Sugiyama & Yazaki, 2014). Flavonoids are also known to regulate plant growth through their effects on auxin transport and localization in nodules as well as root galls formation (Wasson *et al*., 2009). In the current study, the differential and concentration-dependent responses of J2s to flavonoids corroborate other studies which showed that at different concentrations they could elicit different responses; repellents, and as mobility and hatching inhibitors for specific nematode species (Wuyts, 2006). Furthermore, the flavonoids kaempferol, quercetin (**4**) and myricetin have been shown to act as deterrents against *Rodopholus similis* and *M. incognita,* while genistein, daidzein and luteolin (**3**) reduced mobility interactioin in *R. similis* (Mierziak *et al*., 2014; Wuyts *et al*., 2006). Quercetin (**4**) was also found to inhibit the enzyme, P13-kinase, which blocks phytohormones on the nematode cuticle and hinder reproduction of *Meloidogyne javanica* (Dutta *et al*., 2012;Wuyts *et al*., 2006). Luteolin (**3**) has also been associated with inducing nod factors and to serve as chemo-attractants for rhizobium species in the

rhizosphere (Weston & Mathesius, 2013). Collectively, these findings show that flavonoids are not only important for plant defense, but they also mediate plant - microbe interactions including interactions with root knot nematodes.

Alkaloids on the other hand, are secondary metabolites that plants use primarily for defense against microbial infections and herbivore attack (Wuyts *et al*., 2006). In our study, tomatidine (**5**) and solasodine (**6**) induced significant stylet thrusting responses compared to the negative control, indicating that they stimulated the chemosensory receptors of J2s. However, they elicited no significant attraction in J2s of *M. incognita* at all the concentrations tested, except at the highest concentration, 1000 ng/µL, of tomatidine (**5**) where significant deterrence was observed. The structural similarity between tomatidine (**5**) and solasodine (**6**), with the only difference being a cyclic alkene, may account for the similarity in bioactivity of the two compounds. On the other hand, tomatidine (**5**), tested as its hydrochloride derivative was deterrent, possibly due to the presence of the hydrochloride moiety in the molecule. These results indicate that more experiments are required to determine how J2s of *M. incognita* respond to different alkaloids and their derivatives and unidentified components in fractions 1 and 2. It is possible that alkaloids may have other roles other than host signaling in J2s. For instance, alkaloids identified in the root exudate of *Ficus sycomorus* increased egg hatching of *M. javanica* but also had nematicidal effects on the J2s (Jada *et al.*, 2013). In addition, pyrrolizidine alkaloid containing plants, have been shown to suppress development of *M. hapla*, while the isoquinoline alkaloids from *Macleaya cordata*, sanguinarine, chelerytherine and allocryptopine, have been found to have nematicidal activity against *M. incognita, C. elegans and Bursaphelenchus xylophilus* (Kui *et al*., 2012). Furthermore, two 4-quinoline alkaloids isolated from aerial part of *Triumfetta grandidens*, waltherione A and waltherione E were also found to have nematicidal activity against *M. incognita* (Jang *et al.*, 2015). All these findings including the results of the present study, lend support to the fact that alkaloids may not support J2 host plant finding but rather play a role in plant defense against nematode attack (Thoden *et al*., 2009).

5.2 Conclusions

In conclusion, this study shows that root exudates of the tomato varieties have chemical components that plays a role in nematode host seeking process. Profiling of the root exudates showed similarity in compound composition, but ratios of this compounds could differ in each tomato variety. The differential responses of *M. incognita* J2s to the root exudates of the different tomato variety can be attributed to the ratios and composition of the bioactive compounds. Fractionating the most preferred root exudate showed that polar fractions have differential influence on the J2s behavior. On identifying the compounds and determining their bioactivity, specific polar compounds differentially influenced *M. incognita* J2s host seeking behavior. Two compounds (zeatin and quercetin) showed attractiveness of *M. incognita* J2s. The phytohormone zeatin (**1**) could possibly be among the compound that is utilized by J2s to determine the site of host root invasion and subsequent penetration while, quercetin could act as one of the possible short distance signaling compounds at low concentration but deter J2s at higher concentrations**.**

5.3 Recommendations

Further research to fractionate and identify the components of the other tomato root exudates could bring more insights into the bioactive compounds that influence J2s host seeking behavior. Investigate structure-activity relationship in root exudates metabolites that influence RKNs chemosensory detection and host seeking behavior. In addition to identifying more compounds in the bioactive fraction and evaluating the responses of *M. incognita* to single or blends of the identified compounds. These could shed more light into the ecological roles of these compounds which may lead to new avenues for developing or improving existing RKN management tools.

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