Interactions Between Below-ground Organisms on Sugar Beets

A Thesis

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Abstract

Plant-parasitic nematodes are detrimental agricultural pests, inflicting substantial economic losses on a global scale. While there is a substantial body of knowledge concerning plant-nematode interactions, there is a dearth of information regarding plant-pathogen-pathogen interactions when multiple plant-parasitic nematode (PPN) species concurrently exist around plants. This master's thesis study employed sugar beet plants as a model system to investigate the impact of PPN-exposed and non-exposed plant exudates and volatiles (VOCs) on the orientation behaviors of two significant PPN species, root knot nematode (RKN) *Meloidogyne incognita* and root lesion nematode (RLN) *Pratylenchus neglectus*. Additionally, it explored how the exometabolomes of these PPN types influenced each other's behavior, both conspecifically and heterospecifically.

For the root exudate testing, hydroponically grown sugar beet plants were inoculated with either 1000 RKN or RLN individuals, with incubation periods of 1-2 and 7 days. Nematode-free plants, incubated for the same duration, served as the control group. Two-choice petri dish experiments were conducted using collected root exudates, and the nematodes' preferences were determined. Similar experiments were established using nematode exometabolomes (metabolic footprints). To test impact of VOCs, olfactometer experiments were conducted by placing 7-day control and nematode-exposed plants at the end of olfactometer arms to assess the nematodes' choices over an 18-hours for RKN and 48-hours for RLN.

Notably, no significant trend was observed in the choices of RLN in the root exudate experiments. In contrast, RKN exhibited a preference for the clean plant root exudate in only one application each in the 1 DPI clean versus RLN exposed and 2 DPI empty versus clean. Similarly, no significant differences were found for both nematode species in exometabolome experiments. Similar to root exudate experiments, RKN, in olfactometer trials, selected the non-exposed option over PPN-infected plants. In the case of RLN trials, significant differences were detected, but no specific trend could be established. RLN individuals exhibited a preference for PPN-infected plants in some groups, while choosing the side with empty or clean plants in other groups.

This thesis study highlights the significant impact of root exudates and VOCs on the conspecific and heterospecific behavior of plant-parasitic nematodes. However, the strength of this effect varies depending on the plant and nematode species and days post inoculation. While the results obtained in this thesis study offer insights into the influence of plant root exudates and VOCs on PPNs, further research is essential in the future to gain a deeper understanding of these interaction

Acknowledgments

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Chapter 1: Introduction to Plant-mediated Belowground Interactions

Plant-parasitic nematodes (PPNs) are microscopic roundworms that infest numerous plant species worldwide (Siddique et al., 2022). The diversity of PPNs is remarkable, with over 4,100 known species, each demonstrating preferences for different plant tissues, such as flowers, stems, leaves, and roots. The estimated annual global crop damage caused by PPNs is around $80 billion (Nicol et al., 2011). However, this figure is likely to be a conservative estimate, particularly in developing nations, where many growers may lack awareness of the presence of PPNs. Furthermore, the symptoms associated with PPN infestation, including root galling, discoloration, necrosis, and excessive root branching, can often be mistaken for other diseases or nutrient deficiencies (Khan, 1993). Based on a survey findings, when PPNs were scientifically and economically ranked, RKNs were the most damaging/important followed by cyst nematodes, and RLN (Jones et al., 2013).

All PPN species are obligate biotrophic parasites, typically engaging in the alteration of the cytoplasm of host cells through the secretion of salivary compounds before the extraction of nutrients (Wyss & Grundler, 1992). Based on their feeding behavior, they are categorized into four groups: ectoparasites, where the nematode remains outside the plant and feeds on plant root cells via a stylet; semi-endoparasites, where the nematodes partially penetrate the plant and feed at certain stages of their life cycle; migratory endoparasites, which spend a significant portion of their life cycle moving through root tissues and feeding destructively on plant cells; sedentary endoparasites, where the nematode resides predominantly within the plant tissue, establishing a highly specialized parasitic relationship. In general, a nematode life cycle consists of six stages: egg, four juvenile (J1, J2, J3, J4) and adult (Wharton, 1986). The transition from the J1 to J2 stage typically occurs within the egg, and following this initial molt, the egg hatches, releasing the J2, which is considered the infective stage for the majority of PPNs (Perry & Moens, 2011). J2s typically initiate the invasion process by entering the host tissue in the case of endoparasitic nematodes. They use their stylet to puncture plant cells and extract nutrients. For sedentary endoparasitic nematodes, such as RKN, the following juvenile stages and the adult stage develop within the host tissue (Jones et al., 2013). In contrast, migratory endoparasites like RLN exhibit a distinct behavior where they repeatedly move out of and re-enter the host tissue at multiple points during their life cycle (Castillo & Vovlas, 2007; Jones & Fosu-Nyarko, 2014). This feeding behavior damages the root system and creates wounds that are entry points for other plant diseases. For example, in apple (*Malus domestica*) Back et al., 2002 reported that RLN are often associated with fungal and oomycete pathogens, such as *Rhizoctonia,*

Fusarium, Pythium, and *Phytophthora*, forming disease complexes, that could be responsible for replant diseases.

Interactions between phytophagous nematodes and disease complexes can manifest as both synergistic and antagonistic relationships. While competition for space and resources can lead to antagonism, synergistic interactions have the potential to inflict greater damage on plants, including crops (Zhang et al., 2020). Based on Back et al., (2002) review, the pioneering observation of a nematode-fungus interaction was documented by Atkinson (1892), revealing that fusarium wilt in cotton (caused by *Fusarium oxysporum* f.sp. *vasinfectum*) became more severe in the presence of root-knot nematodes (*Meloidogyne* spp.). Conversely, Edin et al. (2019) found that the severity of stem canker (*Rhizoctonia solani*) did not escalate in the presence of *Pratylenchus penetrans* on potato plants and the *P. penetrans* population was decreased in the presence of *R. solani*. In potato fields, Björsell et al. (2017) reported higher stem canker (*R. solani*), called dry rot canker on sugar beet, severity in patches infested with stubby-root nematodes and potato cyst nematodes, but not with root-lesion nematodes. Moreover, at 6 weeks post-planting of potato plant, a positive correlation was observed between infestations of *G. rostochiensis* and stolons of the plants infected by *R. solani* (Back et al., 2006).

Furthermore, the interaction between phytophagous nematodes and pathogenic bacteria can vary depending on the preceding infection agent or simultaneous infection. For example, tomato plants cultivated in *Meloidogyne* spp. infested soil were significantly more susceptible to attack by *Pseudomonas solanacearum* compared to those in nematode-free soil. Conversely nematode reproduction was decreased by prior inoculation of the bacteria as documented by Siddiqui et al. (2012). Similarly, prior infection of *Pectobacterium carotovorum*, causing soft rot decay on sugar beet, significantly decreased *M. incognita* population on potato (Siddiqui et al., 2014)

Simultaneous infection of tomato plants with the root-knot nematode *M. incognita* and tomato mosaic virus resulted in a synergistic reaction, causing more extensive damage than infection by either pathogen alone. Notably, pre-inoculation by either pathogen adversely affected the reproduction of the other (Alam et al., 1990). Naqvi et al. (1977) reported that 2- and 3-week prior inoculation of spinach mosaic virus on sugar beet decreased multiplication of *M. incognita* and *Tylenchorhynchus brassicae* (stunt nematode). Based on these sources, the interactions between phytophagous nematodes and plant pathogens can vary depending on the specific pathogen combination and the initial infecting agent in the plant.

Understanding the mechanism underlying the attraction, repulsion, and entry of PPNs into host plants represents a crucial role in their management (Wang et al., 2021). In the context of belowground plant-arthropod interactions, volatile organic compounds (VOCs) are known to be important attractants or repellents for arthropods that feed on roots. Among these, CO_2 emissions by roots are a ubiquitous signal in soils, with low concentrations acting as attractants but high concentrations causing disorientation. However, the orientation of insects within CO_2 gradients can be influenced by other non-volatile or volatile olfactory stimuli exuded by roots (Banerjee & Hallem, 2020). PPNs depend on plant signals for host and feeding site localization. CO_2 emissions play a vital role in nematode attraction, but secondary metabolites can initiate and guide PPN attraction (Rasmann et al., 2012). Secondary metabolites are part of root exudates, which can contain as much as 20-40% of the carbon fixed by the plant through photosynthesis (Canarini et al., 2019). These exudates consist of sugars, amino acids, carboxylic acids, and phenolics with their composition varying among plant species, and these chemicals regulate the rhizosphere organisms in two ways; they can attract or repel certain species (Ma et al., 2022). Herbivore-induced terpene VOCs have been identified as possible attractants for PPNs, with several terpene compounds, such as α-pinene, β-pinene, limonene, geijerene, and pregeijerene showing activity. Kihika et al. (2017) reported that *Meloidogyne incognita* (Kofoid and White, 1919) Chitwood, 1949 (Rhabtidita: Meloidogynidae) is attracted to pinene, limonene, tridecane, and 2-methoxy-3-(1-methylpropyl)-pyrazine secreted by *Capsicum annum* L. roots, while being deterred by thymol. Considering these findings, compounds released by plant roots play a significant role in modulating herbivore behavior, influencing their ability to locate their favored host plants.

Plants exhibit diverse responses to herbivore attacks, employing a range of morphological, biochemical, and molecular mechanisms to counter the impact of herbivory. These defensive mechanisms are varied, marked by high dynamism, and encompass both direct and indirect forms of defense (Howe & Jander, 2008; van Dam & Heil, 2011). Plants have an innate immune system that responds to biotic agents' signals, triggering the production of specific defensive chemicals, and alterations in plant morphology, as for example in back mustard, trichome amount increased in response to *Pieris rapae* (L.) (War et al., 2012). They exhibit responses to pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) through the production of the phytohormones salicylic acid (SA) and jasmonic acid (JA), as part of their systemic defense mechanisms. PPNs also have specific molecules that have been identified as key players initiating signaling cascades within the plant, thereby activating the plant's innate immune responses. These molecules are referred to as Nematode-Associated Molecular Patterns (NAMPs) (Choi &

Klessig, 2016), and are secreted by PPNs during feeding and infection, and they are recognized by plants, activating a defensive cascade (Siddique et al., 2022).

The pathways of reactive oxygen species (ROS) and phytohormones such as SA and JA signaling pathways are activated and translocated through the plant's vascular system, in order to protect the plant from the threat of herbivory (Ripa et al., 2023). Wang et al., (2019) reported that ROS and JA concentrations peak in the leaves at 24 h after RKN inoculation to tomato plants. As the RKN infection progresses, after 14 days post inoculation (DPI), RKN downregulates the expression of genes related to the expression of SA and JA as the case of the plant *Arabidopsis*' roots (Hamamouch et al., 2011). In an investigation, the impact of methyl jasmonate (MeJA) on inducing plant defense compounds in oats that were infected with nematodes, specifically *Pratylenchus neglectus* Filipjev, 1936 (Rhabditida: Pratylenchidae), *Heterodera avenae* Wollenweber, 1924 (Rhabditida: Heteroderidae), and *Ditylenchus dipsaci* , (Kühn, 1857) Filipjev, 1936 (Rhabditida: Anguinidae) was examined. The findings revealed that MeJA-treated plants exhibited reduced susceptibility to invasion by RLN and *H. avenae*, while also demonstrating increased plant growth indices (Soriano et al., 2004).

The nematodes, RKN and RLN, have the ability to trigger or inhibit host defense mechanisms, by production of toxic compounds, cell wall-degrading enzymes (especially β-1,4-endoglucanases), the secretion of effectors, including proteins and ascaroside #18 (Vieira et al., 2020). Additionally, some effectors are implicated in the modulation of host developmental pathways, aimed at initiating and sustaining the nematode feeding sites. While sedentary plant nematodes introduce various effectors into host cells or tissues, RLNs, which do not induce long-term feeding sites, may not produce effectors to complete their life cycle (Jones & Fosu-Nyarko, 2014). Although the majority of effector studies have been conducted on RKNs and cyst nematodes (CNs), there has been a growing focus on effector research in other nematode species, including the pinewood nematode, as well as root lesion and burrowing nematodes. In the case of RKN and CN, effectors are typically secreted from the esophageal glands (both dorsal and subventral) through the stylet and into the plant; however, they can also be secreted from other organs, such as the amphids or cuticle (Vieira & Gleason, 2019). (Manohar et al., 2020) reported that pre-treating roots with 10 nM or 50 nM ascr#18 for 48 h before inoculation with RKN significantly enhanced resistance in the *Arabidopsis thaliana* wildtype plants. In contrast, ascr#18 treatment exhibited no detectable effect in the acx1, acx5 mutant ones (*acx* genes have a role in JA production). Zhou et al., reported (2023), effector Minc03329 expressed in the subventral esophageal gland of RKN suppressed host defenses and promoted pathogenicity.

This study addresses a notable gap in our knowledge concerning about belowground interactions mediated by plants involving PPNs. To achieve our research objectives, we conducted a series of experiments using sugar beet as a model plant species and two generalist plant-parasitic nematodes, RKN and RLN. The primary aim was to investigate the impact of root exudates collected from both infected and uninfected plants on RKN and RLN. The second objective was to gain insights into how nematode exometabolomes (metabolic footprints) affect the behavior of nematode juveniles, considering both conspecific and heterospecific effects. Lastly, we desired to see how volatiles produced by infected and uninfected plants influence the behavior of plant-parasitic nematodes, considering conspecific and heterospecific effects. We hypothesized that nematode-associated molecular patterns (NAMPs) produced by plant parasitic nematodes that infect plants first should elicit plant defenses and reduce the preferences and rate of the plant pathogenic nematodes that infect plants second.

Chapter 2: Two-way Behavioral Conspecific and Heterospecific Bioassays of RKN and RLN

Materials and Methods

Sugar Beet Cultures

Sugar beets (*Beta vulgaris*) were cultivated from seeds procured from Seed Kingdom (North Miami Beach, Florida, US) and were germinated inside plastic bags. Fifty to sixty seeds were placed between moistened paper towels in a plastic bag and left on a heating mat at 30 °C for 24 hours (Figure 1a). After 24 hours, the seeds were transferred to a growth chamber at 25 °C and were left four more days. Five-day old seedlings were transferred to a hydroponic system (Figure 1b) that measured 45.72 cm (Length) x 25.4 cm (Width) x 9.52 cm (Height) (12-liter volume) for an additional 25 days. Aerogarden® Liquid Plant Food 4-3-6 was added to the water at the manufacturer's recommended rate (8 ml/2.6 l water). Seedlings were maintained at 22 °C under 16/8 LD light cycle until the experiments ended (Figure 1b).

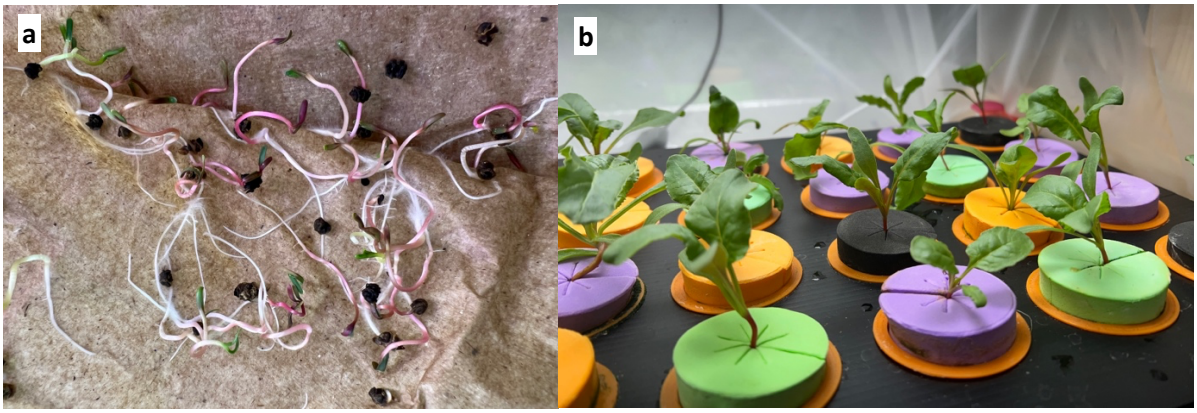


Figure 2.1 a) Germinated sugar beet seeds on paper towels. b) Sugar beet seedlings after insertion to the hydroponic system.

Nematode Cultures

Root-knot nematodes (*Meloidogyne incognita*) were cultured on tomato plants (*Solanum lycopersicum* cv. Rutgers) (Figure 2b) under greenhouse conditions maintained at approximately 28 °C. After at least 90 days, eggs were extracted from infected roots by cutting them into 1 cm pieces and rinsing them with 1% bleach for 1 min. A 25-micron sieve was used to collect the eggs. Eggs were collected and placed into Baerman funnels to allow collection of second stage-juveniles (J2s)

after hatching. J2s were stored in tissue culture flasks at 15 °C no more than 10 days following their collection until use.

Root-lesion nematodes (*Pratylenchus neglectus*) were obtained as a sterile carrot disc culture from Montana State University (Dr. Alan Dyer). The culture was maintained on sterile carrot discs at 25 °C. Carrots were gently scrubbed with soapy water after which they were submerged into 10% sodium hypochlorite solution for 3 min. Following this treatment, the carrots were dried in a laminar flow hood, peeled, and immersed in 70% ethanol for a second round of surface sterilization. Carrots were flamed and then sliced into 1 cm thick discs with a sterile scalpel. Each disc was placed within a 6 cm diameter Petri dish and sealed with Parafilm™ (Figure 2a). The discs were monitored for contamination for 2-4 days. After this observation period, RLN were collected from the current carrot disk culture and rinsed with sterile deionized water several times. All individuals were placed in a watch glass embryo dish for 1 hour in a 2000 ppm streptomycin sulfate solution for surface sterilization. Following surface sterilization, the nematodes were rinsed one more time with sterile deionized water and transferred to the new carrot discs at approximately 50 mixed stage nematodes/ per disc. Depending on carrot quality, the production process duration varies but typically spans 4-6 months. At the end of the period, thousands of individuals can be harvested from uncontaminated carrot discs.

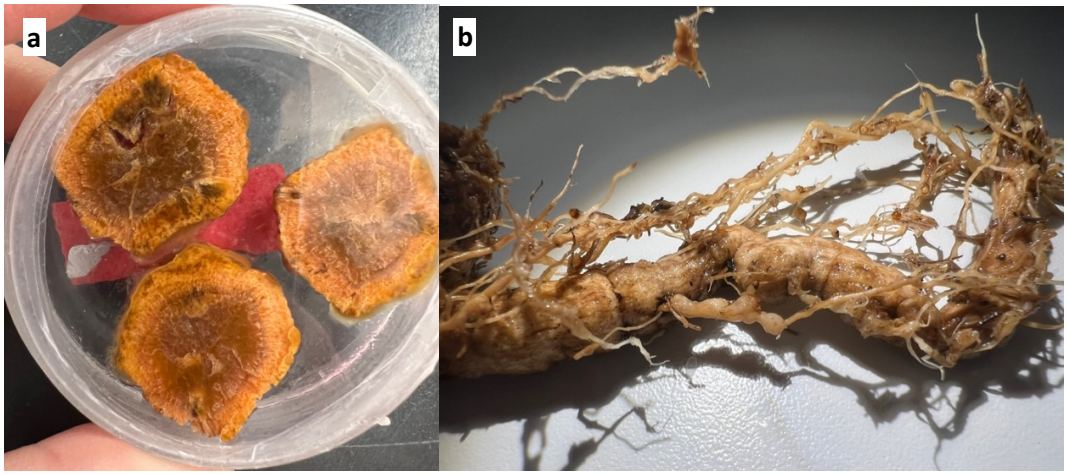


Figure 2.2 a) *Pratylenchus neglectus* carrot discs culture, b) Tomato roots infected with *Meloidogyne incognita.*

Rhizosphere and Nematode Exometabolome Collection

Plant root exudates were collected from hydroponically produced plants. Plants were placed into 30 ml glass bottles with 20 ml fertilizer and DI water solution. The same concentration of fertilizer as was used with hydroponic production was used in the experiment for exometabolome collecting bottles. For nematode exposed plants, 1000 of either RKN J2 or mixed stages of RLN nematodes were added to the solution. As plants were grown in a hydroponic system, a gravel substrate was added to facilitate nematode infection of the roots. 3-D printed custom bottle lids, 12 mm height and 35 mm diameter, were used to make the plant and air tubing stable. Air tubing was placed into the bottle through a hole in the lid to provide oxygen for nematodes and the roots from an air pump. The bottles were covered with a piece of aluminum foil to prevent light from reaching the root system. Additionally, each bottle was carefully nestled inside 709 ml plastic cups containing 50 ml of water, effectively preventing solution evaporation from the glass bottles. The bottles were left for specific durations (1, 2 and 7 days) at 25 °C in an environmental chamber (Darwin Chambers) (Figure 3). The solutions within the bottles were replaced at 24-hours before collecting root exudates. Plants were grown under blue-red 16/8 LD light cycle. After root exudate collection, the nematodes inoculated plant roots were stained with acid fuchsin to determine a successful nematode infection of the roots. Bottles were arranged in sets of three for each treatment group, and there were four replicates in total. For the root exudate analysis, 1 ml was extracted from each bottle and combined in a glass tube. This solution was utilized to minimize the variability in root exudates. All the root exudate solutions were stored in 50 ml centrifuge tubes at -20 °C until used in experiments.

Figure 2.3 Collecting plant root exudates hydroponically.

Nematode exometabolomes (Manosalva et al., 2015) were collected from 1000 nematodes maintained in 20 ml DI water for seven days at 25 C. Each species flasks were prepared as four flasks. After 7 days, the nematode+DI water solutions were filtered with 20-micron filter paper to separate the nematodes from the exometabolome solution. The exometabolome solution was stored in 50 ml centrifuge tubes at -20 °C until used in experiment

Two-choice Petri Dish Tests

The tests were conducted in 6 cm diameter glass Petri dishes filled with 2% water agar. To test the effect of plant root exudates and nematode exometabolomes on nematode behavior, two wells connected with a canal were created by a custom 3-D printed bar (Figure 4). One hundred ml of plant exudate or nematode exometabolome solutions were placed into wells then a nematode was released on the center point of the canal between the test solutions. Each nematode was observed for 30 minutes; RLN were checked every 10 minutes whereas RKN J2s were checked every 5 min. Preference of the nematodes was noted; 40 individuals were tested for each group. If a nematode crossed the centerline, it was recorded as a response. In the case of RLN, its head direction was also considered to indicate a directional choice (Lewis et al., 2006). To determine head directions, individual nematodes were released from the centerline and observed until they oriented themselves in one direction. The side toward which the nematode primarily oriented was recorded as the preference for RLN. Non-responsive nematodes were replaced with a new one after they failed to move for 30 minutes. The experiments were conducted with 1,2, 7-day post inoculation clean, RKN-exposed and RLN-exposed plant root exudates. Seven-day-old nematode exometabolome was tested to determine preference (Table 2.1). Root exudates and nematode exometabolomes were collected in four different times. 10 petri dish experiments were conducted with each time exudates and exometabolomes. Total 40 petri dish experiments were carried out for each treatment group of root exudate and nematode exomatabolome testing.

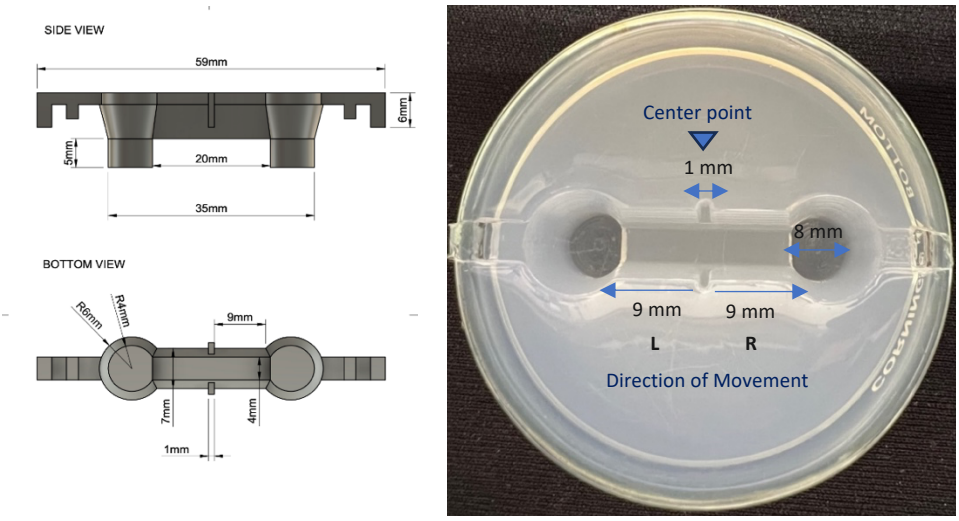


Figure 2.4. 3-D printed bar drawings on the right, petri dish experiments design made with 2% agar

Table 2.1 List of the treatment groups of root exudates and nematode exometabolome testing

Root Exudate and Olfactometer Testing Groups	
Well 1	**Well 2**
Empty	Empty
Empty	Clean Plant
Empty	RKN Exposed
Empty	RLN Exposed
Clean Plant	RKN Exposed
Clean Plant	RLN Exposed
RLN Exposed	RKN Exposed

Nematode Exometabolome Testing Groups	
Well 1	**Well 2**
Empty	Empty
Empty	RKN Exa.
Empty	RLN Exa.
RKN Exa.	RLN Exa.

Data obtained from binary choice tests are expressed actual numbers and the data were analyzed by Chi-square (X^2) goodness of fit to assess a) root exudates collected from clean and nematode exposed plants effects on RKN J2s and RLN juveniles, b) nematode exometabolomes conspecific and heterospecific impact on RKN J2s and RLN juveniles. Statistical analyses were performed by GraphPad Prism 9 software at 5% significance level.

Two-choice Olfactometer Tests

RKN and RLN Olfactory Tests

To investigate the behavior of PPNs in belowground interactions, particularly in response to plant volatiles, olfactory tests were conducted using two-arm olfactometers. The olfactometers were custom made of glass and comprised of a centerpiece (2 cm) and two equally spaced outer pots which held treatments and two middle arms (8.6 cm) connected by Teflon^{TM,} each containing a metal screen to prevent PPNs from reaching and infecting the test plant roots (Figure 5).

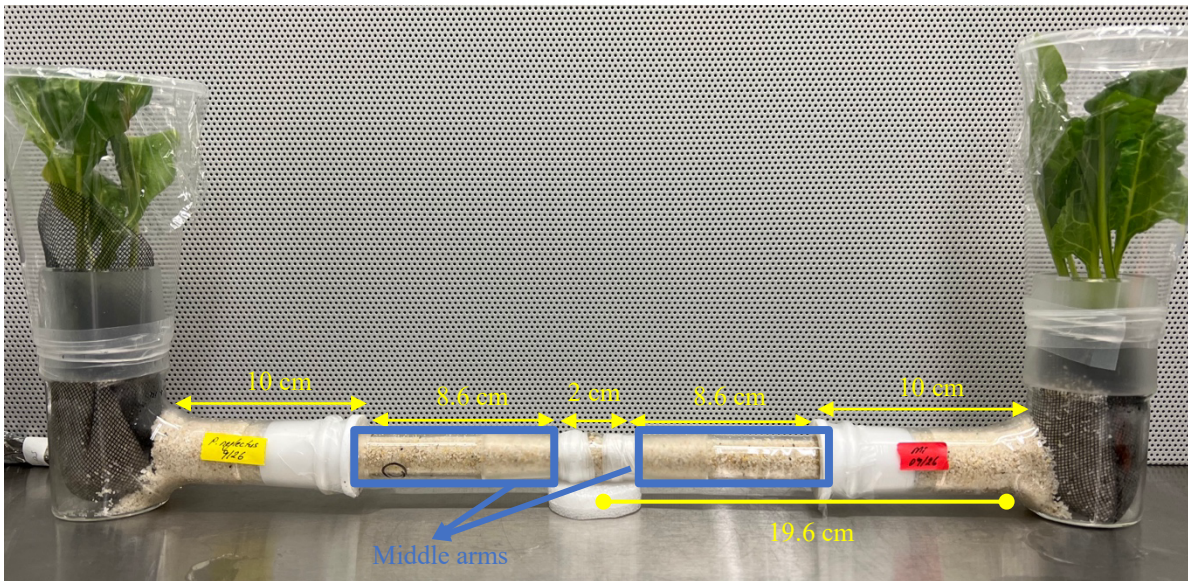


Figure 2.5 Two-way olfactometer was used to test RKN and RLN

Hydroponically grown 23-25 days old sugar beet seedlings were transferred to 100 ml beakers filled with %10 moisture w/w 20/30 silica sand (Lane Mt. Company™ Valley, WA). A 15x15 cm screen mesh was placed at the bottom of the beaker to enable easy transfer of the plant to outer pot arm of the olfactometers. As a precaution, the planted beakers were placed into 709 ml plastic cups to prevent inducing defensive responses (Karban et al., 2013). On the first day, plants were irrigated with 2 ml fertilizer solution (Aerogarden® Liquid Plant Food, 8 ml/2.6 l water). On the second day after transplant to beakers, a solution of 1000 nematodes/4 ml was inoculated into each beaker. The same amount of DI water was applied to clean plants. The plants were taken out from the beakers after 5 days for transplanting to outer pots of the olfactometers and they were allowed to adapt to the new environment for 2 days in the glass arms. To prevent cross-inducing among plants, 591 ml plastic cups bottom were cut and placed on top of the outer pots then covered with plastic wrap. Seven days post inoculation (DPI) of the plants, the olfactometers were assembled (Figure 5), and 1000 RKN J2s /4 ml DI water were released into the center and allowed to respond to plants for 18 hours. For RLN 2000 mixed stages nematodes/ 4 ml DI water was applied the same way and were allowed to respond to plants for 48 h. At the end of the test period, olfactometers were disassembled then the sand taken from middle arms was placed into Baermann funnels for 24 hours after which 25 ml water was collected to 50 ml centrifuge tubes and the nematodes were counted. The plants taken out from outer pots, were stained to ensure that nematode inoculated plants were infected (Figure 6).

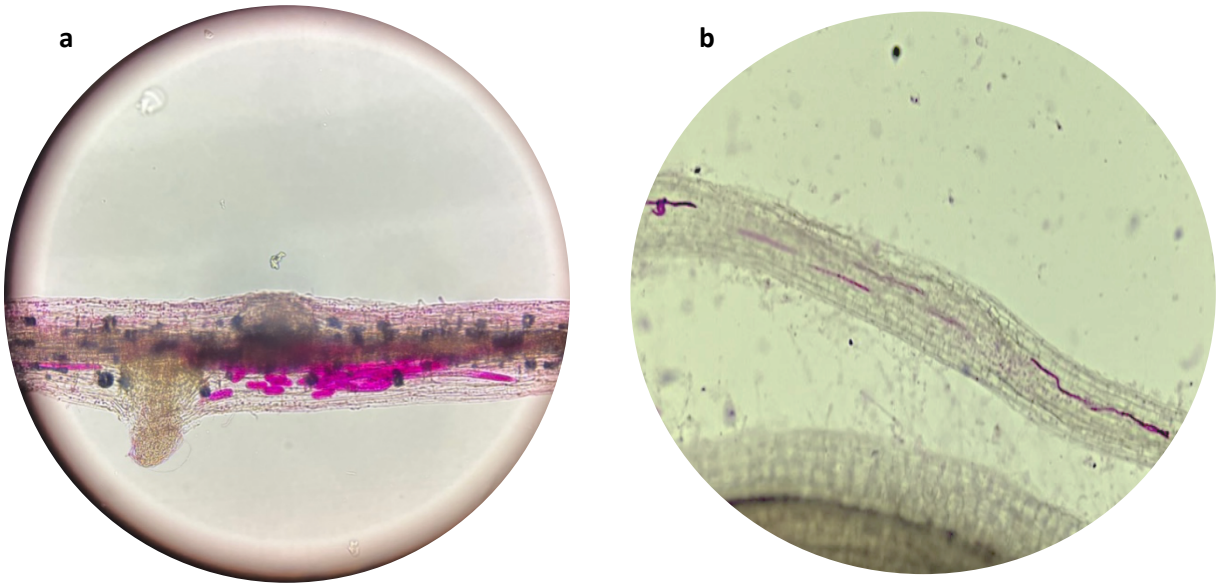


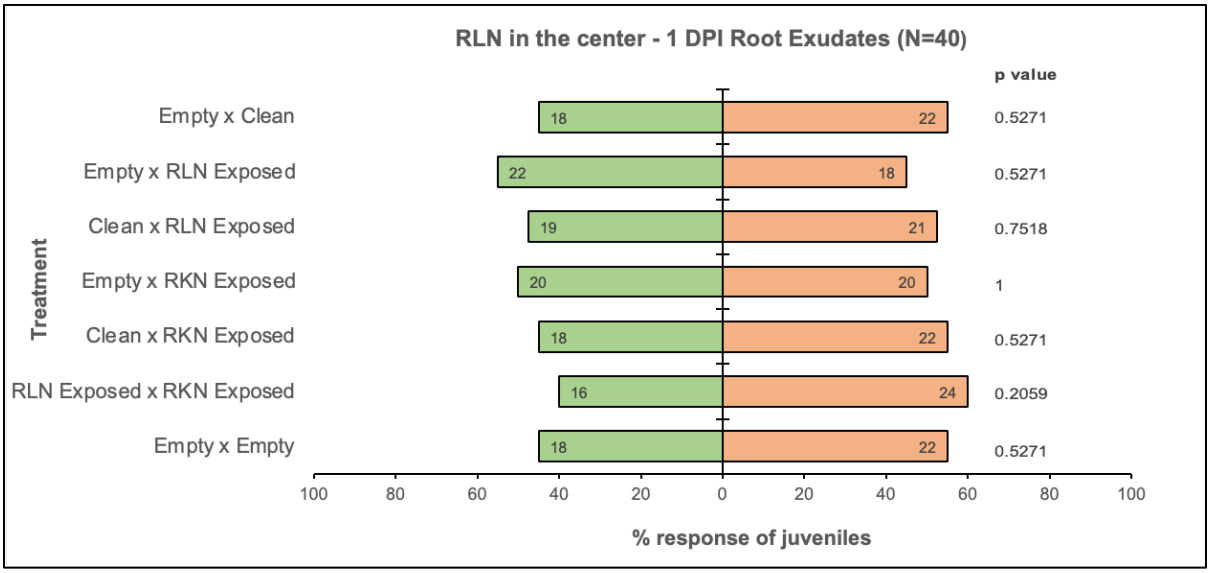
Figure 2.6 RLN (a) and RKN (b) in sugar beet roots

One dual choice olfactometer was considered as an experimental block and the experiment were set up according to randomized completed experimental design and replicated 5 times for each treatment group. In each replicate, the seven treatments group experiments were carried out simultaneously. The data from the dual choice olfactometer assays were analyzed as actual numbers. Only the nematodes that responded and were recovered from middle arms were calculated then also stated as percentages on the graphs. The data analyzed using Chi-square (X^2) goodness of fit to test null hypothesis in which RKN and RLN were distributed in 1:1 ratio between treatment and control. To compare the dispersal rate of RKN and RLN, Student's t-test were used.

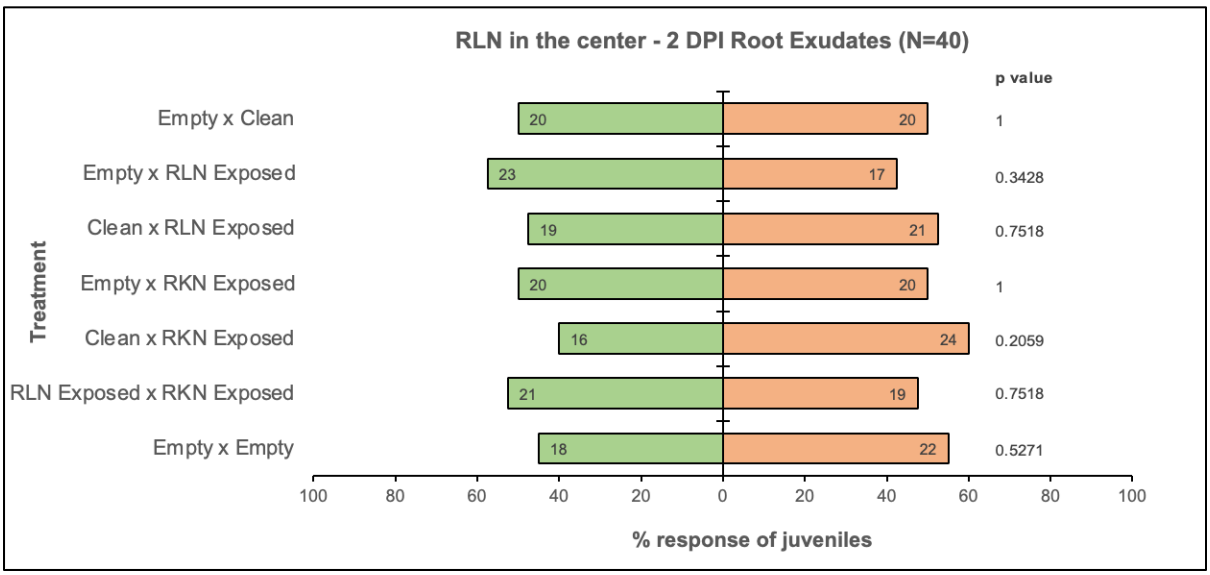
Results

Two-choice petri dish tests of RLN with plant root exudates

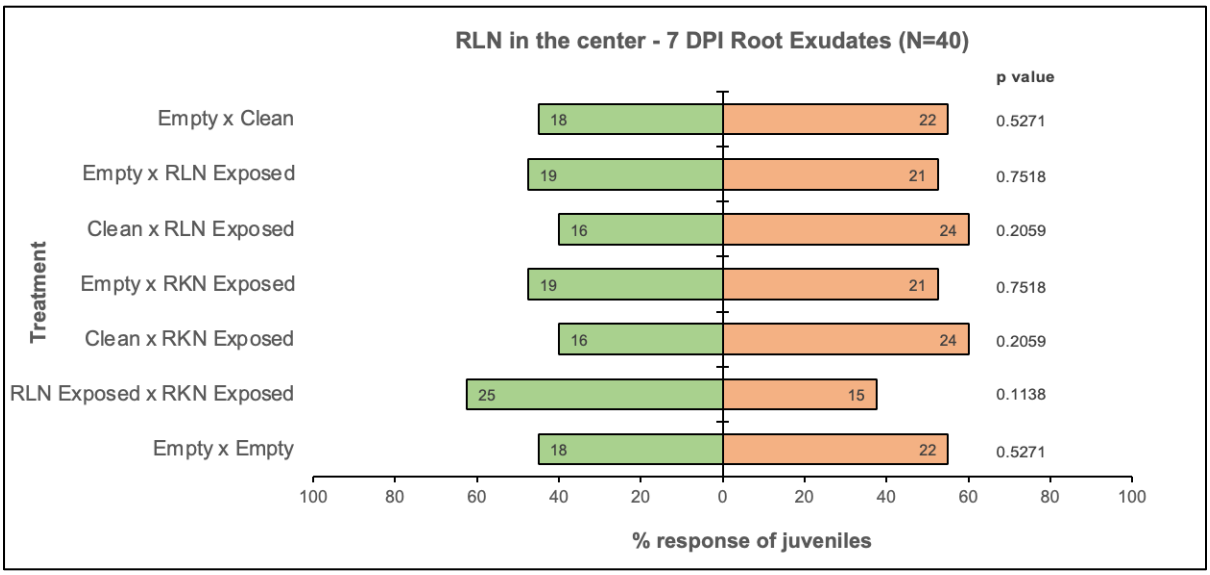
For the petri dish experiments, an individual was released from centerline then the nematode preferences were recorded. In all treatments, Day 1, 2 and 7 root exudates were used, no significant differences in nematode choice were detected (Fig. 7 a, b, and c). No treatment affecting the choice of nematodes was found in comparisons made with clean plants, nematode-exposed plants, and empty groups.



(a)



(b)

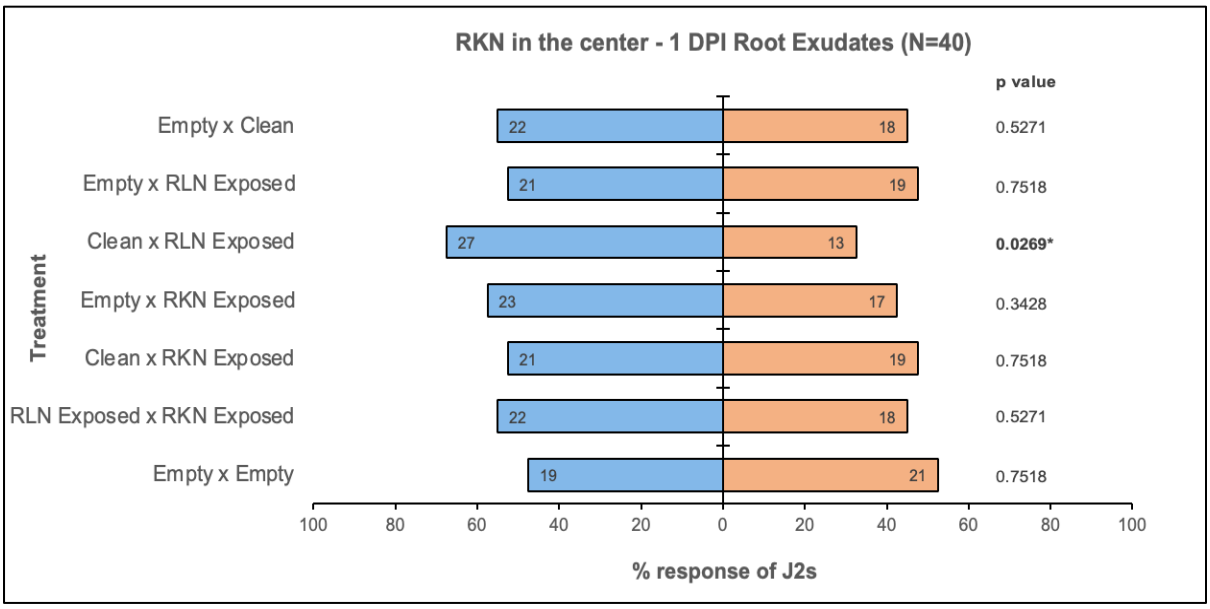


(c)

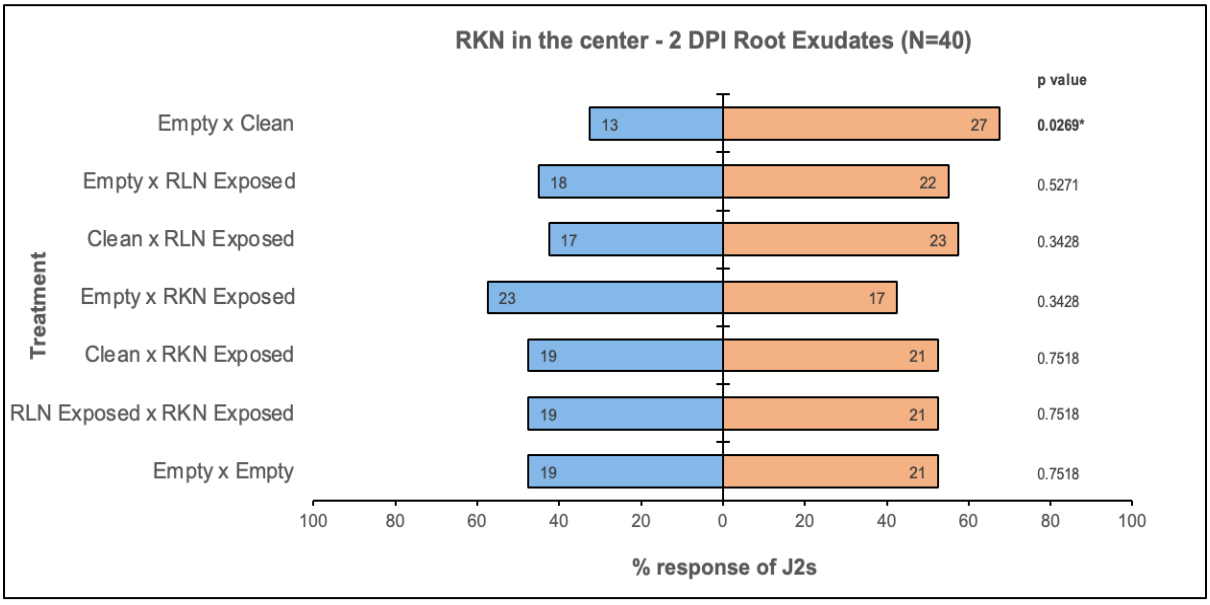
Figure 2.7 Responses of RLN juveniles to root exudates collected from clean and nematode exposed plants. (a) 1 DPI root exudates, (b) 2 DPI root exudates, (c) 7 DPI root exudates were tested with N= 40 individuals of RLN . Numbers within the bars represent individual counts. No statistically significant differences in nematode choice were observed ($p < 0.05$).

Two-choice petri dish tests of RKN with plant root exudates

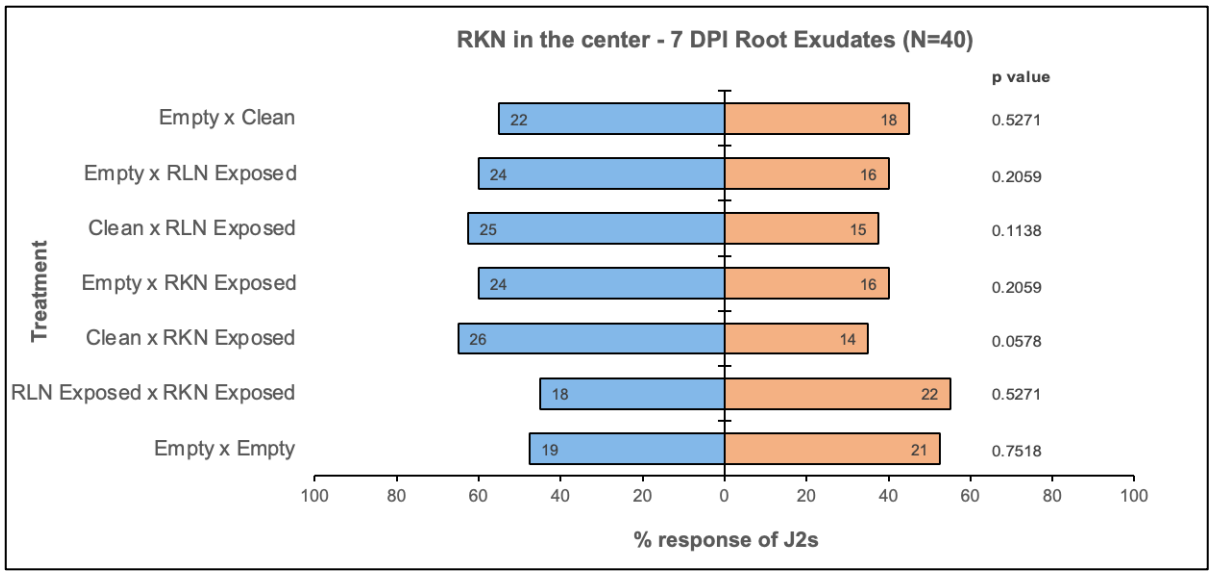
No significant preference was observed in J2 during the Day 7 trials. In the Day 1 and Day 2 trials, a significant preference was only found in one comparison each. Specifically, RKN J2s exhibited a preference to the clean plant in the comparison between RLN-exposed and clean plants at Day 1 (67.5%, $X2= 4.9$, $df = 1$, $p < 0.05$) and once more preferred the clean plant in the comparison between empty and clean plants at Day 2 (67.5% , $X2= 4.9$, $df = 1$, $p < 0.05$).


RKN in the center - 1 DPI Root Exudates (N=40)
p value
Empty x Clean
22
18
0.5271
Empty x RLN Exposed
21
19
0.7518
Clean x RLN Exposed
27
13
0.0269*
Empty x RKN Exposed
23
17
0.3428
Clean x RKN Exposed
21
19
0.7518
RLN Exposed x RKN Exposed
22
18
0.5271
Empty x Empty
19
21
0.7518
Treatment
100 80 60 40 20 0 20 40 60 80 100
% response of J2s

(a)


RKN in the center - 2 DPI Root Exudates (N=40)
p value
Empty x Clean
13
27
0.0269*
Empty x RLN Exposed
18
22
0.5271
Clean x RLN Exposed
17
23
0.3428
Empty x RKN Exposed
23
17
0.3428
Clean x RKN Exposed
19
21
0.7518
RLN Exposed x RKN Exposed
19
21
0.7518
Empty x Empty
19
21
0.7518
Treatment
100 80 60 40 20 0 20 40 60 80 100
% response of J2s

(b)

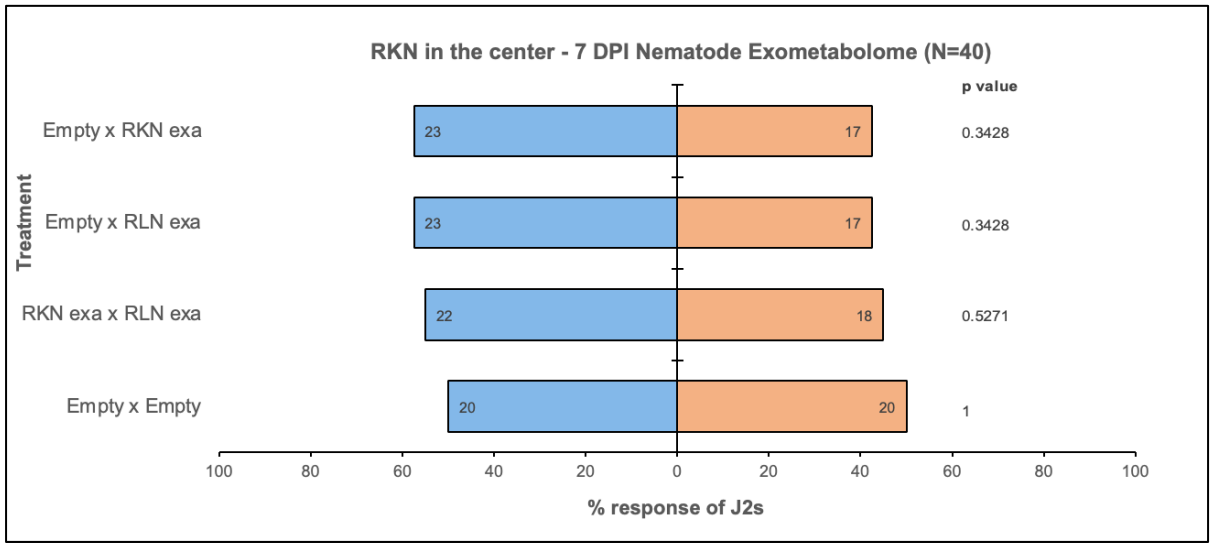


(c)

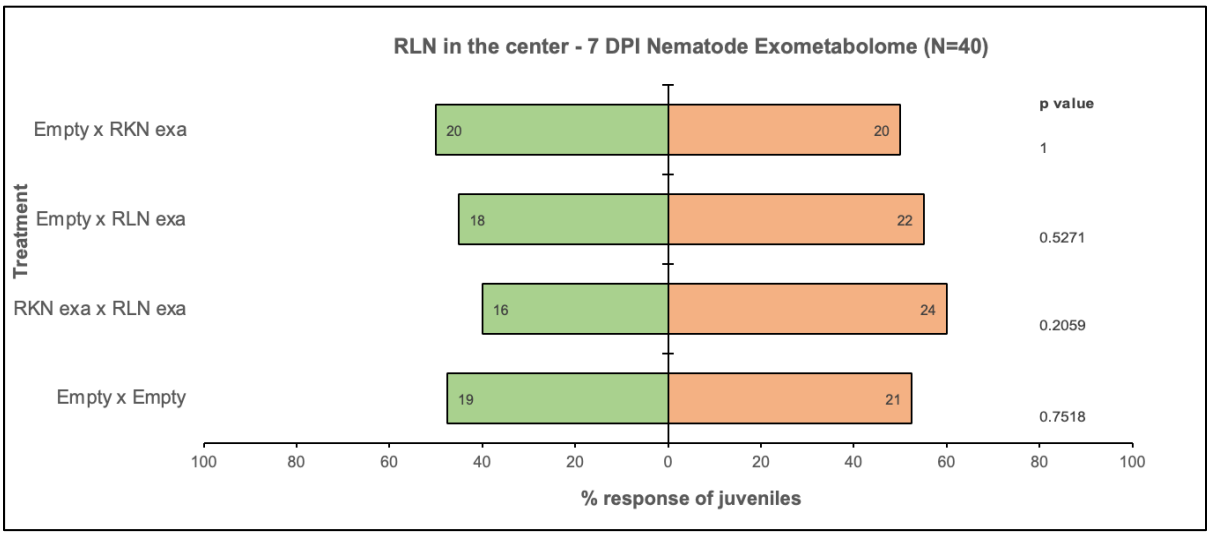
Figure 2.8 Responses of RKN J2s to root exudates collected from clean and nematode exposed plants. (a) 1 DPI root exudates, (b) 2 DPI root exudates, (c) 7 DPI root exudates were tested with N= 40 individuals of RKN. Numbers within the bars represent individual counts. *$p < 0.05$.

Two-choice petri dish tests with nematode exometabolomes

Although RKN J2s exhibited a tendency to move away from both their own and RLN exometabolomes, no significant preference in nematode choices were observed in experiments involving Day 7 nematode exometabolomes. Likewise, no significant distinctions were identified in RLN choices.



(a)



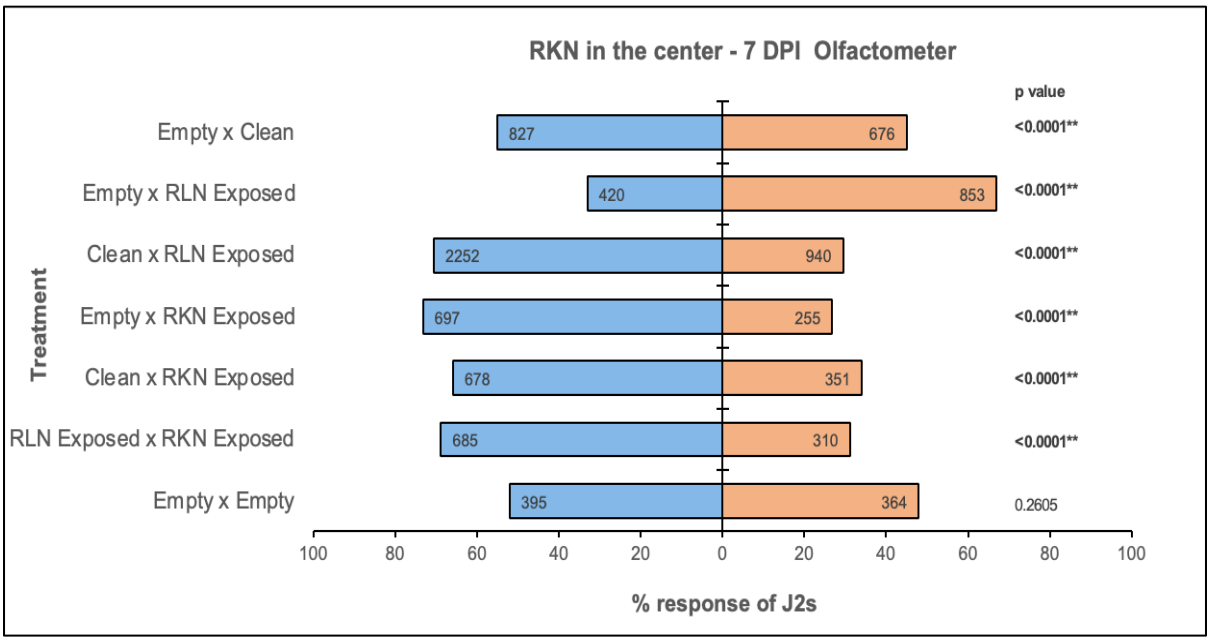
(b)

Figure 2.9 Two-choice petri test of root-knot (a) and root-lesion nematodes (b) in different treatments using 7 DPI nematode exometabolomes. Numbers within the bars represent individual counts. No statistically significant differences in nematode choice were observed ($p < 0.05$).

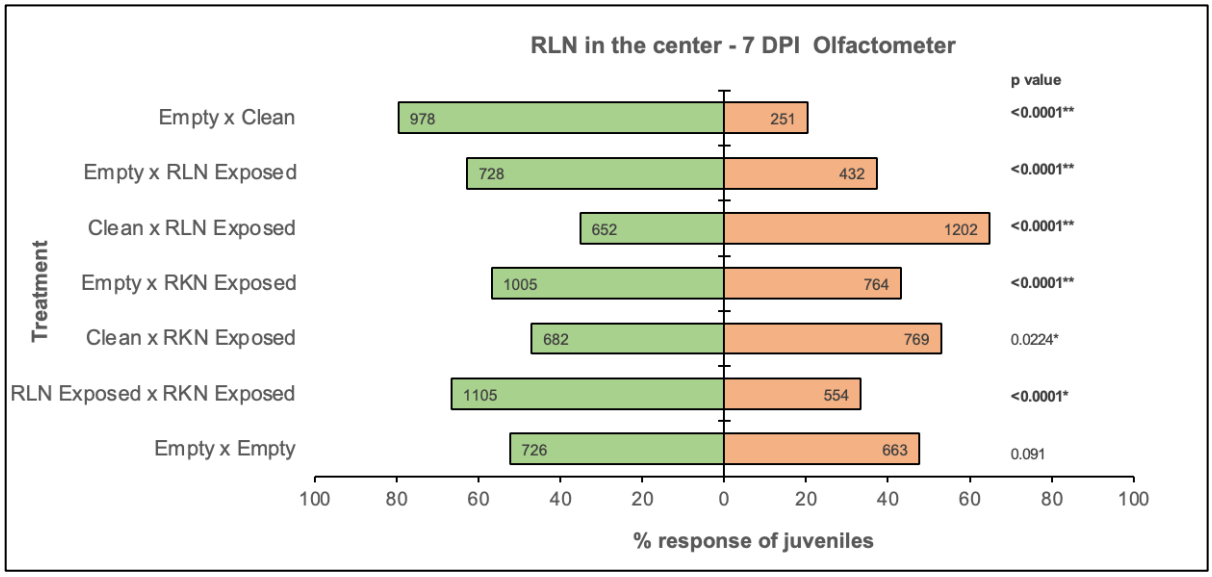
Two-choice olfactometer tests of nematodes

In the RKN olfactometer trials, a statistical difference was observed in choices in all comparisons except the control. It was found that RKN J2s tend to move away from plants infected by conspecific individuals (Fig. 10a). Similarly, in the comparison between the clean plant and the RLN-exposed plant, the clean plant was the preference by most of the J2s (70.6%, $X^2 = 539.3$, df =1, $p < 0.0001$). However, conversely, in the comparison between the empty side and RLN-exposed plants, J2s preferred the infested plant (67%, $X^2 = 147.3$, df =1, $p < 0.0001$). In the comparison between empty and clean plants, the empty side was preferred (55%, $X^2 = 15.17$, df =1, $p < 0.0001$).

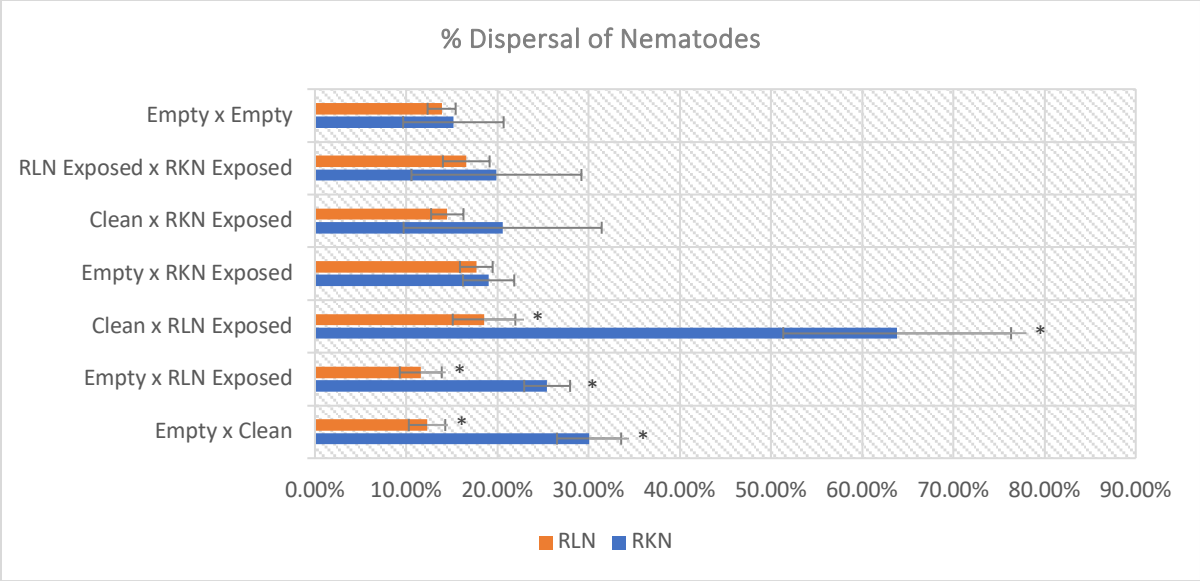
Due to the distinct and slower behaviors exhibited by RLNs (Fig.10c) compared to RKNs, RLN dispersal was observed to be lower than RKNs in the olfactometers. Analyzing the dispersal rate, it was noted that RLN exhibited a preference (Fig. 10b) for the empty side when presented with empty versus plant groups. In the case of clean versus RLN-exposed (64.8%, $X^2 = 163.2$, df =1, $p < 0.0001$) and clean versus RKN-exposed groups (53%, $X^2 = 5.216$, df =1, $p = 0.0224$), the nematodes showed a preference for nematode-exposed plants. In the comparison of RLN-exposed and RKN-exposed group, the nematodes tended to move to the conspecific side (66.6%, $X^2 = 183$ df =1, $p < 0.0001$).


RKN in the center - 7 DPI Olfactometer
p value
Empty x Clean
827
676
<0.0001**
Empty x RLN Exposed
420
853
<0.0001**
Clean x RLN Exposed
2252
940
<0.0001**
Empty x RKN Exposed
697
255
<0.0001**
Clean x RKN Exposed
678
351
<0.0001**
RLN Exposed x RKN Exposed
685
310
<0.0001**
Empty x Empty
395
364
0.2605
Treatment
100 80 60 40 20 0 20 40 60 80 100
% response of J2s

(a)


RLN in the center - 7 DPI Olfactometer
p value
Empty x Clean
978
251
<0.0001**
Empty x RLN Exposed
728
432
<0.0001**
Clean x RLN Exposed
652
1202
<0.0001**
Empty x RKN Exposed
1005
764
<0.0001**
Clean x RKN Exposed
682
769
0.0224*
RLN Exposed x RKN Exposed
1105
554
<0.0001*
Empty x Empty
726
663
0.091
Treatment
100 80 60 40 20 0 20 40 60 80 100
% response of juveniles

(b)



(c)

Figure 2.10 Two-choice olfactometer to test RKN and RLN behavior in different treatments using 7 DPI plants. Numbers within the bars represent individual counts. ($p < 0.05$). (a) For RKN tests, 1000 J2 were used. (b) For RLN tests, 2000 mixed stage nematodes were used. (c) Dispersal rate of the nematodes in the olfactometers *$p<0.05$, **$p<0.0001$.

Discussion

During the seven days of plant infection, plant-mediated interactions between RKN and RLN can influence each other's choices, both conspecifically and heterospecifically. The primary objective of these belowground choice-test experiments was to investigate the influence of a prior exposure to one PPN on the subsequent preference of the second PPN, whether conspecific or heterospecific.

Plant secondary metabolites play diverse roles, serving as nematicidal agents, nematode attractants, repellents, hatching stimulants, or inhibitors, thus underlying different strategies based on their effects (Sikder & Vestergård, 2020). It is known that soil microorganisms and nematodes can induce plants, leading to the upregulation or downregulation of defense-related phytohormones, such as SA, JA, and ET biosynthesis (Xie et al., 2022). According to Kyndt et al., (2012), the JA pathway has a crucial role in defending rice against the sedentary endoparasitic nematode *Meloidogyne graminicola* Golden & Birchfield (1965) (Rhabditida: Meloidogynidae) whereas defense against the migratory endoparasitic root nematode *Hirschmanniella oryzae* (van Breda de Haan) Luc & Goodey (Rhabditida: Pratylenchidae) induces the coordinated activation of SA, JA, and ET biosynthesis pathways. Moreover, application of ethephon and methyl jasmonate to rice has shown reduced infestation by *M. graminicola*. In a separate study, the introduction of *Trichoderma harzianum* (T-78), which is known for inducing SA-related plant defenses, to tomato plant roots led to a significant decrease in gall numbers upon subsequent inoculation with RKN, compared to the control 3 and 7 weeks after inoculation (Martínez-Medina et al., 2017). Furthermore, an experiment involving PPN and ET-insensitive mutant tomato plants (Never Ripe) suggested that these mutants were more attractive to *Meloidogyne hapla* Chitwood (1949) (Rhabditida: Meloidogynidae) J2s than the wild-type, indicating that downstream products of ET signaling reduced the attractiveness to *M. hapla* (Fudali et al., 2013). Our speculation, olfactory test results for RKN could also be attributed to stress phytohormones that are induced by RKN and RLN such as SA, JA and ET (Nahar et al., 2011). On treatments of the clean versus nematode exposed plants, RKNs were strongly repelled by infected plants. But species-specific responses are more complicated to explain.

Plant induction rate or secondary metabolite alterations depend on several factors such as plant species, nematode species, PPN biology and days post inoculation. According to our results, RKN and RLN exhibited different behaviors in response to the treatment groups. RKNs preferred mostly non-exposed plants or heterospecific exposed plants, whereas RLNs chose predominantly conspecific exposed treatment sides. Furthermore, when considering the feeding behavior and life cycles of the PPNs in our study, RKN J2s make choices only once during their life cycle, whereas RLN individuals have the capacity to make multiple choices throughout their various life stages

(Sikder & Vestergård, 2020). Given these insights, *M. incognita* would be expected to make choices with greater caution, since the fitness cost of a poor decision would be significant. It was observed that RLN showed less specificity in response to alterations in phytohormones (SA, JA, ET) synthesis and signaling pathways when compared to *Meloidogyne hapla*, even though both species have close interactions with plant root tissues (Sikder et al., 2021).

Time post inoculation is an important factor influencing plant induction and accumulation of secondary metabolites in the tissue. For instance, glyceollin I, is a flavonoid that enhances soybean (*Glycine max*) resistance to RKN, glyceollin I was exhibited higher levels at 3 DPI compared to 7 DPI (Desmedt et al., 2020). It is particularly noteworthy that sedentary nematodes can modulate plant responses based on J2s existing inside within the plant tissue. Based on that, at 1 DPI, RKN J2s invade the tissue and migrate intercellularly to their target site. By 2 DPI, they induce feeding cells and become sedentary and, after 7 days, the nematodes become completely sedentary with the females feeding from the syncytium that they have induced (Bartlem et al., 2014). According to the knowledge, root exudates were decided to be collected on day 1, 2 and 7 in my thesis research to test nematode behaviors.

Root exudates play a significant role in influencing the physical and chemical properties of the rhizosphere (Hinsinger et al., 2005). Cluster roots, for example, can release substantial quantities of organic acids, leading to a reduction in soil pH. This alteration in pH has implications for the growth and colonization of microorganisms in the rhizosphere, effectively regulating the attraction or repulsion of some species (Ma et al., 2022).Various organic acids, including hydrochloric acid, sulfuric acid, hypochloric acid, mesonic acid, acetic acid, formic acid, propionic acid, lactic acid, succinic acid, and citric acid, have been identified as attractants for *M. hapla* (C. Wang et al., 2009). Additionally, malic acid, oxalic acid, 4-aminobenzoic acid, and lactic acid have been observed to exhibit chemotactic effects on RKN J2, further emphasizing the intricate interactions between root exudates and plant-parasitic nematodes (Wang et al., 2021). On the other hand root exudates can stimulate stylet thrusting responses and host related expression levels of *Pc-eng-1* (β-1,4-endoglucanase) gene and *Pc-xyl* (β-1,4-endoxylanase (Bell et al., 2019). In our investigation, we sought to understand the differential attractiveness of root exudates from plants exposed to nematodes compared to non-exposed plants, specifically examining their impact on RKN and RLN. We did not detect any notable variance in preference by RLN. However, it is important to note that in significant cases, RKN exhibited a distinct preference for the root exudates of non-exposed plants.

Ascarosides, nematode pheromones, secreted by wide range of nematode species (Choe et al., 2012) play key roles in nematode development and behavior, especially on dispersal and foraging as

well as inducing plant defenses (Kaplan et al., 2020; Manosalva et al., 2015). Ascarosides are secreted in different blends based on nematode species. For instance, Ascr#18 is the most abundant for repellence of nematodes, whereas Ascr#9 is responsible for mating and dispersal (Yang et al., 2023). Depending on the relative quantities of ascarosides in their blend, these behaviors may vary in strength. These blends can influence the infection behaviors of entomopathogenic nematodes, either within or between species. Koppenhofer et al. (1995) noted a remarkable 94% reduction in *Steinernema carpocapsae* progeny production when coinfected with *Steinernema glaseri*. Considering these insights, we conducted experiments to examine the exometabolomes of PPNs in both conspecific and heterospecific interactions. I did not observe any significant directional impact on behavior of RKN and RLN exometabolomes conspecific or heterospecifically. This result may be attributed to the fact that PPNs typically secrete a relatively small amount of ascarosides compared to their entomopathogenic counterparts (Choe et al., 2012). On the other hand, ascarosides elicit plant defenses. Exogenously applied ascr#18 on *Arabidopsis thaliana* induce the JA pathway, reduced the infection of RKN and repelled J2s significantly (Manohar et al., 2020)

Examining the impact of plant-mediated interactions involving plant-parasitic nematodes (PPNs) holds the potential to yield invaluable insights for the development of sustainable agricultural management strategies. Our study, centered on plant mediated belowground interactions with PPNs, not only forms a foundational knowledge base for innovative nematode control approaches but also contributes new insights on the evolution of chemical signaling especially on RLN in this context.

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