Two-way Plant-mediated Interactions Between a Plant Parasitic Nematode and a Foliar Herbivore Arthropod

A Thesis

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by

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Abstract

Interactions between belowground and aboveground heterotrophic communities that have no direct physical contact can be connected through the plant as the mediator of these interactions. Herbivores use different types of cues to find suitable host plants, and plants respond to herbivore attack by producing a suite of defensive compounds that can affect the choice and performance of herbivores. The aim of this study was to explore two-way, plant-mediated interactions between two herbivores: the belowground plant-parasitic root knot nematode (RKN) *Meloidogyne incognita* (Tylenchida: Heteroderidae), and the two-spotted spider mite, *Tetranychus urticae* Koch (TSSM) (Acari: Tetranychidae), which is an aboveground folivore. Two different host species were used, Lima bean (LB) (*Phaseolus lunatus* cv. Henderson), which is an optimal host for TSSM, and tomato (*Solanum lycopersicum* cv. Rutgers), which is a sub-optimal host for TSSM, but optimal for RKN.

For the belowground experiments, to test RKN preference, we used two-way glass olfactometers to determine the response of RKN to a LB plant exposed to TSSM versus a LB plant that was not exposed to TSSM. RKN penetrated the roots of non-exposed plants at a greater rate than the TSSM exposed plants.

For the aboveground experiments, we used leaf discs and two-way olfactometers to conduct choice tests of the response by TSSM to an RKN-infected plant at 1, 2 and 25 days post-inoculation (DPI) with RKN against a plant of the same age that had not been exposed to RKN. More TSSM were found on non-infected LB plants compared to RKN-infected plants but only at 25 DPI. On tomato plants, TSSM significantly preferred RKN infected plants at 1 DPI. We also tested the effect of the inoculation on the rhizosphere of tomato plants (1 DPI) of the soil-dwelling entomopathogenic nematode (EPN) *Steinernema carpocapsae* (Rhabditida: Steinernematidae) and found that TSSM preferred the EPN inoculated plants compared to non-inoculated tomato.

We carried out a no-choice performance test for TSSM on both LB and tomato plants to determine the performance of the TSSM on plants inoculated with RKN versus clean plants. There was no observed effect of RKN exposure on TSSM performance.

This thesis research shows that plants can mediate interactions between below and aboveground herbivores that share the same plant.

Acknowledgments

I would like to express my deepest gratitude to my supervisor, Dr. Edwin Lewis, for giving me the chance to be a member of his team, guiding me with the patience to advise me how to overcome the issues that arose during this project, teaching me how to become a better professional, this is something that I will treasure for the rest of my life. I could not have undertaken this task without the support of Dr. Michael Parrella who introduced me to the University of Idaho and the College of Agriculture and Lifesciences; it is really an honor to have the chance to be here. I am also thankful of Dr. Sanford Eigenbrode, and I feel lucky that I was able to take his classes that are a critical component of my education as an entomologist. Also, I would like to thank the entire Lewis Lab, as they always were there helping me in anything that I needed and creating a nice work environment. Finally, I am deeply indebted to my family, my parents and sisters; no matter what the issue or time was, they are always there for me.

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Chapter 1: Introduction to Two-way Plant-mediated Interactions

Plants must meet a series of environmental requirements to grow and reproduce. Even in ideal conditions, there are threats to a plant throughout its life that can negatively affect performance. These threats can be classified into two types: biotic and abiotic. They are all dynamic events. Biotic stress, such as attack by herbivores or pathogens, and abiotic stress, such as drought, high temperatures and wind, all induce plants to initiate defensive responses to reduce the impact of these events (Kohli et al., 2013). Most plants exposed to stressors produce a variety of phytohormones which induce a series of complex physiological responses (Smekalova et al., 2014). Additionally, defensive compounds are induced in response to biotic stress in the attacked tissue and even in distant locations that have not been attacked. These plant-wide effects prepare the plant for the upcoming herbivore attack in a dynamic form of plant immunity (Howe & Jander, 2008). These defense mechanisms are fine-tuned, through an ongoing coevolutionary arms race between plants and herbivores, where both can recognize, elicit, and even suppress plant defenses (Mithöfer & Boland, 2012)

Belowground and aboveground heterotrophic communities with no direct physical contact can be connected by the plant as a mediator of these interactions. Insect herbivores, plant pathogens, nematodes, and rhizobacteria can induce responses that trigger the production of several signaling chemicals that are transported by the plant and cause changes in the content of defensive compounds in yet undamaged plant tissues (Howe & Jander, 2008). This systemic response often crosses the below-above ground barrier (van Dam & Heil, 2011). Plants generally respond to microbial infections and herbivore damage by producing the plant hormones salicylic acid (SA), jasmonic acid (JA), and ethylene (ET). The primary hormone response depends on the nature of the inducer and the compounds that trigger defensive responses in the plant. If the attacker is an herbivore, the compounds are called Herbivore-Associated Molecular Patterns (HAMPs). They are molecules produced in oral secretions, cuticle, feces or oviposition secretions of herbivorous arthropods that elicit plant defenses with the task of reducing the arthropod's performance. For example, glucose oxidase (GOX) is a HAMP present in the saliva of the corn earworm *Helicoverpa zea* (Lepidoptera: Noctuidae) and depending on the plant species, can suppress or elicit direct defenses, as in the case of tobacco and tomato plants respectively

(Acevedo et al., 2015). In the case of plant-parasitic nematodes (PPNs) the molecules responsible for triggering the signaling cascade finally activating the plant's innate immune responses labeled as Nematode-Associated Molecular Patterns (NAMPs), or generally referred as to Pathogen-Associated Molecular Patterns (PAMPs) (Choi & Klessig, 2016). In general, the SA pathway is triggered in response to biotrophic pathogens and piercing-sucking herbivores (Glazebrook, 2005), whereas the JA pathway controls the responses to chewing-biting herbivores (Walling, 2000). Necrotrophic pathogens include the JA/ET pathway (Biere & Goverse, 2016). The pathways of JA and SA are strongly interconnected. For example, SA can inhibit the synthesis of JA. This crosstalk can result in a tradeoff that translates into reduced plant response to herbivore damage that would initiate the JA-signaling pathway when the SA-signaling pathway is already activated or the other way around (Bostock, 2005). However, not all herbivore feeding triggers a defensive response in the plant. Some herbivores (especially herbivore specialist) suppress plant defenses, which can result in an adaptive advantage for the specialist against generalist herbivores (Goverse & Smant, 2014; Sarmento et al., 2011). Also, some plants can respond to volatile cues emitted by damaged neighboring plants, leading to an increased resistance to herbivory (Karban et al., 2013). It is important to recognize that systemically induced resistance responses are not always a net benefit for the plant due to the investment of resources to promote resistance in unaffected tissues that may prevent the plant's response to future enemies that may never arrive in the future (van Hulten et al., 2006).

The present study focused on two extreme generalist herbivores and their impacts on each other's performance and preference, using the plant as a conduit to connect their very different lifestyles; the folivorous two-spotted spider mite, *Tetranychus urticae* Koch (TSSM) (Acari: Tetranychidae), and the root-feeding PPN, *Meloidogyne incognita* known as a root-knot nematode (RKN) (Tylenchida: Heteroderidae). These two herbivores often share the same host plant, as they are both extremely polyphagous and widespread. For example, TSSM can feed on more than 1,100 documented different host plant species (Dermauw et al., 2013), and the RKN host range includes more than 3000 species (Abad et al., 2003). This extreme host breadth elevates these herbivores as major economically important pests across multiple crops due to their rapid lifecycles and the economic injury they can cause.

The life cycle of the TSSM is very short and it takes approximately 14 days from egg to adult (Liu et al., 2020) but it can be as short as 6.5 days at 30 ºC (Herbert, 1981). The TSSM goes through five stages; egg, larva, two nymphal stages (protonymph and deutonymph) and adult. The TSSM feeds on individual cell contents of leaves by using its stylets to penetrate the leaf mesophyll, without damaging the epidermal cell layer (Bensoussan et al., 2016). Feeding by relatively low TSSM densities and the production of webbing can lead to loss of plant production – high densities can kill plants. TSSM feeding elicits a defensive response related to both JA and SA pathways (Iida et al., 2019; Kawazu et al., 2012). Proteins in the salivary glands of the TSSM known as “tetranins”, elicit these plant defense responses (Iida et al., 2019).However, not all TSSM strains trigger these defensive pathways in all plant species. There are examples of TSSM strains that can reduce the expression of defenses, leading to their increased performance on tomato plants (Alba et al., 2015; Kant et al., 2008).

The RKN is a sedentary, endoparasitic PPN since most of its life cycle is completed inside the host plant roots, moving through plant tissue, establishing and developing while attached to a single cell. The life cycle of the RKN is divided into two phases, endophytic inside the roots and exophytic in the soil. The life cycle begins with eggs that, by embryogenesis, progress to the first-stage juvenile (J1) inside the egg. The J1 molts into a second-stage juvenile (J2) while inside the egg, which is the infective stage that disperses and invades host plant roots. After infecting the roots, the J2 moves through the vascular system, and induces the formation of a permanent feeding site. It molts into a third-stage juvenile (J3), fourth-stage juvenile (J4), and then the adult (Cao et al., 2015). A generation can be completed in 20 days at 30 ºC (Ploeg and Maris, 1999). Using effectors, the RKN supports a successful infection by suppressing the plant defense response and by converting the feeding site cells into enlarged, multinucleated cells called syncytia, or giant cells (Rutter et al., 2022). In *Arabidopsis,* genes related to the expression of SA and JA were down-regulated in the leaves of RKN-infected plants at 5 days post-inoculation (Hamamouch et al., 2011). Although, during early infection of tomato plant roots by RKN triggers a systemic signaling and production of reactive oxygen species (ROS), that travel from the roots to the shoots (Wang et al., 2019). This signaling system induces the biosynthesis of JA in the leaves, which is transported back to the roots aiding in the plant’s defensive response against the RKN. Accumulation of JA in leaves peaks 24 h after inoculating the plant with RKN (Wang et al., 2019).

Predicting the outcomes of plant-mediated below-aboveground interactions between a PPN and a folivorous arthropod is challenging, because the host plant, PPN, and folivore are each variable, including with respect to their impact on the other players. Cabbage plants infected with the PPN *Heterodera schachtii* (Tylenchida: Heteroderidae) reduced the aphid *Brevicoryne brassicae* (Hemiptera: Aphididae) performance, growth, and reproduction due to an increased concentration of glucosinolates in the leaves (Hol et al., 2013). Similar results were observed by Kaplan et al. (2011) in a system with RKN and the aphid *Myzus persicae* (Hemiptera: Aphididae) on tobacco plants. One cannot generalize about these outcomes. For example, contrasting results were obtained with an infection by *Meloidogyne arenaria* (Tylenchida: Heteroderidae) that increased the reproduction of *Myzus. persicae (Hemiptera; Aphdidae)* on tobacco (Li et al., 2020). These variable effects illustrate the complexity of the possible interactions and the need for additional studies with generalist herbivores sharing the same plant.

To address this gap, we focused on a system composed of the belowground RKN and the aboveground TSSM, using an optimal and sub-optimal host for each of the two herbivores. Lima bean (*Phaseolus lunatus* cv. Henderson) is optimal for TSSM, but not RKN and tomato (*Solanum lycopersicum* cv. Rutgers) is optimal for RKN, but not for TSSM. In one of the experiments, we included the non-herbivorous entomopathogenic nematode (EPN), *Steinernema carpocapsae* (Rhabditida: Steinernematidae) to see if it had an impact on the plant-mediated effects on TSSM preference in comparison to RKN.

Based on the preference-performance hypothesis, commonly referred to as the '*mother knows best'* hypothesis, female insects should prefer to oviposit on plants that maximize the survival and fitness of their offspring (Clark et al., 2011). We hypothesized that TSSM females prefer plants that offer the highest potential fitness to their offspring, in this case, clean plants without RKN infection contrasted against a RKN infected plant. Similarly, RKN belowground, also should prefer plants that maximize their survival and fitness. Therefore, we hypothesized that RKN prefer and infect clean plants over plants infested with TSSM.

Chapter 2: Plant-mediated Behavioral Bioassays of TSSM and RKN

Materials and Methods

Plants

Lima bean (cv. Henderson) (LB) plants were grown from seeds obtained from Isla's Garden Seed Company (Eagle, ID) and sown in 20/30 pure sand (Lane Mt. Company™ Valley, WA) to minimize root damage during transplanting. For RKN tests, seedlings were maintained at 30 °C under a 16/8 LD light cycle for ten days before transplanting into the segments of the two-way olfactometers for RKN bioassays (Figure 1). For above-ground TSSM choice bioassays, LB was grown in a soil mix composed of PRO-MIX™ BX M growing medium (Premier Horticulture, Quakertown, PA) and 20/30 sand in equal parts. LB seeds were sown to 164 mL cone-tainers (Greenhouse Megastore, Danville, IL) and grown for 10 days at 30 °C under a 16/8 LD inside a BugDorm™-2120 insect rearing tents, (MegaView Science Co., Ltd. Taiwan).

Tomato (cv. Rutgers) were grown from seeds obtained from Seed Kingdom (Lubbock, TX) and were sown using the same soil mix and cone-tainers as LB. Tomato seeds were grown for 25 days at 30 °C under a 16/8 LD, inside a BugDorm™ insect rearing tent (MegaView Science Co., Ltd. Taiwan).

Spider Mite and Nematode Cultures

Two-spotted spider mites *(T. urticae* Koch) were maintained on LB cv. Henderson plants on a 16/8 LD cycle at 28 ºC, for more than one hundred generations using the same plant host species.

Root-knot nematodes *(M. incognita*) were maintained under greenhouse conditions (22-28 ºC) on tomato (cv. Rutgers) plants. Eggs were obtained by cutting infected tomato roots into 1 cm length pieces and immersing them in 1% bleach for 1 min. Eggs were collected using a 500-mesh sieve. Eggs were placed in a Baermann funnel to collect hatching second-stage juveniles (J2s) used for the experiments and stored in culture flasks at 15 °C for a maximum of 4 days before use.

Entomopathogenic nematodes *(S. carpocapsae)* were maintained on the insect host *Galleria mellonella* (Lepidoptera: Pyralidae) last-instars obtained from Speedy Worms™ Inc, (5116 Co Rd 82 NW Alexandria, MN). Infective juveniles (IJs) were collected using a White trap (White, 1927) and stored in culture flasks at 15 ºC until use no more than 10 days after collection.

Belowground bioassays

Two-way choice test root penetration of *M. incognita*. Olfactometers were custom made of three glass cylinders with a total volume of roughly 815 cm^3 (Figure 1) when assembled. Ten-day-old LB plantlets were transferred into 15.3 cm by 5.4 cm (349 cm^3) glass cylinders that were used as "pots" filled with 20/30 mesh particle size pure sand at 10% moisture w/w and kept at 28 °C on a 16/8 LD cycle. After 24 hours, treatment plantlets were exposed to 15 female TSSM which were caged on one true leaf for 48h whereas control plantlets were protected from exposure to TSSM. Three days after transplanting, TSSM and any eggs were removed from the exposed plants, and the foliage of each plant was covered using Organza mesh to prevent possible cross-contamination between treatments (TSSM exposed plant and Control plant). Tests were conducted by connecting the two glass cylinders holding plantlets together with a 5 cm by 5.4 cm (116 cm^3) glass tube centerpiece filled with 20/30 mesh pure sand at 10% moisture w/w and held together by wrapping the joint with Parafilm™. Approximately 1000 J2 RKN contained in 10 mL of deionized water were added to the centerpiece opening (Fig.1). Nematodes were allowed 7 days to move freely among the three sections, at 28 °C under a 16/8 LD plant growing light. At the end of the experiment, the plantlets were recovered, and the roots stained to visualize nematodes using the acid fuchsin technique modified from Daykin and Hussey (1985). Nematodes that had penetrated the roots were then counted under a stereomicroscope for each treatment. One two-way olfactometer was considered as an experimental unit. Experiments were conducted 18 times.

Figure 1. Two-way choice-test root penetration of Meloidogyne incognita olfactometers setup.

Aboveground bioassays

Leaf disc choice-test of TSSM. An experimental arena, adapted from Greco et al. (2005), was used to test TSSM choice between leaf discs from infected versus uninfected plants. Four equidistant 2.54 cm diameter leaf discs taken from the first pair of true leaves of plants that were either infected by RKN (Infected) or not (Control) were placed equidistantly in Petri dishes (90 mm diam) (Fig. 2). A filter paper disc saturated with 0.125 mL of DI water was placed below each leaf disc to reduce leaf disc dehydration.

Leaf discs were obtained from Lima bean plants at 1, 2, and 25 days post-inoculation (DPI) with RKN. Each treatment was tested individually against leaf discs from uninfected plants. Ten-day-old LB plants were inoculated with a population of 1000 RKN J2s for 1 and 2 DPI and 300 J2s for 25 DPI, whereas control plants received a mock inoculation of DI water using the same volume of water needed to inoculate the respective number of RKN J2s of the paired treatment.

Fifteen adult female TSSM were collected on young leaves from the mite colony using a vacuum pump attached to a Pasteur pipette tip. TSSM were immediately transferred simultaneously to the center of the Petri dish and left 24h for dispersal. All tests were conducted at 21 ºC and placed under a 16/8 LD LED plant growing light. The Petri dishes were covered and sealed with Parafilm™ to prevent TSSM from escaping. The numbers of TSSM adults and eggs were counted under a stereomicroscope for each leaf disc. To confirm an RKN infection in the plants that were inoculated with nematodes the roots were stained using the acid fuchsin technique (1 and 2 DPI) or the presence of galls (25 DPI) to confirm an RKN infection. One Petri dish was considered an experimental unit. Each experiment had 20 replications at 1 and 2 DPI and 70 replications at 25 DPI

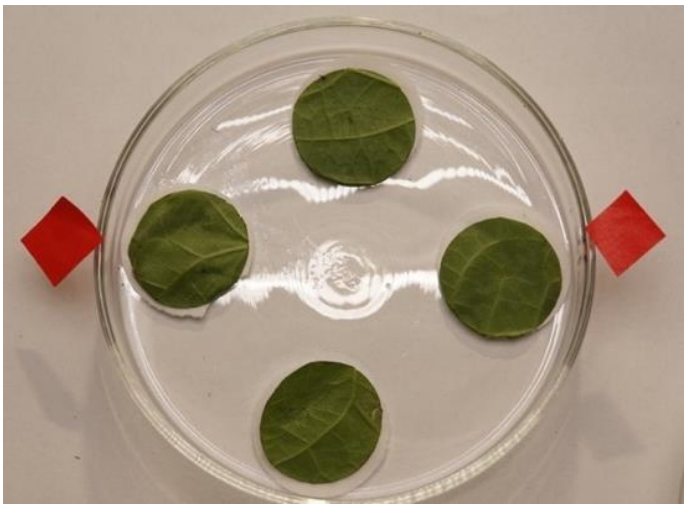


Figure 2. Leaf disc TSSM choice-test of TSSM experiment setup. Red labels indicate the position of the RKN inoculated leaf discs.

Two-way choice-test of TSSM. The same custom-made glass olfactometers (815 cm^3) used for the two-way choice-test root penetration of RKN were used for these experiments. The choice tests were conducted by releasing 15 adult female TSSM into the centerpiece of the olfactometer with the arms containing either an RKN-infected plant or a Control plant, where the whole aerial part of the plant was placed inside the olfactometer (Fig. 3). The stem of each plant was carefully placed through an N° 11 rubber stopper that provided a seal that prevented TSSM from escaping the olfactometer (Fig. 3). A bag made from Organza mesh containing 20 g of moisture-absorbent silica gel granules (Interteck Packaging Silica Gel, Orchard Park, NY) was placed at the base of the stem of each plant, to reduce the buildup of condensation on the inner walls of the olfactometer, allowing TSSM

to disperse. The centerpiece of the olfactometer and the two external segments were held together by wrapping them with Parafilm™. The direction of the olfactometers was randomized and all tests were conducted at 21 ºC and placed under a 16/8 LD LED plant growing light.

The first set of assays was done using ten-day-old LB plants that were inoculated with 1000 RKN J2s. Plants were grown in 164 mL cone-tainers (Greenhouse Megastore, Danville, IL) and used in the choice-test 1 DPI. For the experiments at 7 DPI, an inoculation of 200 J2s RKN was used, since a higher inoculum would have had detrimental effects on the plant.

The second set of assays consisted of twenty-five-day-old tomato plants inoculated with 1000 RKN J2s or 1000 IJs *S. carpocapsae.* Both treatments were tested at 1 DPI. Each inoculated plant with RKN or S. *carpocapsae* was paired individually against an unexposed control plant. Olfactometers were maintained at 21 ºC under a 16/8 LD LED plant growing light for the duration of the experiment. Each plant was examined under a stereoscope to count the number of TSSM adults and eggs. The roots of RKN inoculated plants were stained with acid fuchsin and the number of RKN was counted. One two-way olfactometer was considered as an experimental unit or replication. Experiments had 18 replications.

Figure 3. Top view of a two-way choice-test of TSSM olfactometers containing tomato plants, red label indicates the position of the RKN inoculated plants.

Non-choice performance test of TSSM. To assess the effect of inoculation and early development of RKNs on the performance (fecundity and survival) of TSSM sharing the same plant, experiments were carried out on ten-day-old LB plants and on twenty-five-day-old tomato plants, kept inside BugDorm™-2120 insect rearing tents. Plants were grown in 164 mL cone-tainers before being inoculated with 1000 RKN J2s and kept for the duration of the experiment planted inside a BugDorm™ at 32 ºC and 25% R.H. under a 16/8 LD (Fig.4). Each plant was spaced to prevent the canopies from touching each other, and the walls of the BugDorm™. Control plants were exposed to the same conditions and watering regime as the RKN inoculated plants. A mock inoculation of the same volume of DI water was applied to each control plant.

At 1 DPI of the RKN, 5 female TSSM were transferred to one of the true leaves of each plant. The TSSM used for the experiment were previously age-synchronized using a modified method from Suzuki et al. (2017).

Four and eight days after transferring the TSSM, eggs, nymphs and adults were counted on each plant. Roots were stained using the acid fuchsin technique for the RKN inoculated plants. Each plant was considered an experimental unit. Experiments were conducted 8 times at 4 and 8 DPI.

Figure 4. Non-choice performance test of TSSM on RKN inoculated Tomato plants, inside a BugDorm™ insect cage. Canopies were carefully placed, so they would not touch any surface or contiguous plants.

Statistical analysis

Data from all binary choice tests are expressed as percent. The percent responding to each treatment was calculated using only the TSSM or eggs and RKN J2s recovered in each treatment. The recovery rate, for TSSM adults was 68% and 20.3% for RKN J2s that penetrated the roots. TSSM that did not disperse were not considered in the analysis. For the two-way choice-test root penetration experiments, the percent responding to each treatment was calculated from the number of RKN J2s counted.

Data from the two-way choice-test olfactometers and leaf disc experiments were analyzed used using Chi-square (χ^2) goodness of fit, testing the null hypothesis in which RKN and TSSM were distributed in a 1:1 ratio between the treatment and the control. Data from the non-choice performance experiments of TSSM were analyzed using Student's t-test.

All the statistical analyses were completed using the software RStudio version 2022.02.0+443.

Results

Belowground bioassays

Two-way choice-test root penetration of *M. incognita*. Nematodes penetrated the unexposed plants at a greater rate than the TSSM infested plants (61.39%, χ^2 = 190.24, df = 1, P = <2.2e-16) (Fig. 5)

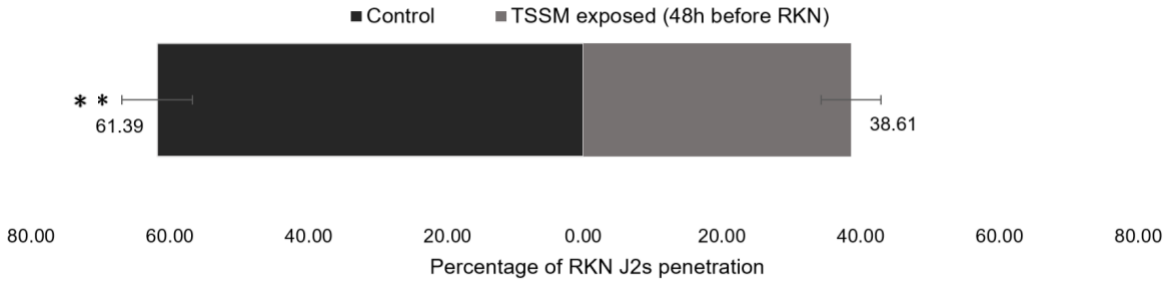
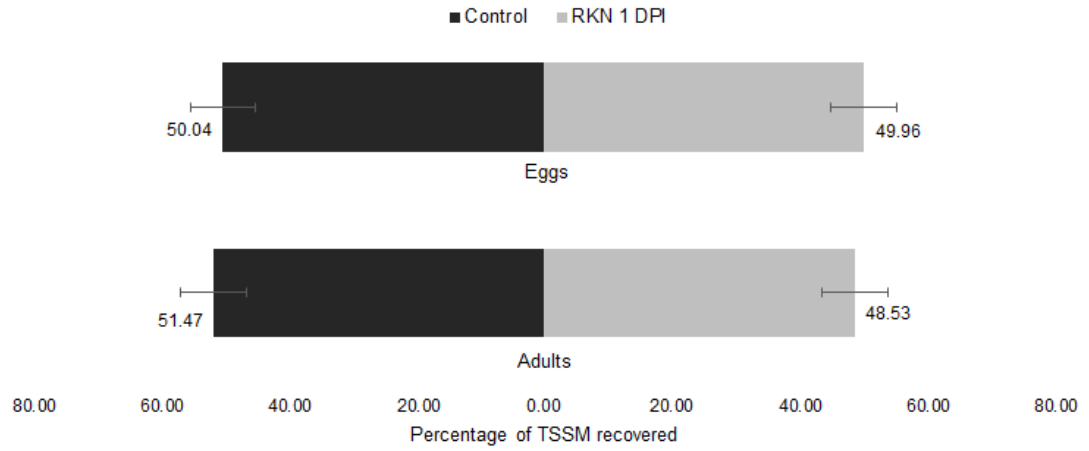


Figure 5. RKN J2s root penetration two-way choice test. A significantly larger mean percent of J2s penetrated the untreated control plants than the TSSM exposed plants ** P < 0.0001.

Aboveground bioassays

Leaf disc choice-test of TSSM. The settling and oviposition of TSSM female on leaf discs from LB plants either infected with RKN or not infected was determined on plants at 1, 2, or 25 DPI. There were no differences in the TSSM responses at either 1 DPI or 2 DPI with 1000 RKN J2s (Fig. 6, a and b). Twenty-five days after exposure to 300 J2s, control leaf discs were preferred for settling by TSSM adults (63.76%, χ^2 = 68.32, df = 1, P = < 2.2e-16) and more eggs were found on the control leaf discs (66.72%, χ^2 = 68.32, df = 1, P = < 2.2e-16) (Fig. 6, c). All RKN inoculated plants were stained using the acid fuchsin technique (1 and 2 DPI) or the number of galls assessed (25 DPI) to confirm a successful RKN infection.



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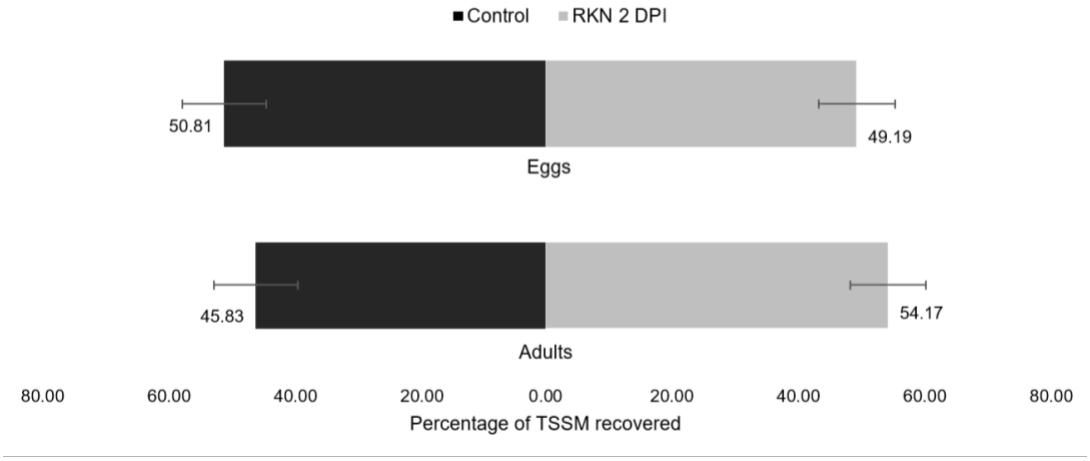

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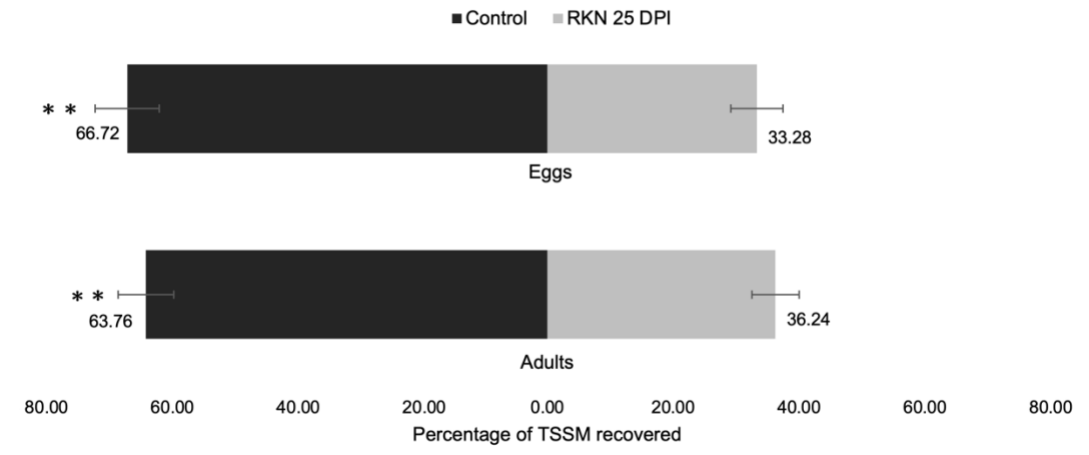
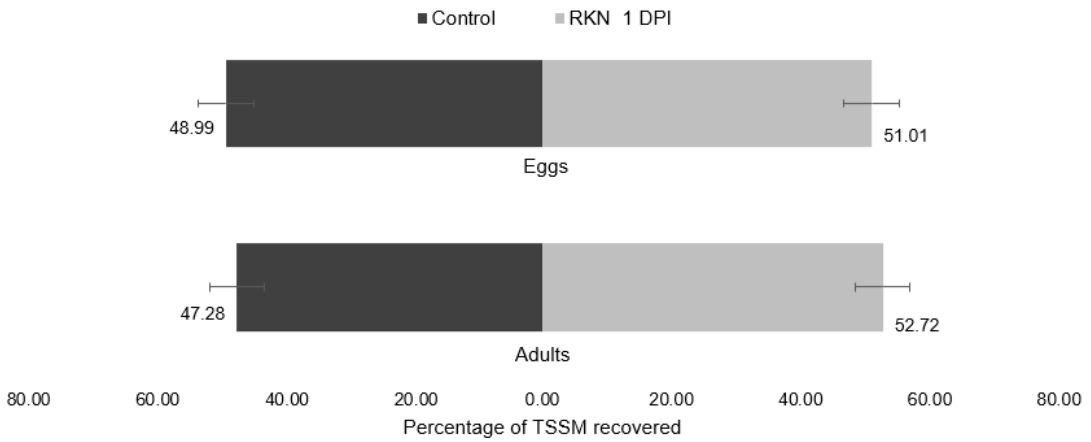


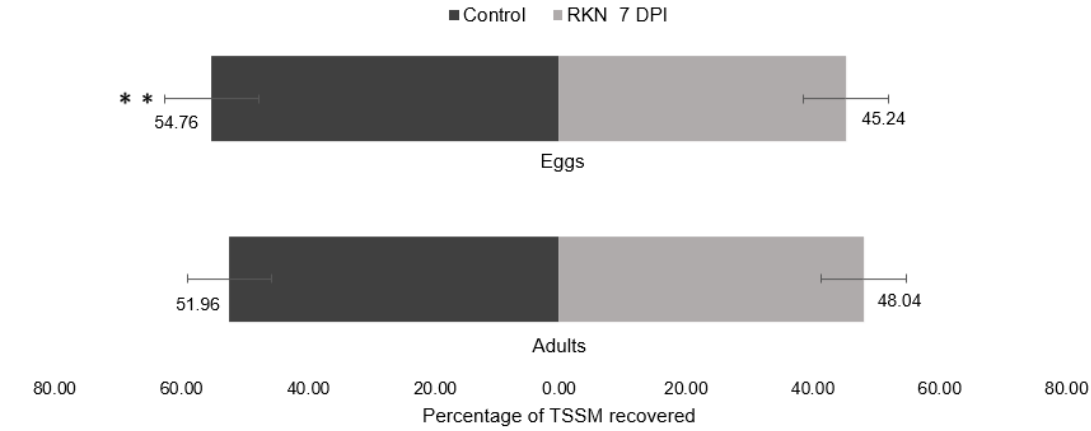
(c)

Figure 6. Responses of TSSM leaf discs from control and RKN infected Lima bean. (a) At 1 DPI using 1000 J2s, (b) at 2 DPI - 1000 J2s and (c) at 25 DPI - 300 J2s. ** $P < 0.0001$.

Two-way choice-test of TSSM – Lima bean. Spider mite adult females were given a choice between the aerial part of an uninfected or an RKN infected LB plant at 1 and 7 DPI using two-way olfactometers. There were no significant differences between the responses of the TSSM to the RKN inoculated plant and the clean plant at 1 DPI (Fig 7, a). Interestingly, at 7 DPI, significantly more TSSM eggs were found on the control plants (54.76%, χ^2 = 16.83, df = 1, P = 4.08e-05) (Fig 7, b). All the RKN inoculated plants were fuchsin stained to confirm the nematode infection.



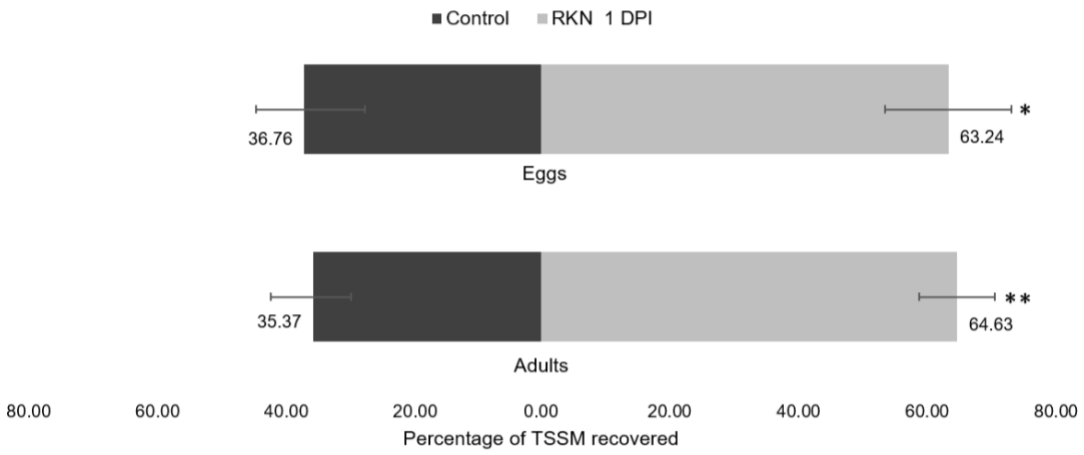
(a)



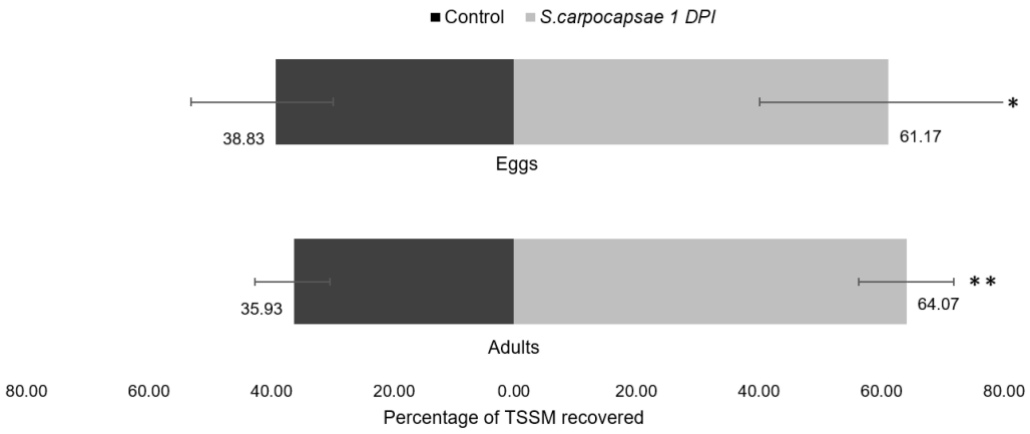
(b)

Figure 7. Two-way olfactometer choice-test of TSSM on Lima bean plants. Responses of TSSM to Lima bean RKN infected plants at two different DPI. (a) At 1 DPI using 1000 J2s and (b) at 7 DPI - 200 J2s. **P< 0.0001.

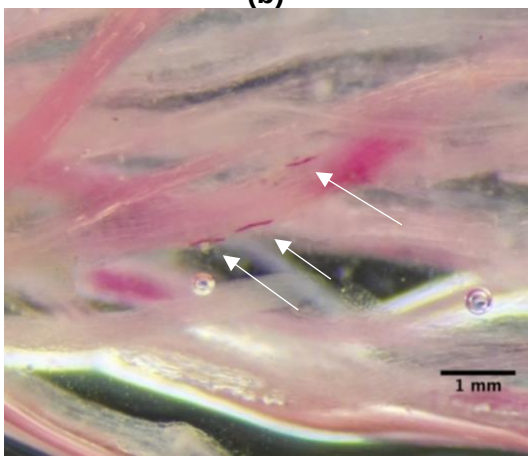
Two-way choice-test of TSSM – Tomato. One day post-inoculation with RKN or *S. carpocapsae*, experiments were conducted using tomato plants in the same two-way olfactometers as described for LB plants to compare the settling and oviposition by TSSM. Both nematode species elicited a similar response, where the TSSM significantly preferred the nematode-inoculated plants to the control plants. In the case of the RKN-inoculated plants, TSSM females settled more on the nematode-inoculated plants (64.63%, χ^2 = 14.05, df = 1, P = 0.00017), and significantly more eggs were found on the nematode- inoculated plants (63.24%, χ^2 = 25.95, df = 1, P = 3.5e-07) (Fig. 8, a). The same effect on TSSM was observed in plants inoculated with *S. carpocapsae*, where TSSM females preferred nematode-inoculated plants (64.07%, χ^2 = 16.83, df = 1, P = 4.08e-05) (61.17%, χ^2 = 5.13, df = 1, P = 0.023) (Fig. 8, b). Both RKN and *S. carpocapsae* inoculated plants were stained using the acid fuchsin technique to confirm a nematode infection since *S. carpocapsae* IJs are capable to penetrate roots in very low numbers (Fig. 8, c)



(a)



(b)



(c)

Figure 8. Two-way olfactometer choice-test of TSSM on Tomato plants. Responses of TSSM to tomato RKN and *S. carpocapsae* inoculated plants at 1 DPI. (a) At 1 DPI using 1000 RKN J2s, and (b) 1 DPI using 1000 *S. carpocapsae* IJs, (c) White arrows pointing to three *S. carpocapsae* IJs inside tomato roots. * P<0.05, ** P< 0.0001.

Non-choice performance test of TSSM. These tests were conducted to determine the performance of the TSSM on plants inoculated with approximately 1,000 RKN versus plants that were not exposed to RKN. On Lima bean plants, no significant differences were found in the average number of eggs, nymphs, or adults, at 4 or 8 days after infestation with TSSM adult females (Fig. 9, a). No correlation between the number of TSSM eggs and the number of RKN J2s was found. On tomato plants, at 4 DPI of the TSSM, the number of adults TSSM (p-value=0.042), but not in the number of eggs or nymphs. No differences were found at 8 DPI (Fig. 9, b). No correlation between the number of TSSM eggs and the number of RKN J2s was found.

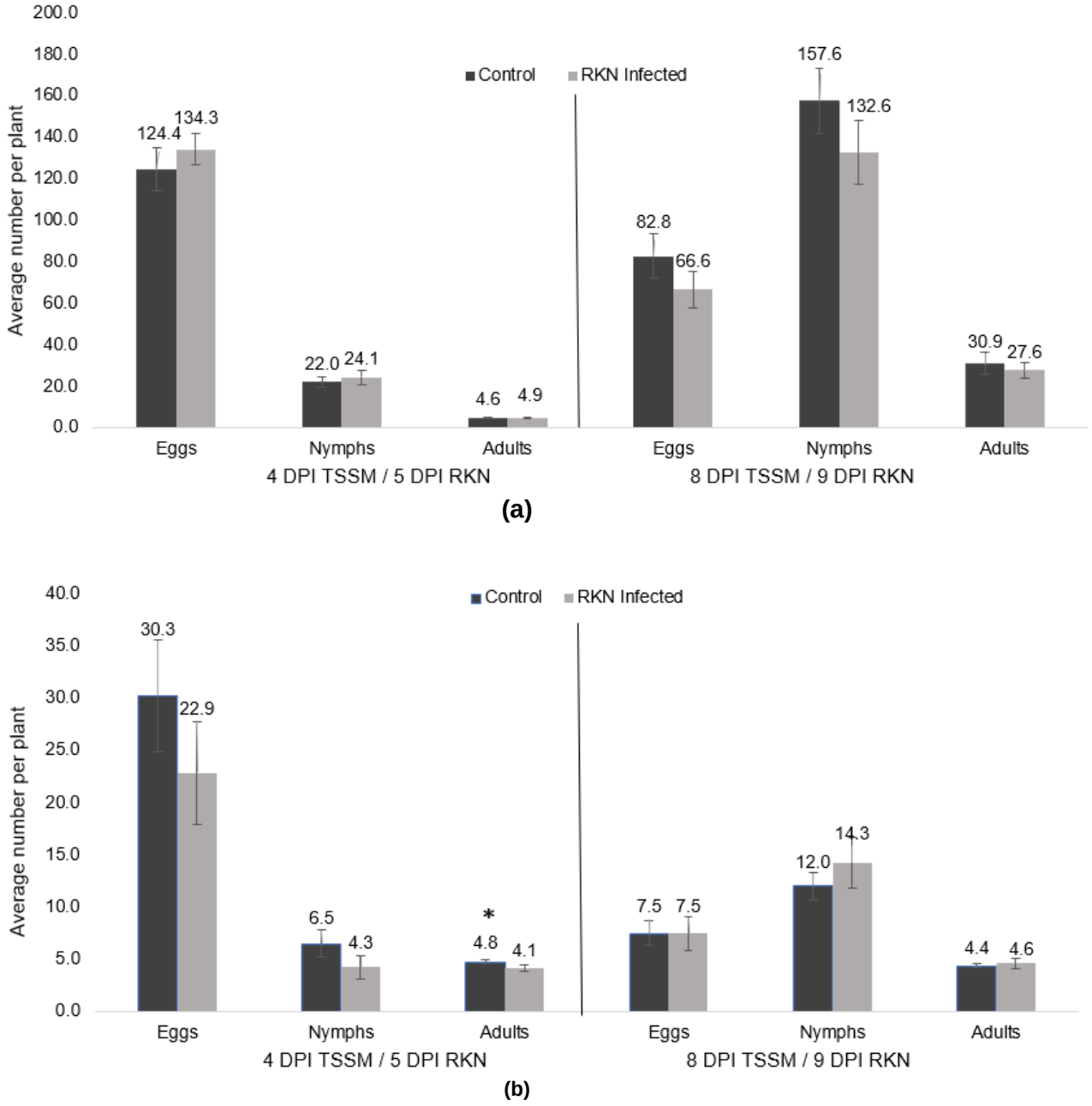


Figure 9. No-choice test of TSSM settling and oviposition on Lima bean and Tomato plants after different treatments of inoculation with RKN. Graphs show the average number of TSSM life stages in control and plants inoculated with 1000 J2s. Plants were inoculated with RKN; the following day they were inoculated with TSSM; assessments were done 4 days or 8 days later. Data were analyzed using a t-test. TSSM = “two-spotted spider mite”; Nymphs include larvae, protonymphs, and deutonymphs; DPI = “days post inoculation”; RKN = “root knot nematode”; * above a pair of bars denotes P< 0.05 based on a Student’s t-test for data within that TSSM stage.

Discussion

Our experiments showed plant-mediated interactions between RKN and TSSM affected each other's choices about invading roots by RKN and settling on leaves for the TSSM. RKN inoculation affected TSSM settling in the early phase of infection in tomato plants, but not in older infections on LB plants. We did not measure the effect on an older infection on tomato plants. TSSM affected the preference and penetration for RKN in LB plants. The aim of our belowground choice-test was to measure the impact of a previous TSSM infestation on the preference and subsequent penetration (invasion) of the RKN on LB plants using two-way olfactometers.

The mechanisms behind these plant-mediated interactions between belowground nematode and aboveground arthropods are still uncertain (Hauri and Szendrei, 2022). Feeding by TSSM induces both the JA and SA pathways. This has been reported in citrus, tomato, *Arabidopsis,* and pepper among other plant species (Santamaria et al., 2020). SA was induced by TSSM feeding in *Phaseolus vulgaris,* increasing levels by about 500% (He et al., 2007). TSSM feeding triggered the systemic activation of both the SA and JA marker genes in leaves not being fed upon on *Arabidopsis thaliana* (Kiełkiewicz et al., 2019). In our study, LB plants that were exposed to TSSM were significantly less infected by RKN compared to the non-infested plants (Fig. 5). This effect could be attributed to a higher content of systemic JA in the roots, among other secondary metabolites, reducing the success of the penetration and establishment of the RKN J2s. Likewise, feeding of the caterpillar *Manduca sexta* (Lepidoptera: Sphingidae) on tomato plants reduced RKN galling in plants with both RKN and *M. sexta* (Martínez-Medina et al., 2021). This effect was attributed to jasmonate mediated induction of defense-related metabolites, such as glycoalkaloids in the roots, as a systemic response to the caterpillar feeding (Martínez-Medina et al., 2021). Our results could also be attributed to stress phytohormones that are induced by TSSM feeding, such as Abscisic Acid (ABA) and Ethylene (ET). LB plants infested by TSSM produced elevated ET, JA, and SA (Arimura et al., 2002). In a choice experiment for PPN using ET-insensitive mutant tomato plants (Never Ripe), were more attractive than wild type plants to *Meloydogine hapla* (Tylenchida: Heteroderidae) J2s, suggesting that ET or downstream products of ET signaling reduced the attractiveness to *M. hapla* (Fudali et al., 2013*)*. Infestation by TSSM led to the systemic accumulation of ABA in local and distant leaves of tomato plants (Gawronska and Kielkiewicz, 1999). ABA also

plays an important role in plant physiology during drought stress, leading for example, to stomatal closure to preserve water, which could affect the release of root exudates that are used as cues by the RKN to find suitable roots.

In the leaf disc choice test of TSSM, no significant differences were observed at 1 or 2 DPI in the preference or oviposition (Figs.6 a,b), but there were differences at 25 DPI (Fig. 6c). Since these experiments were performed with LB plants which is already an optimal host and used for the TSSM colony, for hundreds for generations. TSSM were fully adapted to LB, and the subtle differences in the leaves of the RKN infected plants were not enough to have a significant impact on the plant choice or oviposition by TSSM, perhaps because LB is an optimal host for the TSSM (van den Boom et al., 2003). The nutritional quality of the host plays an important role in the preference performance hypothesis, and at 25 DPI, RKN infected LB plants might have suffered from the reallocation of photosynthates to the RKN galls (McClure, 1977), even though the plants didn't show an apparent difference in leaf color or overall vigor. These nutritional differences between infected plants and clean plants may explain why the TSSM preferred to feed and oviposit on leaf discs taken from clean plants, which would be consistent with the preference performance hypothesis

The infection of tomato plant roots by RKN triggers a systemic signaling and production of reactive oxygen species (ROS), that travel from the roots to the shoots (Wang et al., 2019). This signaling system induces the biosynthesis of JA in the leaves, which is transported back to the roots aiding in the plant's defensive response against the RKN. Accumulation of JA in leaves peaks 24 h after inoculating the plant with RKN (Wang et al., 2019). Based on this information, we chose 1 DPI to add 1000 RKN J2 to the pot as the most likely point during the test to elicit plant-mediated effects aboveground for both tomato and LB plants in the two-way choice-test of TSSM.

The RKN treatment increased the settling of TSSM on tomato plants one DPI. Surprisingly, the EPN treatment was also more attractive to TSSM in tomato plants (Fig. 8 a-b). These results are consistent with the work done by Kammerhofer et al. (2015), where TSSM females preferred *Arabidopsis thaliana* plants that were inoculated with sugar beet cyst nematode, due to elevated levels of endogenous JA, SA and the auxin (indole-3-acetic acid, IAA), all of which were found in the CN inoculated plants at 1 DPI. The time frame selected for the TSSM behavioral assays after nematode inoculation matches the time

frame used for most of our experiments. This timing also matches the accumulation of JA in the leaves, which peaks at 24 h after inoculation the RKN in tomato plants (Wang et al., 2019), and could help explain the settling of TSSM on RKN infected plants. Similarly, LB plants that were treated with exogenous applications of SA and JA, were more attractive to TSSM females, in leaf disc experiments (Wei et al., 2014). Black mustard (*Brassica nigra*) plants infected with *Meloidogyne hapla,* were more attractive to the aphid *Brevicoryne brassicae* in a choice test compared to a clean plant (van Dam et al., 2018). In contrast, our experiments with two-way choice-test of TSSM on LB plants at 1 DPI didn't show a significant difference between treatments (Fig. 7, a), but at 7 DPI, TSSM preferred the clean plants over the RKN infected plants (Fig. 7, b). Differing levels of host plant susceptibility to RKN should be considered as a factor, and further studied in plant-mediated interactions, since the response of the TSSM varied between LB and tomato RKN plants. It would be reasonable to expect tomato plants to have a stronger initial plant-mediated response to the RKN infection than LB plants, considering that Rutgers tomato variety used for our experiments is highly susceptible to RKN.

Most species of nematodes studied produce a mixture of pheromones called ascarosides that regulate nematode behavior. Ascarosides are produced by EPNs and regulate dispersal (F. Kaplan et al., 2012), by free-living nematodes, such as *Caenorhabditis elegans* (Rhabditida: Rhabditidae), PPNs (RKN and cyst nematodes), and even parasites of mammals like *Pelodera strongyloides* (Rhabditida: Rhabditidae) (Choe et al., 2012). Ascarosides are widely produced by PPNs and have a variety of effects including repelling other nematode species and can also act as a plant immunity modulator (Manohar et al., 2020). Ascaroside #18 (10R)-10-[(3,6-dideoxy-alpha-L-arabino-hexopyranosyl) oxy] undecanoic acid) elicits plant defenses including SA and JA-mediated responses in leaves and roots and is the most abundant ascaroside among PPNs (Manosalva et al., 2015). *Steinernema carpocapsae* produces ascaroside #18 among others (Hartley et al., 2019). After assessing the tomato roots in the two-way choice-test of TSSM on tomato, we found *S. carpocapsae* IJs to be capable of penetrating the roots (Fig. 8c) in some of the replicates. This behavior has also been reported for other EPN species in the genus *Steinernema*, but not for *S. carpocapsae* (Fallon et al., 2002). Since not all the tomato plants inoculated with EPNs had nematodes inside the roots, ascarosides contained in the exometabolome in which the EPN IJs were contained, and inoculated to the plants, are likely to be driving these

plant-mediated interactions and therefore, eliciting plant responses that affected the TSSM response towards the inoculated plants.

In the non-choice performance test of TSSM, there was no strong effect of the RKN infection on TSSM reproduction on either LB or tomato (Fig. 9, a-b). Experiments with the green peach aphid (*M. persicae*) and RKNs in different plant hosts had contrasting results. On tobacco plants infected with RKN, the performance of the aphid was decreased (Kaplan et al., 2011) but a different study of the same system found contrasting results; with infection by *Meloidogyne arenaria* increased the reproduction of *M. persicae* on tobacco (Li et al., 2020). RKN infection of tomato plants increased *Spodoptera exigua* (Lepidoptera: Noctuidae) performance during galling stage of RKN (15 DPI), but not at invasion (5 DPI) or reproduction (30 DPI)**.** They saw as the reason for the increased caterpillar performance, a higher C/N ratio in the leaves of RKN infected plants at galling stage (Mbaluto et al., 2021).

As the RKN infection develops, the nematode suppresses plant defenses in a systemic manner, which affects aboveground tissues (Mbaluto et al., 2021). In *Arabidopsis,* genes related to the expression of SA and JA were down-regulated in the leaves of RKN-infected plants, at 5-14 DPI (Hamamouch et al., 2011). In our non-choice performance test TSSM were exposed at the beginning to an adverse situation with the plant expressing a defensive response to the RKN, expressing JA response in the leaves, that should have negatively affected TSSM performance. As the RKN infection progressed and suppressed the plant defenses this could have benefited the TSSM. Our method of assessing performance could have missed fine-grained changes in plant quality.

The results of these interactions differ for each herbivore and can be negative or positive to different trophic levels, depending on the perspective in which the system is studied (van Dam & Heil, 2011). In a higher trophic level, the carnivorous *Phytoseiulus persimilis* (Acari: Phytoseiidae) which is a specialist predatory mite that feeds on TSSM, significantly preferred LB plants that were exposed to TSSM, and preferred LB plants that were treated with methyl-jasmonate over non-infested plants (Dicke et al., 1999).

Plant-mediated interactions between below and aboveground communities are extremely difficult to predict since the complexity of these interactions depends on many factors. Since high density infestations of TSSM cause serious injury to plants and result in defoliation or even the death of the plant (Helle and Sabelis, 1985). In less severe TSSM infestations, plant-mediated responses could impact belowground herbivores, as RKN infections can debilitate the plant and lower the yield.

Studying the effects on the plant (and plant-mediated effects) on below and aboveground microbial and arthropod communities at different trophic levels can provide valuable information in creating sustainable agricultural management systems, since generally growers know the history of pest problems that they have had, and based on these type of studies, it would be reasonable to expect a certain outcome for other major pests, either below or aboveground, providing information to manage better and more efficient way.

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