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Identification of Key Root Volatiles Signaling Preference of Tomato over Spinach by the Root Knot Nematode *Meloidogyne incognita*

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ABSTRACT: The root knot nematode, *Meloidogyne incognita* (Kofoid and White) Chitwood, is a serious pest of tomato (*Solanum lycopersicum)* and spinach (*Spinacea oleracea*) in sub-Saharan Africa. In East Africa these two crops are economically important and are commonly intercropped by smallholder farmers. The role of host plant volatiles in *M. incognita* interactions with these two commodities is currently unknown. Here, we investigate the olfactory basis of attraction of tomato and spinach roots by the infective second stage juveniles (J2s) of *M. incognita*. In olfactometer assays, J2s were attracted to root volatiles from both crops over moist sand (control), but in choice tests using the two host plants, volatiles of tomato roots were more attractive than those released by spinach. Root volatiles sampled by solid phase microextraction (SPME) fiber and analyzed by gas chromatography/mass spectrometry (GC/MS) identified a total of eight components, of which five (2-isopropyl-3-methoxypyrazine, 2-(methoxy)-3-(1-methylpropyl)pyrazine, tridecane, and α- and β-cedrene) occurred in the root-emitted volatiles of both plants, with three (δ-3-carene, sabinene, and methyl salicylate) being specific to tomato root volatiles. In a series of bioassays, methyl salicylate contributed strongly to the attractiveness of tomato, whereas 2-isopropyl-3-methoxypyrazine and tridecane contributed to the attractiveness of spinach. *M. incognita* J2s were also more attracted to natural spinach root volatiles when methyl salicylate was combined than to spinach volatiles alone, indicating that the presence of methyl salicylate in tomato volatiles strongly contributes to its preference over spinach. Our results indicate that since both tomato and spinach roots are attractive to *M. incognita*, identifying cultivars of these two plant species that are chemically less attractive can be helpful in the management of root knot nematodes.

KEYWORDS: *Kairomone, Meloidogyne sp., root volatiles, semiochemical, Solanum lycopersicum, Spinacea oleracea*

INTRODUCTION

Tomato (*Solanum lycopersicum* L.) (Solanaceae) and spinach (*Spinacea oleracea* L.) (Amaranthaceae) are economically important vegetable crops and commonly intercropped by smallholder farmers in East Africa. Both crops are common hosts for plant parasitic nematodes such as root knot nematodes (RKNs) (*Meloidogyne* spp.) with infection causing up to 50−100% yield losses in both plants.[1] The infective second stage juvenile (J2), which is the host seeking stage, of RKNs attack host plants to exploit the plant nutrients for development,[2,3] causing damage and suppressing plant growth.[4] Plants that are infected with RKNs are also more susceptible to infection by soil and foliar pathogens, leading to additional physical and economic damage.[5] Smallholder farmers in Africa are often unaware of RKNs or the damage they cause directly but recognize reducing yields and therefore use crop rotation to manage the situation.[6]

Despite the economic importance of these crops, there is limited knowledge of the efficacy of plant volatiles in the management of RKNs. In a recent study on RKN−plant root interactions, we demonstrated the importance of olfactory cues mediating the host-seeking process of the tropical species *Meloidogyne incognita* (Kofoid and White) Chitwood.[7] We identified α-pinene, limonene, 2-methoxy-3-(1-methylpropyl)-pyrazine, methyl salicylate, and tridecane as occurring in the root volatiles of four different pepper cultivars, *Capsicum annuum* L. (Solanaceae). These compounds were found to be important components of the attractant blend, especially methyl salicylate, which was identified as a key attractant for *M. incognita*. On the other hand, thymol, identified as specific to one of the cultivars (AVDRC PP0237), elicited avoidance behavior by *M. incognita*. This indicates that disrupting the host location process using different repellent plants may lead to the semiochemical management of RKNs. Moreover, smallholder farmers in Africa could benefit from this approach since it is compatible with their crop production practices and would provide a cost-effective and environmentally sensitive option to control plant parasitic nematodes.

In addition to our previous findings on the interaction between RKNs and *Capsicum*, in the current study we

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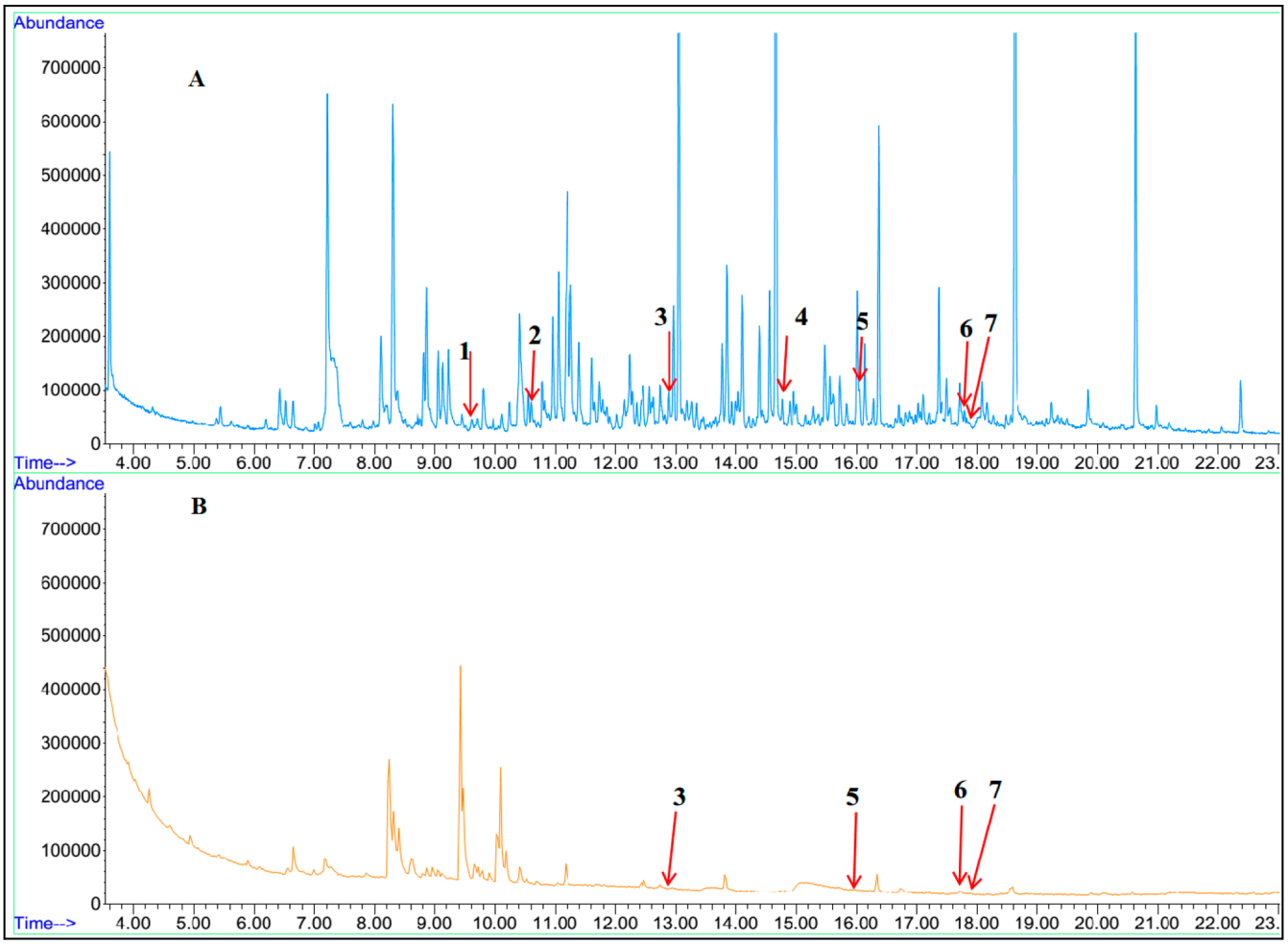

Figure 1. GC/MS chromatogram showing identified root volatiles of (A) tomato and (B) spinach collected with Super Q adsorbent traps: δ-3-carene (**1**)*, sabinene (**2**), 2-isopropyl-3-methoxypyrazine (**3**), 2-methoxy-3(1-methylpropyl)pyrazine (**4**), methyl salicylate (**5**), tridecane (**6**), α-cedrene (**7**)*, and β-cedrene* (* = tentatively identified).

hypothesized that root-emitted volatiles of two commonly intercropped host crops, tomato and spinach, influence the host preference of RKNs. We tested this hypothesis using a bioassay-guided chemical analysis to (i) evaluate the response of *M. incognita* to root volatiles of tomato and spinach and (ii) identify root volatile components mediating these responses.

MATERIALS AND METHODS

Host Plants. Tomato (cv. "Cal J") and spinach (cv. "Fordhook Giant") seeds were purchased locally (Simlaw Seeds Company Ltd., Nairobi, Kenya), surface sterilized in 1% sodium hypochlorite for 10 min, and rinsed three times in distilled water for 5 min. They were sown in seed-growing trays (24 cells) containing sterile sand (autoclaved at 121 °C for 40 min) and maintained in a screen house (27 ± 2 °C; 60−70% RH and 12:12 h L:D photoperiod) at the International Centre of Insect Physiology and Ecology (*icipe*), Duduville Campus, Nairobi (01° 13′ 25.3″ S, 36° 53′ 49.2″ E; 1600 m ASL). Seedlings were transplanted into sterile sand in 5 L plastic pots (29 cm depth) at 4 weeks after sowing. Plants were watered daily with a nutrient solution (macronutrients: calcium nitrate tetrahydrate 653 g/L; magnesium sulfate heptahydrate 399 g/L; potassium nitrate 184 g/L; ammonium phosphate dibasic 108 g/L and iron(II) sulfate heptahydrate, 10 g + 72 mL of ethylenediaminetetraacetic acid (pH 4) /L; and the micronutrients: manganese(II) chloride tetrahydrate 1.81 g/L; copper(II) sulfate pentahydrate 0.1 g/L; zinc sulfate heptahydrate 0.22 g/L; boric acid 2.86 g/L; molybdic acid 0.02 g/L).[7,8] Plants were used for the experiments 2−3 weeks after transplanting.

Nematodes. A pure culture of *M. incognita* was provided by George Kariuki (Department of Agricultural Science and Technology, Kenyatta University), which originated from a single female egg mass collected from tomato grown in Taita Taveta County (3.3161° S, 38.4850° E), Coastal Region, Kenya, and subsequently maintained on spinach cv. "Fordhook Giant" in a screen house (27 ± 2 °C, 60−70% RH, with 12:12 h L:D photoperiod) at *icipe*, using a single egg mass to inoculate individual pots. At 6 weeks after inoculation, plants with galled root systems were removed and used to collect egg masses. Plants were established and nourished with a nutrient solution as described above. Roots were gently rinsed free of sand, stained with Phloxine B (0.15 g/L water) for 20 min to highlight the egg masses,[9] and then roots were destained and rinsed under running tap water for 5 min and placed in distilled water. Egg masses were individually removed from roots using a fine needle under a stereomicroscope (Leica M125, Leica microsystems, USA), placed in 24-well culture plates filled with 2 mL of distilled water and placed in a dark cabinet for 2−5 days until the J2s hatched. Since J2s emerged from the egg within 2 days,[10] on day 4, they were collected using a 1 mL plastic transfer pipet to a counting dish and numbers determined under a stereomicroscope. They were thereafter transferred into 15 mL falcon tubes containing 1 mL of distilled water until their use for bioassays.

Dual-Choice Assays with Tomato and Spinach. The response of *M. incognita* J2s to root-emitted volatiles of tomato and spinach was tested in a dual-choice olfactometer as described previously.[7] For the assays, 10 plants of either tomato or spinach were placed in growth

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chambers (85 mm diameter × 140 mm depth) containing 300 g of sterilized sand for 4−5 days prior to the experiment in a room maintained at 23 ± 2 °C and 65 ± 5% RH. They were watered with 20 mL of the nutrient solution daily. The control growth chamber contained 300 g of sterile sand moistened with 50 mL of nutrient solution. Nematodes were assayed for plant odor perception to the two host plants in the same room using two methods: (i) each plant species was assayed against a control (sterilized moist sand), and (ii) the two plants were assayed against each other in pairwise sets. Prior to assays, J2s were tested with sterile moist sand in paired growth chambers to compensate for asymmetry and exclude bias in the setup. A total of five replicates each comprising 500 J2s were used in the experiments. After 4 h, the olfactometer was disassembled, and nematodes in each detachable collection section[7] were recovered over 24 h using a modified Baerman sieving technique[9] and counted under a stereomicroscope. The olfactometer was afterward cleaned using Teepol detergent, rinsed with acetone, and dried in an oven at 70 °C for 24 h for use in the subsequent bioassay with a fresh batch of moist sand.

Identification of Host Plant Root-Derived Volatiles. Ten 2−3-week-old tomato and spinach plants were brought to the laboratory and maintained in glass chambers with moistened sterile sand for 3−5 days prior to the experiments. Thereafter, they were gently uprooted and their roots washed under running tap water and rinsed with distilled water. For the analyses of volatiles, the roots of respective plants were cut off at the base of the stem under liquid nitrogen[11] to increase detection of compounds identified in trace amounts by gas chromatography/mass spectrometric (GC/MS) analysis. A preliminary GC/MS analysis of volatiles sampled for 24 h on precleaned (dichloromethane and white spot nitrogen dried) Super Q (30 mg; Analytical Research System, Gainesville, FL) traps attached to a steel probe (17 cm long; 0.5 cm i.d.; vacuum 170 mL/min) inserted into the sterilized sand near the plant's root zone in the plant growth chamber[7] was dominated by sand-associated contaminants and trace amounts of root volatiles (Figure 1). The frozen roots were then pulverized in a precooled mortar in liquid nitrogen within 1 min, and 0.5 g of the resulting powder placed in an airtight clean glass beaker cooled by immersing in a box of ice and covered with 3−4 layers of aluminum foil held tightly to the beaker with three tight-fitting rubber bands. To adsorb the volatiles, a precleaned (via thermal desorption at 250 °C for 30 min to remove any ambient contaminants) 65 μm polydimethylsiloxane/divinylbenzene (PDMS/DVB) solid phase microextraction (SPME) fiber (Supelco, Bellefonte, USA) was inserted through the aluminum foil into the beaker immersed in ice for 1 h at 25 ± 2 °C. The experiment comprised three replicates with roots from 10 plants comprising one replicate.

The SPME collected volatiles were analyzed using a GC/MS with a HP-7890A series gas chromatograph (Agilent Technologies, Wilmington, DE) linked to a HP 5975C mass spectrometer (Agilent, Wilmington, DE) operated in the electron ionization mode. The fiber was inserted manually into the injector port (250 °C), desorbed, and chromatographed on a nonpolar HP-5MS capillary column (30 m × 0.25 mm i.d.; 0.25 μm film thickness, J & W Scientific, Folsom, USA) with 5%-phenyl methyl polysiloxane as the stationary phase. Helium was used as the carrier gas at 1.2 mL/min. After fiber insertion, the column temperature was maintained at 35 °C for 5 min, increasing to 280 °C at 10 °C/min. The injector and the detector were held isothermal at 280 °C for 10.5 min. The ion source temperature was 230 °C while electron ionization mass spectra were acquired at 70 eV within a mass range of 38−550 Da (Da) during a scan time of 0.73 scans/s. Volatile compounds were identified using their retention times and mass fragmentation spectra against authentic standards of 2-isopropyl-3-methoxypyrazine and 2-(methoxy)-3-(1-methylpropyl)-pyrazine, sabinene, methyl salicylate, and tridecane analyzed similarly. Quantification was based on calibration curves (peak area vs concentration) generated from authentic standards of identified compounds. The GC/MS conditions for quantitative analyses including injection operation of the standards, capillary column dimensions, and oven temperature were the same as those for host−root volatile analysis. Three components (δ-3-carene, α- and β-cedrene) were identified based on mass spectral library data from Adams[12] and National Institute of Standards and Technology (NIST)[13] (MSD ChemStation E.02.00.493, MS HP, USA) only. Retention times obtained from GC/MS analysis of C8−C31 *n*-alkane standards and tomato and spinach volatiles were used to determine retention indices of identified compounds.[14−16]

Chemicals. The synthetic standards including sabinene, 2-isopropyl-3-methoxypyrazine (IPMP), 2-(methoxy)-3-(1-methylpropyl)pyrazine (MPP), and tridecane were purchased from Sigma-Aldrich, St. Louis, MO. Methyl salicylate was purchased from Sigma-Aldrich, Steinhelm, Germany. The purity of all the chemicals was ≥97%.

Olfactory Choice Assays Using Synthetic Root Volatile Chemicals. To determine which of the compounds identified from the tomato or spinach root volatiles contributed to the behavioral responses in *M. incognita* J2s, we tested synthetic standards using the same olfactometer and procedure described in the dual-choice assays. To quantify nematode response to individual compounds, stock solutions of 100 ng/μL (for 2-isopropyl-3-methoxypyrazine and 2-(methoxy)-3-(1-methylpropyl)pyrazine) and 10 000 ng/μL (for sabinene, methyl salicylate, and tridecane) identified in either tomato or spinach were prepared in hexane as a solvent. The other three compounds (δ-carene, α- and β-cedrene) were not commercially available. Three final concentrations tested were 40, 80, and 160 ng/μL of the respective compounds that were prepared using the stock as the initial concentration and a volume that correlated with the test concentration (Table 1). Similarly, a blend was prepared for the respective compounds using the stock concentration for the initial volume and their ratio in the blend determined (Table 2). From respective compounds being tested, 50 μL was dispensed using a micropipette to the center of the plant growth chamber containing 300 g of sterile sand. The control side was loaded with a similar volume of hexane only. The number of nematodes that migrated to each respective side was determined as in the dual-choice assays after

Table 1. Authentic Standards Showing the Concentrations (ng/μL) and Volumes (μL) of Individual Compounds Used To Prepare and Test the Concentrations of 40, 80, and 160 ng/μL in Bioassays

compounds	stock concentration (ng/μL)	volume picked from stock (μL)	working concentration (ng/μL)
sabinene	10000	5	10
2-isopropyl-3-methoxypyrazine	100	500	10
2-methoxy-3-1 (-methylpropyl) pyrazine	100	500	10
methyl salicylate	10000	5	10
tridecane	10000	5	10

Table 2. Authentic Standards Showing the Concentrations (ng/μL), Volumes (μL), and Ratio of Compounds in the Blend Used To Prepare and Test the Concentrations 40, 80, and 160 ng/μL in Bioassays

compounds	volume (μL) picked from the stock solution	concentration of compound in the blend (ng/μL)	ratio of compound in the blend
sabinene	3.5	7.0	70
2-isopropyl-3-methoxypyrazine	1.5	3.0	30
2-methoxy-3-1 (-methylpropyl) pyrazine	6.5	13.0	130
methyl salicylate	10	20.0	200
tridecane	3.5	7.0	70

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4 h at 23 ± 2 °C. The most attractive individual compounds were used further in paired bioassays.

Further dual-choice assays were conducted to examine the importance of methyl salicylate (MeSA), which elicited the highest attraction among the single compounds (see results section Identification of the Host Plant Attractant) in J2 attraction. Ten spinach plants were conditioned for 7 days in a plant growth chamber with 300 mg of sterilized sand and nourished with the nutrient solution. A 50 μL portion of MeSA of respective concentrations of 0.25, 5, and 10 ng/μL was added at the root zone of spinach plants using a micropipette. The control spinach plants received no MeSA. A total of 500 J2s were introduced at the center of the dual-choice olfactometer (as described previously in Dual-Choice Assays with Tomato and Spinach) and counted after 4 h under a stereomicroscope.

Statistical Analysis. The number of nematodes responding to different treatments in the dual-choice olfactometer assays was recorded as means and expressed as percent response according to the formula $[(n/N) \times 100]$, where n is the number of J2s responding to a given treatment, while N is the total number of responding J2s.[7] Non-responding J2s were not included in the analysis. Data from the dual-choice assays were subjected to Chi-square (χ^2) goodness-of-fit analysis testing the hypothesis that nematode choice of odors was in the ratio 1:1. All statistical analyses were conducted in R software version 3.0.2.[17]

RESULTS AND DISCUSSION

Host Root Volatiles Attract *Meloidogyne incognita* Infective Juveniles. In olfactometer assays, J2s of *M. incognita* significantly preferred both tomato (72.3%, χ^2 = 83.75, df = 1, $P < 0.001$) and spinach (66.4%, χ^2 = 46.14, df = 1, $P < 0.001$) root volatiles relative to the control (moist sand) (Figure 2a). In the paired assays, J2s significantly preferred the root volatiles of tomato over spinach (65.2%, χ^2 = 71.32, $P < 0.001$) (Figure 2a). These results suggest that olfactory cues play a role in host attraction and that chemoreception and host discrimination in J2s are host plant dependent. The results of our paired assays also demonstrated that maintaining *M. incognita* cultures on spinach did not trigger learning behavior in J2s. It has been shown in previous studies that plant herbivores differ in their responses to different species, which is due to differences in plant chemistry.[18−20] Recently, we showed that *M. incognita* J2 responses to root volatiles varied with different pepper (*Capsicum annuum*) cultivars.[7] As such, J2 host selection may be dependent not only on the chemistry of host plant species cultivar and host plant developmental stage but also on soil factors including temperature and moisture. Therefore, experimental work is needed to fully understand the importance of these factors in host discrimination and selection by J2s. Nonetheless, in the present study the strong J2 preference for the root volatiles of tomato over spinach suggests either a qualitative or quantitative variation, or both, in the root volatile chemistry of the two host plants.

Analysis of Root Volatiles. Coupled GC/MS analysis of tomato and spinach root volatiles collected on Super Q showed mainly sand-associated contaminants and trace amounts of root volatiles. However, using selected ion monitoring (SIM) mode (m/z 93, 121, 136, 161, 204 for terpenes, m/z 124, 137/138 for pyrazines), several volatiles including α-pinene, β-pinene, limonene, 2-isopropyl-3-methoxypyrazine, 2-(methoxy)-3-(1-methylpropyl)pyrazine, tridecane, and α- and β-cedrene were detected in both tomato and spinach root volatiles, with δ-3-carene, sabinene, camphene, 6-methyl-5-hepten-2-one, myrcene, β-ocimene, and methyl salicylate detected in tomato root volatiles, and the sesquiterpene geosmin detected in spinach root volatiles. Eight of these components including the monoterpenes δ-3-carene and sabinene, the pyrazines 2-isopropyl-3-methoxypyrazine and 2-(methoxy)-3-(1-methylpropyl)pyrazine, the benzenoid methyl salicylate, the hydrocarbon tridecane, and sesquiterpenes α- and β-cedrene were detected in the SPME collected root volatiles of tomato and spinach (Figures 1, 2b; Table 3). Five components (2-isopropyl-3-methoxypyrazine, 2-(methoxy)-3-(1-methylpropyl)pyrazine, tridecane, and α- and β-cedrene) were found to occur in the root volatiles of the two host plants in varying quantities, whereas the three compounds δ-3-carene, sabinene, and methyl salicylate were specific to tomato root volatiles. Four (2-isopropyl-3-methoxypyrazine, tridecane,and α- and β-cedrene) of the shared volatile components were twice as abundant in the root volatiles of tomato than in spinach, while 2-(methoxy)-3-(1-methylpropyl)pyrazine was 33 times more abundant in spinach root volatiles than that of tomato (Figure 2b; Table 3). These results indicate that sample preparation and volatile collection methods determine the composition of volatiles detected, suggesting that the results obtained in the current study should be treated with caution. In the current study, the advantage of using Super Q trap is that, unlike SPME, chemical analysis shows that it is compositionally richer, reflecting the natural situation, but the disadvantage is that it is less precise in the quality of volatiles captured because of the nature of sample used. On the other hand, sampling by SPME improved the quantity of volatiles captured, but the disadvantage is that

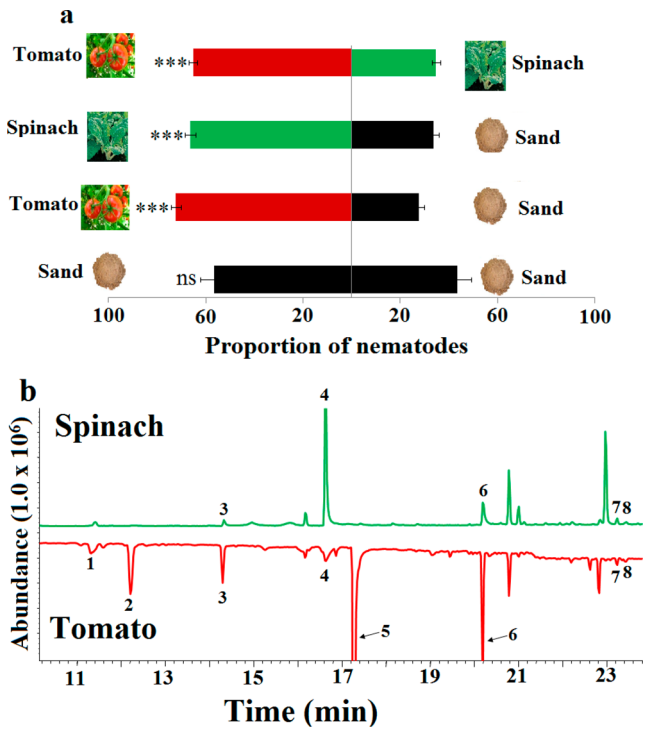

Figure 2. Response of *Meloidogyne incognita* juveniles (J2s) to host plant roots: (a) olfactometer response to tomato and spinach roots compared to a control (moist sand) and (b) chemical composition of tomato and spinach roots volatiles collected after crashing roots in liquid nitrogen: δ-3-carene (**1**)*, sabinene (**2**), 2-isopropyl-3-methoxypyrazine (**3**), 2-methoxy-3(1-methylpropyl)pyrazine (**4**), methyl salicylate (**5**), tridecane (**6**), α-cedrene (7)*, and β-cedrene* (* = tentatively identified).

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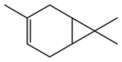
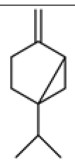
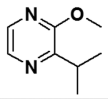
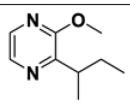
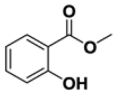
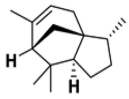
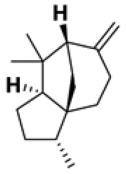
Table 3. Compounds Detected in Super Q and SPME Collected from Tomato (cv. Cal-J) and Spinach (cv. Fordhook Giant) Root Volatiles by GC/MS[a,b]

No.	Compound	Structure	Retention time (min) Super Q	Retention index	Retention time (min) SPME	Mean amount detected (ng/plant/h ± SE)	
						Tomato	Spinach
1	δ-3-carene*		9.52	908	11.31	2.5 ± 1.39	-
2	sabinene[1]		10.64	958	11.66	15.3 ± 5.30	-
3	2-isopropyl-3-methoxypyrazine[1]		11.61	1002	14.29	13.9 ± 2.91	6.7 ± 0.07
4	2-methoxy-3(1-methylpropyl) pyrazine[1]		12.88	1079	16.89	2.4 ± 0.35	78.8 ± 3.34
5	methyl salicylate[1]		14.65	1184	17.41	34.1 ± 3.25	trace
6	tridecane[1]		16.00	1271	20.18	26.5 ± 0.91	12.2 ± 3.66
7	α-cedrene*		17.71	1393	23.23	3.9 ± 1.64	1.6 ± 0.23
8	β-cedrene*		17.82	1401	23.46	1.4 ± 0.66	0.6 ± 0.02

[a]Retention time and retention indices calculated for Super Q trapped volatiles identified in selected ion monitoring (SIM) mode. Mean amount detected (ng/plant/h ± SE) for SPME collected volatiles. – not detected. [b]*Identified by GC/MS library data only; [1]compounds identified by library data and authentic samples.

because samples were crushed in liquid nitrogen, the amounts of volatiles captured may not necessarily reflect the natural situation. Future experiments should be designed to compare both methods in the same setting to optimize volatile collection. However, it is important to note that using both methods increases the precision of volatiles captured and detected.

From an ecological perspective, these results not only confirm our earlier suggestion about diversity in interspecies host plant chemistry but also are consistent with those of our previous studies, which reported that differences in intraspecies chemistry also contribute to J2 host discrimination and selection process.[21,22] Recently, we identified some of these compounds in pepper root volatiles, which is another solanaceous plant, including α-pinene, limonene, β-ocimene, 2-(methoxy)-3-(1-methylpropyl)pyrazine, tridecane, and methyl salicylate.[7] The presence of 2-isopropyl-3-methoxypyrazine in the root volatiles of tomato but not that of pepper cultivars could be due to differences in either volatile collection methods, species, or cultivar differences.[23] Further research is necessary to compare and understand the role that intra- and interspecies root volatiles play in host location in J2s.

Identification of the Host Plant Attractant. In bioassays, with available host plant-specific (sabinene and methyl salicylate) and shared (2-isopropyl-3-methoxypyrazine, 2-(methoxy)-3-(1-methylpropyl)pyrazine and tridecane) components, J2s responded differently to three concentrations of a blend of these five components (specific and shared) and individual compounds against a control (moist sand). J2s showed a concentration-dependent preference to the blend at 40 ng/μL (68%, χ^2 = 55.95, df = 1, P < 0.0001), 80 ng/μL (76%, χ^2 = 103.44, df = 1, P < 0.0001), and 160 ng/μL (85%, χ^2 = 206.04, df = 1, P < 0.0001) relative to the control (Figure 3a). In assays, with individual components, a concentration-dependent response was observed relative to control for the following: sabinene, at 40 ng/μL (48%, χ^2 = 0.42, df = 1, P = 0.5188), 80 ng/μL (56%, χ^2 = 4.72, df = 1, P = 0.0298), and 160 ng/μL (60%, χ^2 = 14.44, df = 1, $P \leq$ 0.0001) (Figure 3b); 2-isopropyl-3-methoxypyrazine at 40 ng/μL (62%, χ^2 = 28.28, df = 1, P < 0.0001), 80 ng/μL (64%, χ^2 = 31.44, df = 1, P < 0.0001), and 160 ng/μL (65%, χ^2 = 38.73, df = 1, P < 0.0001)

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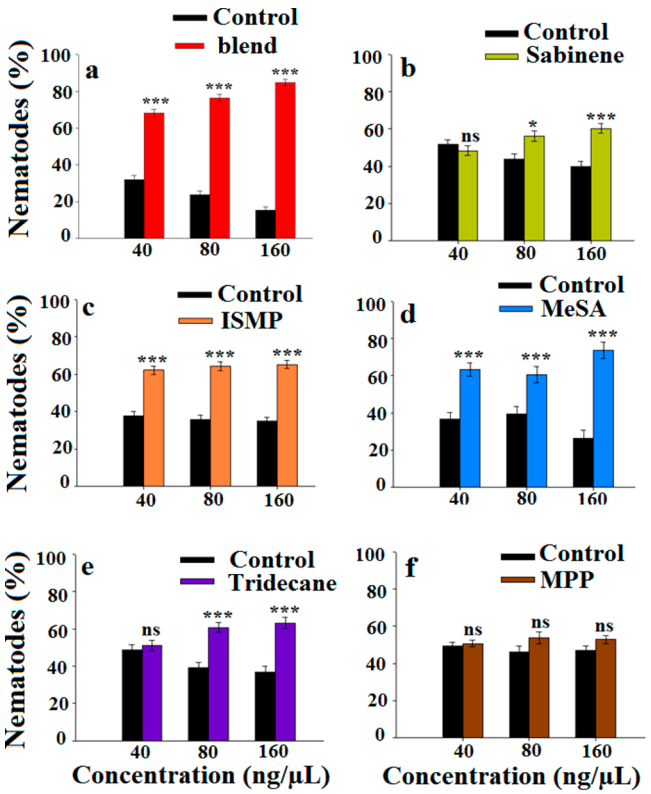

Figure 3. Response of *Meloidogyne incognita* juveniles to the blend and five individual compounds identified from roots of tomato and spinach at three different concentrations compared with the control: (a) blend vs control; (b) sabinene vs control; (c) 2-isopropyl-3-methoxypyrazine (IPMP) vs control; (d) methyl salicylate vs control; (e) tridecane vs control; (f) 2-(methoxy)-3-(1-methylpropyl)pyrazine (MPP) vs control; * = 0.05; *** = 0.0001; ns = not significant.

(Figure 3c); and methyl salicylate at 40 ng/μL (63%, χ^2 = 11.46, df = 1, P = 0.0007), 80 ng/μL (61%, χ^2 = 14.46, df = 1, $P \leq 0.0001$), and 160 ng/μL (74%, χ^2 = 21.37, df = 1, P < 0.0001) (Figure 3d), whereas at 40 ng/μL (51%, χ^2 = 0.15, df = 1, P = 0.7017) tridecane was not preferred; J2s preferred the compound at 80 ng/μL (61%, χ^2 = 13.12, df = 1, P = 0.0003) and 160 ng/μL (63%, χ^2 = 17.17, df = 1, P < 0.0001) (Figure 3e). There was no significant preference of J2s to 2-(methoxy)-3-(1-methylpropyl)pyrazine at the three concentrations 40 ng/μL (49%, χ^2 = 0.08, df = 1, P = 0.7761), 80 ng/μL (46%, χ^2 = 1.33, df = 1, P = 0.2482), and 160 ng/μL (47%, χ^2 = 1.57, df = 1, P = 0.2100) relative to the control (Figure 3f). These results indicate that both the shared- and host plant-specific chemicals contribute to J2 response to tomato and spinach root volatiles over the control, but more importantly, the species-specific chemicals acting alone or as a blend may strongly contribute to the preference of tomato over spinach. Of the pyrazines, the differential response of J2s to 2-isopropyl-3-methoxypyrazine and 2-(methoxy)-3-(1-methylpropyl)pyrazine may be associated with differences in their vapor pressures. On the other hand, methyl salicylate is structurally similar to 2-(methoxy)-3-(1-methylpropyl)pyrazine, but it elicited the highest concentration-dependent response from J2s among the compounds tested, suggesting that substituting the alkyl side chain on the aromatic moiety with an ester group may be optimal for eliciting chemotaxis in J2s. These results also suggest that aromaticity in the molecule may be required for J2 response. However, additional research is needed to explore a wide range of aromatic analogues in *M. incognita* responses.

In bioassays with the four components that elicited attraction in J2s (sabinene, 2-isopropyl-3-methoxypyrazine, methyl salicylate, and tridecane), pairwise comparisons identified methyl salicylate as the most preferred over sabinene at 40 ng/μL (64%, χ^2 = 27.19, df = 1, P < 0.0001), 80 ng/μL (70%, χ^2 = 56.17, df = 1, P < 0.0001), and 160 ng/μL (79%, χ^2 = 128.98, df = 1, P < 0.0001) (Figure 4a); 2-isopropyl-3-methoxypyrazine at 40 ng/μL (60%, χ^2 = 13.79, df = 1, P = 0.0002), 80 ng/μL (63%, χ^2 = 32.36, df = 1, P < 0.0001), and 160 ng/μL (70%, χ^2 = 43.2, df = 1, P < 0.0001) (Figure 4b); and tridecane at 40 ng/μL (69%, χ^2 = 59.82, df = 1, P < 0.0001), 80 ng/μL (70%, χ^2 = 61.62, df = 1, P < 0.0001), and 160 ng/μL (78%, χ^2 = 129.25, df = 1, P < 0.0001) (Figure 4c). These results confirm the significance of the chemical nature, composition, and concentration in J2 attraction, and it appears that in some of the assays all the three variables were vital. Previous studies have shown that these variables play different roles in below-ground plant−herbivore interactions. For example, the sesquiterpene (*E*)-β-caryophyllene, induced by feeding of the Western corn rootworm, *Diabrotica virgifera virgifera* LeConte, on maize roots, attracted the entomopathogenic nematode, *Heterorhabiditis megidis*, Poinar, Jackson, and Klein.[11] Another study showed that field applied sesquiterpene, pregeijerene, which is also a herbivore-induced volatile, increased mortality of the root weevil, *Diaprepes abbreviates* L., by attracting naturally occurring entomopathogenic nematodes.[24] In the current study, taking chemical nature, composition, and concentration together, of the four attractants, methyl salicylate stood out as the potent attractant, which is consistent with our previous study with J2s interacting with the root volatiles of pepper.[7]

Relative Importance of Methyl Salicylate in Host Plant Attraction. Nematodes preferred methyl salicylate at 40 ng/μL (63%, χ^2 = 9.51, df = 1, P = 0.002), 80 ng/μL (67%, χ^2 = 23.90, df = 1, P < 0.0001), and 160 ng/μL (71%, χ^2 = 40.56, df = 1, P < 0.0001) relative to tomato (Figure 4d). Nematode responses were significantly higher in spinach spiked with methyl salicylate at 2.5 ng/μL (56%, χ^2 = 6.66, df = 1, P = 0.009), 5 ng/μL (63%, χ^2 = 29.52, df = 1, P < 0.0001), and 10 ng/μL (77%, χ^2 = 109.39, df = 1, P < 0.0001) relative to the control (spinach without methyl salicylate) (Figure 4e). In addition, J2s preferred a four-component blend (sabinene, 2-isopropyl-3-methoxypyrazine, 2-(methoxy)-3-(1-methylpropyl)pyrazine and tridecane) without methyl salicylate at 40 ng/μL (60%, χ^2 = 14.53, df = 1, $P \leq 0.0001$), 80 ng/μL (70%, χ^2 = 46.89, df = 1, P < 0.0001), and 160 ng/μL (80%, χ^2 = 100, df = 1, P < 0.0001) when compared to a control (sand) (Figure 4f). However, when the four-component blend without methyl salicylate was compared to a full blend (four-component blend plus methyl salicylate), J2s preferred the full blend at 40 ng/μL (55%, χ^2 = 4.91, df = 1, P < 0.0001), 80 ng/μL (60%, χ^2 = 24.89, df = 1, P < 0.0001), and 160 ng/μL (59%, χ^2 = 19.54, df = 1, P < 0.0001) (Figure 4g). These results clearly show that the attractiveness of tomato over spinach is mainly due to the presence of methyl salicylate, while that of spinach is due to 2-isopropyl-3-methoxypyrazine and tridecane. The fact that in bioassays J2s responded to blends and individual compounds belonging to different classes suggests a potential wide host range for RKNs. However, the presence or absence of specific compounds in the root volatile blend may determine host attraction.[25] Results of our previous

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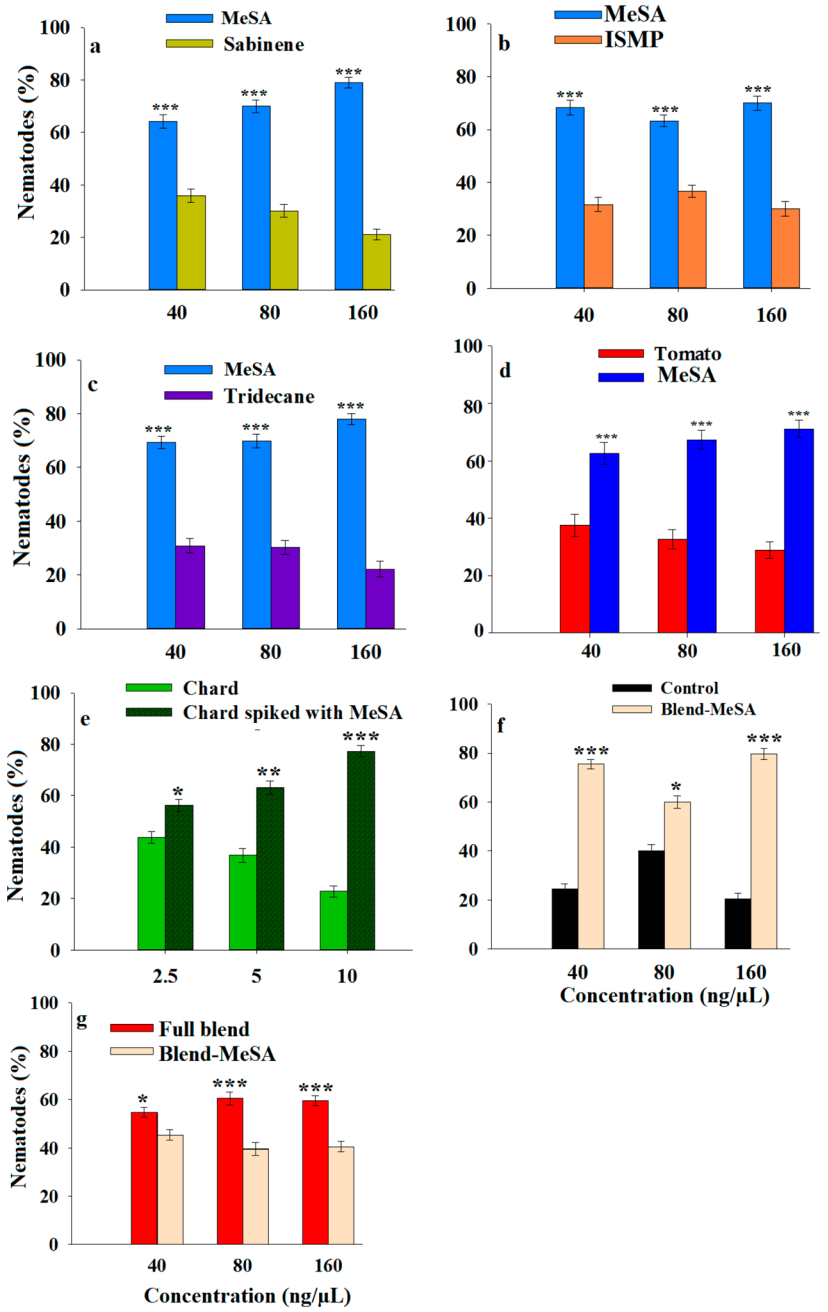

Figure 4. Response of *Meloidogyne incognita* juveniles to volatile compounds identified from roots of tomato and spinach relative to methyl salicylate: (a) response to sabinene relative to MeSA; (b) response to 2-isopropyl-3-methoxypyrazine (ISMP) relative to methyl salicylate (MeSA); (c) response to tridecane relative to MeSA; (d) response to tomato root volatiles relative to MeSA; (e) response to spinach spiked with MeSA relative to natural spinach root volatiles; (f) response to full blend of volatiles without MeSA relative to control (sand) volatiles; and (g) response to full blend without MeSA relative to full blend with MeSA. * = 0.05; *** = 0.0001; ns = not significant.

(pepper) and current (tomato/spinach) studies confirm the important role benzenoids such as methyl salicylate and thymol play in J2 differential host selection-enhanced attraction in the presence of methyl salicylate but avoidance in the presence of thymol,[7] showing that these two compounds are good candidates for further investigation toward RKN management. Additionally, future work should also investigate the role of δ-2-carene and α- and β-cedrene identified in the root volatiles of the two host plants in RKN host discrimination and selection.

Noteworthy, some of the compounds identified in this study have been reported to mediate various plant−herbivore interactions. For example, sabinene, a component of floral volatiles of bitter gourd, *Momordica charantia* L. (Cucurbitaceae), is attractive to the Epilachna beetle *Epilachna dodecastigma* (Weid.) (Coleoptera: Coccinellidae).[26] The pyrazine, 2-isopropyl-3-methoxypyrazine, produced by the multicolored Asian lady beetle *Harmonia axyridis* (Pallas) contributes to off-flavors in wine.[27] It has also been reported as a semiochemical-inducing oviposition in the peach tree borer, *Synanthedon exitiosa* (Say) (Lepidoptera: Sesiidae).[28] The hydrocarbon tridecane, a volatile associated with the brown marmorated stink bug, *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae), acts as a kairomone for the predaceous minute

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pirate bug, *Orius insidiosus* (Say) (Hemiptera: Anthocoridae).[29] In addition to being a below-ground nematode attractant,[30] methyl salicylate has been found to be an herbivore-induced volatile that provides indirect defense to plants by attracting natural enemies of attacking arthropods. In addition, methyl salicylate is also induced in response to pathogen infection (fungi, bacteria, and virus) and abiotic stress, such as wounding.[31,32] Noteworthy, the rhizobacterium, *Bacillus subtilis* has been reported to form mucoid colonies in response to methyl salicylate in order to protect plants under pathogen attack.[33−35] In our current study, it appears that for host location, RKNs may have evolved to eavesdrop on herbivore-induced volatiles, such as methyl salicylate released by plant roots interacting with soil-borne pathogens. As such, it appears that patterns of chemoecological interactions between host plants and below-ground herbivores are like those occurring above-ground, involving release of similar herbivore-induced volatiles. Additional studies with RKN-resistant and susceptible tomato cultivars are needed to determine if methyl salicylate is indeed the only induced signal released by these plants due to soil-borne pathogen attack.

The results of the present study revealed that (i) J2 attraction to tomato and spinach root volatiles is linked to shared volatiles, and (ii) J2 preference of tomato over spinach is linked to the presence of methyl salicylate in tomato root volatiles. These findings form a basis for investigating genes involved in suppressing the production of methyl salicylate in the roots of tomato. Such an approach would have significant ecological meaning if a tomato plant producing sparse amounts of methyl salicylate would be intercropped with spinach or other plants devoid of this compound for protection against RKN infection. Thus, future work is needed to explore a wide range of RKN host plants to guide development of management techniques that are compatible with smallholder farming systems in East Africa.

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Notes

The authors declare no competing financial interest.

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