**Compounds Associated with Infection by Root Knot Nematodes, *Meloidogyne javanica*, influence the Ability of Infective Juveniles to Recognize Host Plants**

Ruth Kihika[†, ‡], David P. Tchouassi[†], Margaret M. Ng'ang'a[‡], David R. Hall[⊥], John J. Beck[§], and Baldwyn Torto*[, †]

[†]Behavioral and Chemical Ecology Unit, International Centre of Insect Physiology and Ecology (*icipe*), P.O. Box 30772-00100, Nairobi, Kenya

[‡]Department of Chemistry, Kenyatta University, P.O. Box 43844-00100 Nairobi, Kenya

[⊥]Natural Resources Institute, University of Greenwich -, Central Avenue Chatham Maritime, Kent ME4 4TB, United Kingdom

[§]Chemistry Research Unit, Center for Medical, Agricultural, and Veterinary Entomology, Agricultural Research Service, U.S. Department of Agriculture, 1700 SW 23rd Drive, Gainesville, Florida 32608, United States

## AUTHOR INFORMATION

***Corresponding Author** (Tel.: +254 20 863 2000. Fax: +254 20 863 2001. Email: btorto@icipe.org)

**ABSTRACT**

Plant root chemistry is altered by parasitism of plant parasitic nematodes (PPN). Here, we investigated the influence of the infective stage juveniles (J2) of *Meloidogyne javanica* in inducing tomato (*Solanum lycopersicum*) root volatiles, and chemotactic effect on conspecifics. In olfactometer assays, J2 avoided roots of 2-day infected plants but preferred 7-day infected tomato compared to healthy plants. Chemical analysis showed a two- to seven-fold increase in the amounts of monoterpenes emitted from tomato roots infected with *M. javanica* relative to healthy roots. In further bioassays, the monoterpenes β-pinene, (+)-(2)-carene, α-phellandrene, and β-phellandrene differentially attracted (51-87%) J2 relative to control. Concurrent reduction and increase in the levels of methyl salicylate and (*Z*)-methyl dihydrojasmonate, respectively, in the root volatiles reduced J2 responses. These results demonstrate that the host plant can alter its root volatile composition to inhibit PPN attack. The observed plant-produced inhibition of J2 warrants further investigation as a potential management tool for growers.

KEYWORDS: *Meloidogyne javanica*, *Solanum lycopersicum*, root volatiles, chemotaxis

## INTRODUCTION

Root knot nematodes (RKNs, *Meloidogyne* spp.) are economically important polyphagous plant parasitic nematodes (PPNs) estimated to incur global crop production losses in excess of US $157 billion each year.[1–3] The second stage infective juveniles (J2) provide a potential weak link for control in the lifecycle because the J2 relies on chemical signals produced by the host plant roots to locate the host.[4–6] After the J2 locate and invade host roots, they complete their life cycle[3] by inducing the formation of specialized feeding sites called giant cells from which they withdraw nutrients using their stylet.[7] They also use their stylet to deposit secretions into the host cells.[3,8] These secretions are known to overcome plant defenses and alter the root chemistry.[9–12] Previous studies have shown that nematode infection increases levels of amino acids, phosphorylated metabolites, sugars and organic acids.[11,12] However, in the PPN-horticultural crop system, there is little understanding of how PPN infection modulates the host root volatile emissions and the consequential inter-species ecological interactions.

In a previous study on RKN-hostplant interactions with solanaceous plants, we identified methyl salicylate (MeSA) as an important attractant for J2 of *Meloidogyne incognita* in the roots of different pepper cultivars and tomato plants.[4,5] Additionally, we identified thymol in the root odor of a resistant pepper cultivar as responsible for disrupting J2 chemoreception in host location.[5] In our investigations we also identified other root volatile compounds including limonene, α-pinene, sabinene, 2-isopropyl-3-methoxypyrazine and tridecane as weakly attractive to J2.[4,5] Other studies have reported the role of non-volatile compounds in the root exudate of tomato on J2 host location.[6,13,14] Among the compounds identified in the tomato root exudate were the cytokinin zeatin, which attracted J2, and the flavonoids, quercetin and luteolin which

reduced J2 responses. In contrast, the alkaloids, tomatidine and solasodine were generally deterrent.[6] Additionally, the roles of exudates from the tips and upper parts of the tomato root on J2 responses have been explored. Specifically, exudates from the root tip attracted J2 compared to those from the upper parts of the roots.[14] Recent studies elucidated the molecular basis of tomato root exudate composition by using Virus-Induced Gene Silencing which showed that knockdown of root expressed ABC transporter genes and Ethylene Response Factor (*ERF*) genes altered the root semi-volatile components and differentially influenced the behavior of PPNs.[15,16] Specifically, knockdown of *ERF-E2* genes increased the attraction of *M. incognita* and *G. pallida* J2 to the root exudates,[15] while knockdown of *ABC-C6* transporter genes caused repellence in the infective J2 of *Meloidogyne* and *Globodera* spp. These findings demonstrate a potential genetic opportunity for reducing the impact of PPN's on crops.[16]

Recent studies in plant-PPN interactions have also explored the influence of these interactions on the behavior and performance of above-ground pests.[17–23] For example, *M. incognita* infection of tomato reduced oviposition and progeny development in the leaf miner, *Tuta absoluta*, attributed to the quantitative reduction of constitutive compounds that commonly attract the insect.[24] Similarly, root parasitism of tobacco by *M. incognita* increased the larval weight of the generalist caterpillar *Trichoplusia ni* but not the specialist caterpillar *Manduca sexta.*[17] This was attributed to reduced amounts (< 2 times) of nicotine, an alkaloid used in defense, that the specialist may be tolerant to.[22] It has been posited that nematode infection impaired the ability of the plant to produce nicotine upon larval feeding. In contrast, work investigating the amounts of gossypol and gossypol-like compounds produced by the cotton plant, *Gossypium hirsutum* showed that parasitism by *M. incognita* neither affected the levels of these compounds in the

plant, nor influenced attraction of the parasitic wasp *Microplitis croceipes* to the plant.[18] Nematode infection of roots increases the severity of pathogenic microbes, shown to be modulated by abiotic factors such as soil pH, which influenced the survival and reproduction of RKNs and consequent impact on the multiplication of the bacteria wilt, *Ralstonia solanocearum*.[19,25] Thus, these studies demonstrate that RKN infection of roots influence hostplant interactions with other herbivores. However, additional research is needed to understand how RKN infection influences J2 behavior.

Given the importance of host root odors for RKN host location, we tested the hypothesis that plant parasitic nematode infection alters root volatiles, and in turn influences J2 behavior. To achieve this, we used the well documented RKN-tomato system, the susceptible tomato cultivar 'Cal J' and infective J2 of the RKN, *M. javanica*.

## MATERIALS AND METHODS

**Plants.** The tomato 'Cal J' cultivar**,** *Solanum lycopersicum***,** used in the present study was obtained locally (Simlaw Seeds Company, Nairobi, Kenya), and the seeds were sown in a rectangular plastic basin (67 cm x 40 cm x 5cm) (Kenpoly Manufacturers Limited, Nairobi, Kenya) containing sterilized sand (autoclaved at 121 $^{o}$C for 40 min) in a screenhouse maintained at 27 ± 2 °C, 60-70% relative humidity (RH) at the International Centre of Insect Physiology and Ecology (*icipe*), Duduville Campus, Nairobi, Kenya (1$^{\circ}$ 13' 18.96"S, 36$^{\circ}$ 53' 47.94"E). After two weeks of germination, the seedlings were transplanted into autoclaved sand in 5 L plastic pots (29 cm depth). Plants were watered daily with 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/L containing 72 mL of ethylenediaminetetraacetic acid (pH 4); and the micronutrients: manganese (II) chloride tetrahydrate 1.81 g/L; copper(II) sulfate pentahydrate 0.1 g/L; zinc sulphate heptahydrate 0.22 g/L; boric acid 2.86 g/L; molybdic acid 0.02 g/L). Plants were used for the experiments 3-4 weeks after transplanting.

**Root-Knot Nematodes.** The inoculum of *M. javanica* was obtained from a nematode population culture maintained on tomato cultivar 'Cal J' in the screenhouse at 27 ± 2 °C, 60-70% relative humidity at *icipe.* Galled root systems were gently washed to remove sand and then stained with Phloxine B (0.15 g/L water) for 20 min to highlight the egg masses. The roots were then de-stained 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) and placed in 24-well culture plates containing 2 mL distilled water. To allow for hatching and emergence of J2, these were kept in a dark cabinet at 27 ± 2 °C for 2 to 5 days.[5,26] The freshly emerged J2 were counted under the stereomicroscope and used to inoculate the plants.

**Behavioral responses of *M. javanica* infective juveniles to infected tomato plants.** The responses of *M. javanica* infective juveniles to root volatiles of RKN-infected and healthy tomato plants (non-infected plants which served as control) were tested separately in a dual choice olfactometer as described previously.[4,5] Briefly, the olfactometer comprised the stimulus and control chambers (85 mm diameter × 140 mm depth) that were linked to detachable connecting arms (20 mm diameter × 70 mm length) with a release arm (20 mm diameter × 60

mm length) at the center where nematodes were introduced. To obtain RKN-infected plants, five plants, three to four weeks old, were placed in a growth chamber (85 mm diameter x 140 mm depth) containing 300 g sterilized sand. The plants were watered daily with 20 mL nutrient solution for 3-5 days prior to conducting the experiments in the laboratory at 25 ± 2 °C after which the plants were inoculated with approximately 1,000 J2. Healthy plants were prepared identically but not inoculated. The control chamber contained 300 g of autoclaved sand moistened with 50 mL nutrient solution. Nematode responses were tested in two different assays: (i) using plants at day 0 (healthy), 2- and 7-days post infection (DPI) compared against a control (sand) and (ii) nematode infected (2-DPI and 7-DPI) vs healthy plants in pairwise tests. Four replicates, each comprising approximately 600 juveniles, were used in each of the experiments. After 4 h the olfactometer was disassembled and the nematodes in each detachable section were recovered over a 48 h period using a modified Baermann sieving method and counted under a stereomicroscope.[4,6] The olfactometer was cleaned after each experiment using soap and tap water, rinsed with distilled water and dried in an oven overnight.

**Identification of volatiles associated with root knot nematode infection.** To characterize the chemical composition of root volatiles released in response to RKN infection, we used solid phase microextraction (SPME) to collect tomato root volatiles from healthy and RKN-infected plant. The plants were prepared as described earlier after which they were gently removed from the sand to avoid damaging the roots. The roots were then washed gently with tap water to remove sand debris and dipped in 0.05% sodium hypochlorite in water for 2 min then rinsed with distilled water. The roots of five intact plants were then placed in a round bottom glass flask (100 mL) containing moist cotton wool at the bottom to avoid desiccation which could lead to plant

stress and thus influence the plant volatiles. The flask was covered with aluminum foil to simulate a dark natural root environment.

Volatiles were collected from roots at day 0 (healthy plant), 2- and 7- DPI to determine root volatile responses associated with root knot nematode infection. To sample root volatiles, a charcoal filter was used to cover the top of the glass to avoid sampling odors from the aerial parts of the plants and the surrounding air. To adsorb the volatiles, a pre-cleaned (via thermal desorption at 250°C for 30 min to remove any ambient contaminants) 65 µm polydimethylsiloxane/divinylbenzene (PDMS/DVB) SPME fiber (Supelco, Bellefonte, USA) was inserted at the side arm of the round bottomed flask for 1 h at 25 ± 2 °C. The experiment was repeated three times, each with five plants per replicate.

The collected root volatiles were analyzed using gas chromatography coupled to mass spectrometry (GC/MS) with a HP-7890B series gas chromatograph (Agilent Technologies, Wilmington, USA) linked to a HP 5977 mass spectrometer (Agilent, Wilmington, USA) operated in electron ionization mode. The SPME fiber was inserted manually into the injector port (250 °C), desorbed and chromatographed on a non-polar HP-5 MS ultra-inert capillary column (5%-phenyl methyl polysiloxane; 30 m x 0.25 mm i.d., 0.25 µm film thickness, J & W Scientific, Folsom, USA). 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 ion source temperature was 230 °C while electron ionization mass spectra were acquired at 70 eV within a mass range of 38-550 Daltons (Da) during a scan time of 0.73 scans/sec. Retention indices (RI) were calculated relative to $C_8$-$C_{31}$ *n*-alkanes. Analytes were

initially identified by comparison of their mass spectra with those in the GCMS library (Library??) and comparison of their RI with literature values. These identifications were confirmed by comparison of RI and mass spectra with those of authentic standards run under the same conditions where possible. Quantification was based on calibration curves (peak area vs. concentration) generated from authentic synthetic standards of identified compounds.

To determine corresponding source amounts, different concentrations (0.2-1,000 ng/µL) of the synthetic standards (1 mL each) were allowed to equilibrate contained in an air-tight 4 mL vial. A pre-cleaned SPME fiber was inserted into the headspace and volatiles were collected for 1 h. Adsorbed volatiles were analyzed by GC-MS using the same conditions as described earlier for the root volatiles.

**Chemicals.** The synthetic standards including *o*-cymene, *p*-cymene, (*R*)-(-)-α-phellandrene (≥97%), (*R*)-(+)-α-pinene (99%), (1*S*)-(-)-β-pinene (99%), 3-isopropyl-2-methoxypyrazine (≥97%), nonanal (95%), tridecane (>95%), and methyl dihydrojasmonate (mixture of *cis* and *trans*) were purchased from Sigma Aldrich (St, Louis, MO, USA). Methyl salicylate (97% purity) was purchased from Sigma Aldrich (Steinhelm, Germany), (+)-(2)-carene (97% purity) from Sigma Aldrich (Switzerland) and (-)-*trans* caryophyllene (99%) from Fluka. For β -phellandrene, we used Angelica seed oil containing 89% (*S*)-(+)-β-phellandrene (SigmaAldrich, Gillingham, Dorset, UK).

**Dual choice bioassays of synthetic compounds in volatiles associated with root knot nematode infection.** We tested the available synthetic standards of constitutive and induced

defense compounds to determine their effect on the behavioral responses of J2 of *M. javanica* using the dual choice olfactometer assay and procedure described in the subsection on behavioral response of *M. javanica* infective juveniles to infected tomato plant. Three concentrations of each compound were prepared in hexane and tested in four replicates. For methyl dihydrojasmonate (MeDiJA), methyl salicylate (MeSA), β-pinene and α-phellandrene, we prepared 55 ng/µL (corresponding to source amount of MeSA detected in a healthy plant), 110 and 220 ng/µL. The doses for (+)-(2)-carene (88, 176 and 682 ng/µL) and β-phellandrene (412, 203 and 1,384 ng/µL) were prepared based on amounts estimated to be present at the three time points of infection (0-DPI healthy, 2-DPI and 7-DPI). The 6-component blend comprised Dose 1 ((+)-(2)-carene (88 ng/µL), β-phellandrene (412 ng/µL), and 55 ng/µL of β-pinene, α-phellandrene, MeSA, and MeDiJA), Dose 2 ((+)-(2)-carene (176 ng/µL), β-phellandrene (203 ng/µL), and 110 ng/µL of β-pinene, α-phellandrene, , MeSA, and MeDiJA) and Dose 3 ((+)-(2)-carene (682 ng/µL), β-phellandrene (1,384 ng/µL), and 220 ng/µL of β-pinene, α-phellandrene, MeSA, and MeDiJA). The treatments were applied by dispensing 50 µL aliquots into the stimulus chamber containing 300 g of sterilized sand while a similar volume of hexane was dispensed in the control chamber. Another experiment assessed the effect of spiking the plant at 2-DPI with MeSA vs 2-DPI (control) and healthy plant with MeDiJA vs healthy plant (control) at the same concentrations tested for individual compounds.

**Statistical analysis.** All analyses were performed using R software 64 (version 3.5.1) and the R Studio graphical user interface (version 1.1.383).[27] 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) x 100], where n is the number of J2

responding to a given treatment, while N is the total number of responding J2. Non-responding J2 were not included in the analysis. Data were subjected to Chi-square ($\chi^2$) goodness-of-fit analysis testing the hypothesis that nematode choice of odors was in the ratio 1:1 between the treatment and control. Concentration of root volatiles for the different time points of RKN-infected and healthy plants were expressed in ng adsorbed on the SPME fiber and subjected to analysis of variance (ANOVA) followed by Student-Neuman-Keuls (SNK) post hoc multiple comparisons tests for mean separation after checking for normality using Shapiro-Wilk test ($P > 0.05$). All statistical analyses were considered significant at $P < 0.05$.

## RESULTS AND DISCUSSION

**Root knot nematode-induced volatiles influence chemotactic responses of *M. javanica* infective juveniles.** Soil olfactometer assays showed that root volatiles of healthy tomato significantly attracted J2 of *M. javanica* (97%, $\chi^2 = 599.2$, df = 1, $P < 0.001$) (Figure 1A) relative to sand controls. This observation corroborates a previous study in which the J2 of *M. incognita* also preferred the same tomato cultivar[4] when compared to a control. The response of J2 to RKN infected tomato varied depending on the time points assayed. At 2-DPI, J2 significantly avoided infected plants (86%, $\chi^2 = 599.2$, df = 1, $P < 0.001$) whereas the converse pattern was observed at 7-DPI with significant preference to the treatment (98%, $\chi^2 = 1384.6$, df = 1, $P<0.001$) compared to a sand control (Figure 1A). Similarly, in the paired assays, J2 significantly avoided the root volatiles of 2-DPI tomato (71%, $\chi^2 = 221.36$, df = 1, $P < 0.001$) but preferred the 7-DPI plant (58%, $\chi^2 = 21.43$, df = 1, $P < 0.05$) over healthy plants (Figure 1B).

These results suggest that at 2-DPI the plants released defensive or inhibitory root volatiles, which interfered with chemoreception of the nematode and affected their behavior to the host

plant. Specifically, J2 avoided the plants during early stages of RKN-infection (2-DPI) that correspond to intercellular migration of J2 before formation of feeding sites.[7] Consequently, the J2 may associate these chemical signals with diminished food resources and therefore avoid this treatment to prevent competition when too many J2 infect the plant. Conversely, nematodes preferred the plants after formation of feeding sites, at 7-DPI even in the paired experiments. These disparate responses could be associated with the quality of the root volatiles both in composition and ratio of attractants and repellents released by the healthy and infected plants at the different post-infection times. This may lead to suppression or masking of the attractive signals by the defense compounds upon J2 penetration in the roots. Thus, the nematodes may produce different chemical signals for nematode-nematode communication during attraction or avoidance to different treatments, which would require further research.

**Constitutive and induced volatiles of 'Cal J' released due to *M. javanica* infection.** The volatile profiles of healthy and infected 'Cal J' were obtained using SPME collection followed by GC-MS analysis. We identified 28 compounds that were consistent in the three replicates sampled per treatment and consisted of 13 monoterpenes, nine sesquiterpenes, two aldehydes, a pyrazine, an alkane, a benzenoid, and a jasmonate (Figure 2). The detected compounds and their quantitative variations at the different time points of root infection are shown in Table 1. Statistical variation in the amounts released between the different time points of infection was evident for *o*-cymene (**2**), (*E*)-isolimonene (**3**), β-pinene (**4**), (+)-(2)-carene (**5**), α-phellandrene (**6**), *p*-cymene (**8**), β-phellandrene (**9**), (*E*)-β-ocimene (**11**), nonanal (**15**), valencene (**26**), viridiflorene (**27**) and MeDiJA (**28**). Notably, we found two- to seven-fold increase in the amounts of (+)-(2)-carene (**5**) released in the root volatiles at 2-DPI and 7-DPI, respectively. The

level of β-phellandrene (**9**), the most abundant compound in the root volatiles of healthy plant, decreased two-fold in the root volatiles at 2-DPI, but increased relatively three-fold at 7-DPI. In contrast, 9,10-dehydro-isolongifolene (**24**) was detected in the root volatiles at 2-DPI and neither in the healthy nor 7-DPI plant. We found that (*Z*)- MeDiJA (**28**) was below the detection limit in the volatiles of healthy and 7-DPI plants but detected at 2-DPI. The amount of methyl salicylate (MeSA) (**16**) adsorbed decreased from 7.2 ng with healthy plants to 1.2 ng at 2-DPI and increased to 8.6 ng at 7-DPI. Compounds that did not differ significantly in the volatiles of healthy and infected plants included α-pinene (**1**), α-terpinene (**7**), γ-terpinene (**12**), terpinolene (**13**), decanal (**17**), (*E*)-caryophyllene (**21**), and α-selinene (**25**). Volatiles that were present in trace amounts at varying time points of infection were 3-carene (**10**), 3-isopropyl-2-methoxypyrazine (**14**), α-copaene (**19**), di-epi-α-cedrene (**20**), α-guiaene (**23**) and tridecane (**18**) (Table 1).

Sampling and analysis of volatiles from the intact plant using SPME-GC/MS was a more sensitive technique for us compared to a previously used method that used Super Q as the adsorbent.[4,5] However, the use of Super Q attached to a probe and inserted in sand may provide a more accurate representation of the natural situation where matrix interference from sand/soil compounds is present. The effect of sand-specific compounds on J2 behavior was not evaluated in these studies.[4,5] Also, different compounds may diffuse at different rates in the sand matrix which would influence the concentrations detected and thus differ from the natural concentrations released by the roots. For instance, using selected ion monitoring mode (*m/z* 83, 156, 226) we detected MeDiJA (**28**) in the volatiles of healthy 'Cal J', but this was not reported in a previous study[4] that used the same plant and sampled volatiles from snap frozen roots. In the

current study, volatiles were sampled from the intact plant with the roots retained in moist cotton wool and the sampling was done within a short period (1 h). This was particularly important since this approach helped reduce the amounts of stress-associated volatiles released by the roots. However, the differences in the methods used for collection of volatiles for the roots and authentic standards may affect the accuracy of the quantities determined for the adsorbed volatiles. The presence of other compounds in excised plant parts has been demonstrated previously. For instance, analysis of methanolic extracts of excised plant parts at different times of PPN infection, ranging from five days to two months, identified significant local and systemic variable increases in amino acids, phosphorylated metabolites and some sugars and organic acids.[11,12] These findings revealed that both primary and secondary metabolites played a role in RKN parasitism, suggesting that different sampling and extraction methods could influence the composition and quantity of identified compounds.[4,5,11,12]

Terpenoids are implicated in defense responses of various plant-herbivore interactions[28–31] including root defenses.[32] Additionally, herbivore physiological state and level of infestation may influence the quantities detected. In different tomato-pest systems, plants respond differently depending upon the mode of feeding by the herbivore.[33] The different feeding guilds may also differ in the extent of tissue damage they cause and signal-transduction pathways triggered.[22,29,34,35] RKNs are endoparasitic biotrophs[7] that cause minimal damage during intercellular movement towards the vascular tissue where they induce gall formation[36] in a localized area and use their stylet to withdraw nutrients from living plant cells.[7] Interestingly, infection with *M. javanica* caused significant variation in certain monoterpenes, specifically (+)-(2)-carene (**5**) and β-phellandrene (**9**), but not sesquiterpenes. It is possible that the degree of *M.*

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*javanica* infection and time frame were only enough to trigger a burst of monoterpenes but not sesquiterpenes. Future research should consider different scenarios including the degree of RKN infection over a longer period.

In this study, MeDiJA (**28**), a derivative in the jasmonic acid (JA) pathway, was detected at 2-DPI that corresponded with intercellular migration and commencement of feeding site formation by RKN J2. This could be due to production of specific nematode secretions to counteract plant defense at this stage of RKN parasitism.[8] The biosynthesis of MeDiJA (**28**) in plants has not been fully elucidated but it may be formed through hydrogenation of methyl JA. Alternatively, the JA isomer, (+)-7-iso JA may be hydrogenated to 9,10-dihydro JA, which is then methylated to MeDiJA (**28**). Methyl salicylate (MeSA) (**16**), a derivative of salicylic acid (SA), a constituent of insect- and pathogen-induced plant volatiles[29–31,37–39] is well known to play important ecological role in indirect defense by attracting natural enemies.[40] This compound was reduced at 2-DPI, and the asynchronous quantitative detection of MeSA (**16**) and MeDiJA (**28**) in this study suggests a possible cross-talk between the SA and JA signaling pathways in response to *M. javanica* J2. MeSA (**16**) maybe reduced as it undergoes conversion to its precursor, SA, to facilitate production of other defense compounds.

**Response of *M. javanica* infective juveniles to volatiles associated with RKN-infection.** In bioassays, we tested the available compounds (β-pinene (**4**), (+)-(2)-carene (**5**), α-phellandrene (**6**), β-phellandrene (**9**), MeSA (**16**) and, MeDiJA (**28**)) that showed significant differences at the different time points of root infection. Concentration-dependent responses were observed in the J2 for individual compounds and a blend of the six components tested against a solvent control

(Figure 3A-G). Nematodes preferred MeSA (**16**) at all the tested concentrations; 2.75µg (86%, $\chi^2 = 203.98$, df = 1), 5.5µg (75%, $\chi^2 = 61.77$, df = 1) and 11µg (80%, $\chi^2 = 100.09$, df = 1) (Figure 3E) whereas MeDiJA (**28**) was unattractive at 2.75µg (75%, $\chi^2 = 37.33$, df = 1) but not at 5.5µg (54%, $\chi^2 = 1.62$, df = 1) and 11µg (53%, $\chi^2 = 0.32$, df = 1) (Figure 3F). In testing the importance of MeSA (**16**) and MeDiJA (**28**) in infected and healthy plants respectively, nematodes significantly preferred the roots of the plant at 2-DPI spiked with MeSA (**16**) at 2.75µg (87%, $\chi^2 = 126.68$, df = 1), 5.5 µg (74%, $\chi^2 = 115.43$, df = 1) and 11 µg (83%, $\chi^2 = 242.42$, df = 1) (Figure 3H). Spiking the roots of healthy plant with MeDiJA (**28**) reduced the preference of J2 to the roots of the healthy plant at 2.75µg (59%, $\chi^2 = 21.39$, df = 1), 5.5µg (39%, $\chi^2 = 22.873$, df = 1) and 11 µg (58%, $\chi2 = 6.0036$, df = 1) (Figure 3I).

The attractiveness of MeSA (**16**) to J2 appears to be concentration-dependent given the reduced amounts of MeSA (**16**) at 2-DPI coincided with avoidance behavior, and when the plant was spiked with MeSA (**16**) the roots became more attractive again. Though the reduced amount of MeSA at 2-DPI was not statistically significant in our analyses, the reduction appeared to have ecological significance since it caused an avoidance response in J2. Perhaps, the other volatile compounds associated with RKN-infection mask or interfere with this important kairomonal signal. Additionally, volatiles that were not tested in this study may contribute to the avoidance response observed at 2-DPI. However, this defense response appears not to be sustained long enough to deter further nematode attack. Concentration-dependent attraction has previously been demonstrated for RKNs where ethylene (ET) signaling was found to modulate attractiveness of *M. halpa, M. javanica* and *M. incognita*.[14,41] Specifically, *Arabidopsis* and tomato roots with reduced ET synthesis were more preferred by the J2 of these nematode species than the

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corresponding wild types that constitutively overproduced ET. The J2 may associate the high amounts of ethylene with reduced food resources since its increased production was observed at the second week in *M. javanica*-infected tomato.[42] Similarly, our findings may indicate the importance of SA signaling in the attractiveness of host roots which is consistent with previous work, whereby MeSA (**16**) was identified in tomato and pepper as an important kairomonal signal for *M. incognita* J2.[4,5] Exogeneous shoot application of JA and methyl jasmonate (MeJA) has been found to induce systemic root defenses against RKNs attack in tomato.[43,44] Furthermore, treatment with JA boosts Mi-mediated resistance at high temperatures[43] showing that jasmonates play an important role in protecting crops against RKNs.

Interestingly, β-phellandrene (**9**), the most abundant compound detected in the volatiles of the roots of the healthy plant, reduced two-fold at 2-DPI and increased three-fold at 7-DPI. However, in behavioral assays, J2 were indifferent to this monoterpene at doses of 20.6 µg (51%, $\chi^2 = 0.07$, df = 1), 10.2 µg (55%, $\chi^2 = 3.54$, df = 1) and 69.2 µg **(**60%, $\chi^2 = 11.60$, df = 1) (Figure 3D). The chirality of the β-phellandrene (**9**) produced by the tomato plants was not determined in this study, and only the (*S*)-(+)-enantiomer was tested in the bioassays. However, this result suggests that β-phellandrene (**9**) and other root volatiles may contribute to the attraction of J2 as background signals. These background volatiles warrant further research. On the other hand, the dose of 34.1 µg (+)-(2)-carene (**5**) corresponding to 7-DPI caused significant preference of J2 to the treatment (82%, $\chi^2 = 59.38$, df = 1), while lower doses corresponding to 0-DPI and 2-DPI, respectively, were weakly attractive (4.4 µg: 57%, $\chi^2 = 2.13$, df = 1; 8.8 µg: 56%, $\chi^2 = 1.43$, df = 1) (Figure 3B).

The monoterpenes, β-pinene (**4**) and α-phellandrene (**6**), also differentially attracted J2. β-Pinene (**4**) was very highly significantly attractive at doses of 2.75 µg (83%, $\chi^2 = 113.43$, df = 1) and 11 µg (63%, $\chi^2 = 16.47$, df = 1), but not at the dose of 5.5 µg corresponding to 2-DPI (56%, $\chi^2 = 3.20$, df = 1) (Figure 3A). α-Phellandrene (**6**) was more attractive at doses of 5.5 µg (65%, $\chi^2 = 14.49$, df = 1) and 11 µg (57%, $\chi^2 = 5.79$, df = 1), than at 2.75 µg (56%, $\chi^2 = 2.84$, df = 1) (Figure 3C). The chirality of the β-pinene (**4**) and α-phellandrene (**6**) produced by the tomato plants was not determined and only the (1S)-(-)- and (R)-(-)-enantiomers, respectively, were tested in the bioassays. In rhizosphere and above-ground studies, β-pinene (**4**) was identified as a herbivore induced plant volatile that attracted the citrus root nematode *Tylenchulus semipenetrans*[45] and constitutively attracted the bark beetle, *Hylastus nigrinus*.[46] The roots of pepper and tomato plants have also been shown to release limonene, α-pinene, and sabinene as signals contributing to the attraction of *M. incognita* J2. [4,5] The blend of all six components was attractive to J2 at the highest dose corresponding to 7-DPI (84%, $\chi^2 = 60.93$, df = 1), but not at doses corresponding to 0-DPI (55%, $\chi^2 = 3.15$, df = 1) or 2-DPI (53%, $\chi^2 = 0.59$, df = 1) (Figure 3G). This may have been influenced by the dose of (+)-(2)-carene corresponding to 7-DPI (34.1 µg) that was also highly attractive to the J2 when tested individually.

The monoterpenes appear to have differential attraction effect which is plausible since they are common in numerous host plant species[4,5,47] of these polyphagous nematodes. Nevertheless, the root plant volatiles stimulated more J2 responses than the individual compounds tested alone or in the 6-component blend, suggesting that other yet-to-be identified compounds contribute to J2 attraction. This indicates that J2 chemoreception is attuned to determine a suitable host that will best support its survival and reproduction.

Genetic engineering of plants to enhance indirect defense has shown success in maize cultivar to enhance constitutive production of (*E*)-caryophyllene (**21**) in order to increase recruitment of entomopathogenic nematodes.[48] In plant-PPN interactions, the knockdown of *ABC-C6* transporter genes altered the root exudate composition and reduced the attraction of *Meloidogyne* and *Globodera* spp.[15,16] These studies show potential application of crop improvement to develop cultivars that are resistant to economically important crop pests. Our findings suggest that masking the attractive signal, MeSA (**16**), with MeDiJA (**28**) could provide an avenue for interfering with host plant recognition by the nematodes.

Overall, these results show that RKN-induced root volatiles provide important olfactory cues that disrupt J2 chemoreception that can be exploited to develop alternative management options for RKNs. Future work should identify the genes responsible for production of MeDiJA (**28**) for their manipulation for crop improvement of RKN-resistant tomato cultivars. Additionally, it would be important to determine the impact of such cultivars on other soil pathogens and beneficial microorganisms.

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**Table 1:** Compounds detected in root volatiles from healthy and *Meloidogyne javanica infected* tomato ('Cal-J') plants collected by SPME and analyzed by GC/MS.

| | **RT (min)** | **Compound** | **RI$^{Calc}$** | **RI$^{Lit}$** | **Mean amount adsorbed (ng ± SE)** | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | **Healthy** | **2-DPI** | **7-DPI** | |
| 1 | 9.71 | α-Pinene$^{\vdash}$ | 915 | 918$^{A}$ | 2.95 ± 0.75$^{a}$ | 2.29 ± 0.70$^{a}$ | 6.21 ± 2.32$^{a}$ | ($F_{(2,6)} = 2.05$, $P > 0.05$) |
| 2 | 10.50 | *o*-Cymene$^{\vdash}$ | 951 | 956 $^{A}$ | 2.96 ± 1.33$^{a}$ | 1.61 ± 0.29$^{a}$ | 10.98 ± 1.79$^{b}$ | ($F_{(2,6)} = 15.25$, $P < 0.01$) |
| 3 | 10.73 | (*E*)-Isolimonene$^{*}$ | 961 | 960 $^{A}$ | 0.49 ± 0.19$^{a}$ | 0.19 ± 0.07$^{a}$ | 1.85 ± 0.43$^{b}$ | ($F_{(2,6)} = 10.35$, $P < 0.05$) |
| 4 | 10.94 | β-Pinene$^{\vdash}$ | 971 | 965 $^{A}$ | 0.40 ± 0.07$^{a}$ | 0.23 ± 0.05$^{a}$ | 1.84 ± 0.23$^{b}$ | ($F_{(2,6)} = 37.07$, $P < 0.001$) |
| 5 | 11.12 | (+)-(2)-Carene$^{\vdash}$ | 979 | 981 $^{A}$ | 18.68 ± 9.02$^{a}$ | 34.40 ± 4.89$^{a}$ | 127.03 ± 34.79$^{b}$ | ($F_{(2,6)} = 7.818$, $P < 0.05$) |
| 6 | 11.20 | α-Phellandrene$^{\vdash}$ | 988 | 985 $^{A}$ | trace | 0.32 ± 0.01$^{a}$ | 1.10 ± 0.28$^{b}$ | ($F_{(2,6)} = 15.97$, $P < 0.01$) |
| 7 | 11.42 | α-Terpinene$^{\vdash}$ | 993 | 996 $^{A}$ | 17.86 ± 16.64$^{a}$ | 1.07 ± 0.27$^{a}$ | 10.09 ± 2.68$^{a}$ | ($F_{(2,6)} = 0.75$, $P > 0.05$) |
| 8 | 11.57 | *p*-Cymene$^{\vdash}$ | 1000 | 1000 $^{A}$ | 0.81 ± 0.29$^{a}$ | 0.66 ± 0.34$^{a}$ | 3.14 ± 0.90$^{b}$ | ($F_{(2,6)} = 13.89$, $P < 0.01$) |
| 9 | 11.65 | β-Phellandrene$^{\vdash}$ | 1005 | 1010 $^{A}$ | 78.46 ± 30.53$^{a}$ | 39.66 ± 6.95$^{a}$ | 252.79 ± 50.34$^{b}$ | ($F_{(2,6)} = 11$, $P < 0.01$) |
| 10 | 11.81 | 3-Carene$^{*}$ | 1014 | 1011 $^{B}$ | trace | trace | 0.10 ± 0.01 | |
| 11 | 12.00 | (*E*)-β-Ocimene$^{\vdash}$ | 1024 | 1029 $^{A}$ | trace | 0.13 ± 0.03$^{a}$ | 0.94 ± 0.15$^{b}$ | ($F_{(2,6)} = 29.15$, $P < 0.001$) |
| 12 | 12.20 | γ-Terpinene$^{*}$ | 1036 | 1041 $^{A}$ | 2.08 ± 1.99$^{a}$ | 0.09 ± 0.02$^{a}$ | 1.06 ± 0.23$^{a}$ | ($F_{(2,6)} = 0.74$, $P > 0.05$) |
| 13 | 12.72 | Terpinolene$^{*}$ | 1066 | 1073 $^{A}$ | 1.43 ± 1.29$^{a}$ | 0.1 ± 0.01$^{a}$ | 2.46 ± 0.45$^{a}$ | ($F_{(2,6)} = 2.23$, $P > 0.05$) |
| 14 | 12.82 | 3-Isopropyl-2-methoxypyrazine$^{\vdash}$ | 1075 | 1079 $^{C}$ | 0.02 ± 0.03 | trace | trace | |
| 15 | 12.96 | Nonanal$^{\vdash}$ | 1082 | 1088 $^{A}$ | 0.27 ± 0.25$^{a}$ | 0.69 ± 0.26$^{ab}$ | 1.09 ± 0.08$^{b}$ | ($F_{(2,6)} = 5.42$, $P < 0.05$) |
| 16 | 14.46 | Methyl salicylate$^{\vdash}$ | 1170 | 1176 $^{A}$ | 7.24 ± 0.28$^{a}$ | 1.18 ± 0.03$^{a}$ | 8.62 ± 4.15$^{a}$ | ($F_{(2,6)} = 3.63$, $P > 0.05$) |
| 17 | 14.57 | Decanal$^{\vdash}$ | 1177 | 1183 $^{A}$ | 0.50 ± 0.18$^{a}$ | 0.57 ± 0.24$^{a}$ | 1.11 ± 0.08$^{b}$ | ($F_{(2,6)} = 4.61$, $P > 0.05$) |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 18 | 15.91 | Tridecane[ŧ] | 1234 | 1271 [A] | 0.47 ± 0.63[a] | 0.10 ± 0.09[a] | trace | ($F_{(2,6)} = 3.30$, $P > 0.001$) |
| 19 | 17.09 | α-Copaene[ŧ] | 1348 | 1351[D] | trace | 0.14 ± 0.03[a] | trace | |
| 20 | 17.61 | Di-epi-α-cedrene[*] | 1385 | 1385[E] | trace | 0.06 ± 0.01[a] | 0.15 ± 0.07[a] | |
| 21 | 17.70 | (*E*)-Caryophyllene[ŧ] | 1389 | 1396[A] | 0.10 ± 0.05[a] | 0.07 ± 0.00[a] | 0.17 ± 0.01[a] | ($F_{(2,6)} = 0.58$, $P > 0.05$) |
| 22 | 17.99 | Geranyl acetone[*] | 1411 | 1424[F] | 0.07 ± 0.01[a] | 0.11 ± 0.09[a] | 0.24 ± 0.07[a] | ($F_{(2,6)} = 2.58$, $P > 0.05$) |
| 23 | 18.07 | α-Guaiene[*] | 1419 | 1433[G] | 0.04 ± 0.00[a] | 0.30 ± 0.07[a] | trace | |
| 24 | 18.22 | 9,10-Dehydro-isolongifolene[*] | 1431 | | ND | 0.28 ± 0.07 | ND | |
| 25 | 18.38 | α-Selinene[*] | 1441 | 1475[H] | 0.04 ± 0.00[a] | 0.32 ± 0.06[a] | 0.30 ± 0.06[a] | ($F_{(2,6)} = 4.49$, $P > 0.05$) |
| 26 | 18.62 | Valencene[*] | 1459 | 1484 [G] | 0.03 ± 0.00[a] | 0.42 ± 0.07[b] | 0.30 ± 0.09[b] | ($F_{(2,6)} = 8.37$, $P < 0.05$) |
| 27 | 18.75 | Viridiflorene[*] | 1469 | 1489 [G] | 0.06 ± 0.00[a] | 0.66 ± 0.13[b] | 0.65 ± 0.15[b] | ($F_{(2,6)} = 8.99$, $P < 0.05$) |
| 28 | 20.43 | (*Z*)-Methyl dihydrojasmonate[ŧ] | 1606 | 1655[I] | BDL | 0.11 ± 0.02 | trace | |

Means with different letters for each compound are significantly different from each other (ANOVA followed by SNK post hoc test; $P < 0.05$, $n = 3$). DPI; days post infection, $RI^{Calculated}$ Retention index relative to $C_8$-$C_{31}$ n- alkanes of a HP-5 MS column, $RI^{Literature}$ Retention index obtained from literature. ND, not detected. BDL, below detection limit

[ŧ]Compound whose identity was established based on comparison of retention time and mass spectra data with authentic standard.

[*]Compound identified tentatively based on library data, calculated RI values and comparison to literature: (A)[49], (B)[50], (C)[4], (D)[51], (E)[52], (F)[53], (G)[54], (H)[55], (I)[29]

**FIGURE CAPTIONS**

**Figure 1**. Response of *Meloidogyne javanica* infective juveniles (J2) to tomato "Cal J" root volatiles. (**A**) Healthy (0 days post infection (DPI)) and infected (2- and 7- DPI) versus a sand control (**B**) Healthy vs. RKN-infected. (N corresponds to the total number of responding J2 while n is the number of J2 corresponding to a given treatment; non-responders were not included in the analysis; level of significance is indicated by: ***$P < 0.001$; ns = not significant)

**Figure 2**. Gas chromatography-mass spectrometry chromatograms of root volatiles collected from healthy (Day 0 **(A)**) and *Meloidogyne javanica* infected (Day 2 **(B)** and 7**(C)**) tomato ('Cal-J') plants by SPME with compounds numbered as in Table 1. **(D)** Chemical structures of the identified compounds numbered as in Table 1 (**1**) α-pinene, (**2**) *o*-cymene, (**3**) (*E*)-isolimonene, (**4**) β-pinene, (**5**) (+)-(2)-carene, (**6**) α-phellandrene, (**7**) α-terpinene, (**8**) *p*-cymene, (**9**) β-phellandrene, (**10**) 3-carene, (**11**) (*E*)-β-ocimene, (**12**) γ-terpinene, (**13)** terpinolene, (**14**) 3-isopropyl-2-methoxypyrazine, (**15**) nonanal, (**16**) methyl salicylate, (**17**) decanal, (**18**) tridecane, (**19**) α-copaene, (**20**) di-epi-α-cedrene, (**21**) (*E*)-caryophyllene, (**22**) geranyl acetone, (**23**) α-guaiene, (**24**) 9,10-dehydro-isolongifolene, (**25**) α- selinene, (**26**) valencene, (**27**) viridiflorene (**28**) (*Z*)-methyl dihydrojasmonate. Asterisk (*) indicates column contaminants.

**Figure 3**. Response of *Meloidogyne javanica* infective juveniles (J2) to compounds associated with RKN infection at different doses of (**A**) β-pinene, (**B**) (+)-(2)-carene, (**C**) α-phellandrene, (**D**) β-phellandrene, (**E**) methyl salicylate (MeSA), (**F**) methyl dihydrojasmonate, and (**G**) 6-component blend vs. sand control. Dose 1 ((+)-(2)-carene (4.4 µg), β-phellandrene (20.6 µg), and 2.75 µg of β-pinene, α-phellandrene, MeSA, and MeDiJA), Dose 2 ((+)-(2)-carene (8.8 µg), β-

phellandrene ( 10.2 µg), and 5.5 µg of β-pinene, α-phellandrene, MeSA, and MeDiJA) and Dose 3 ((+)-(2)-carene (34.1 µg), β-phellandrene (69.2 µg), and 11 µg of β-pinene, α-phellandrene, MeSA, and MeDiJA); (**H**) 2-DPI plant spiked with MeSA vs. 2-DPI plant (control), (**I**) healthy tomato spiked with different doses of MeDiJA vs healthy tomato (control). (N corresponds to the total number of responding J2 while n is the number of J2 corresponding to a given treatment; non-responders were not included in the analyses; level of significance is indicated by: ***$P < 0.001$, $^{*}P < 0.05$, ns = not significant)

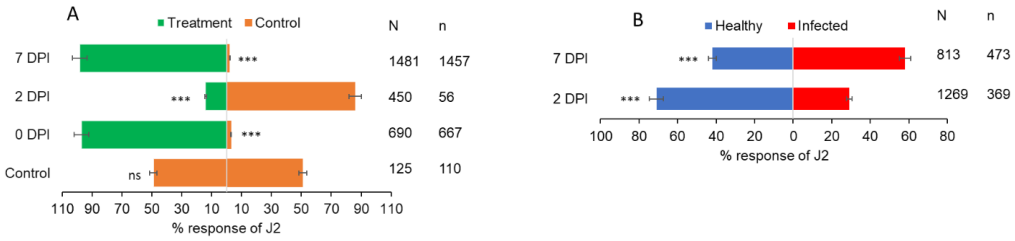
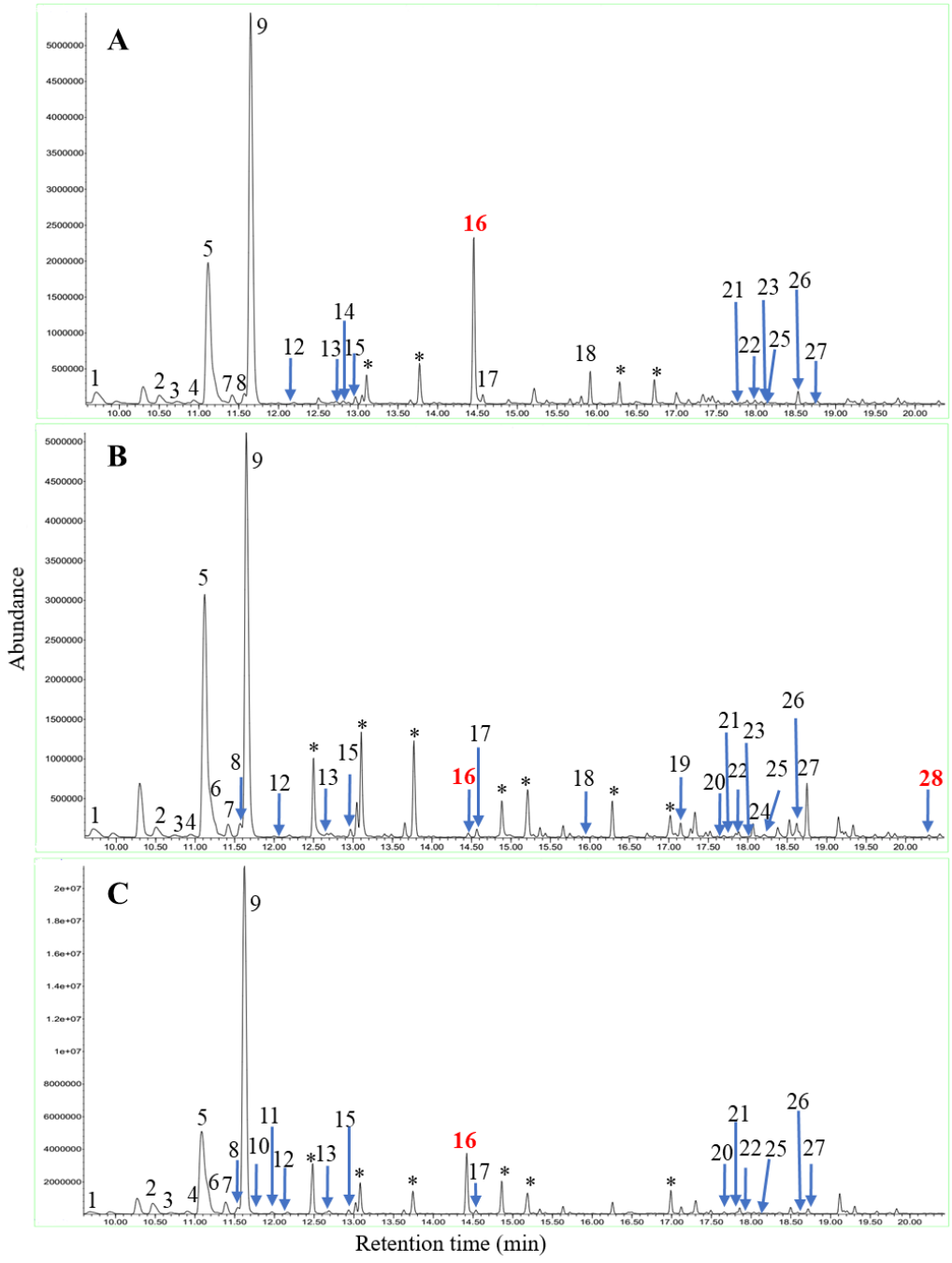

A
Treatment
Control
N
n
7 DPI
***
1481
1457
2 DPI
***
450
56
0 DPI
***
690
667
Control
ns
125
110
110 90 70 50 30 10 10 30 50 70 90 110
% response of J2
B
Healthy
Infected
N
n
7 DPI
***
813
473
2 DPI
***
1269
369
100 80 60 40 20 0 20 40 60 80
% response of J2

**Figure 1**


A
B
C
Abundance
Retention time (min)

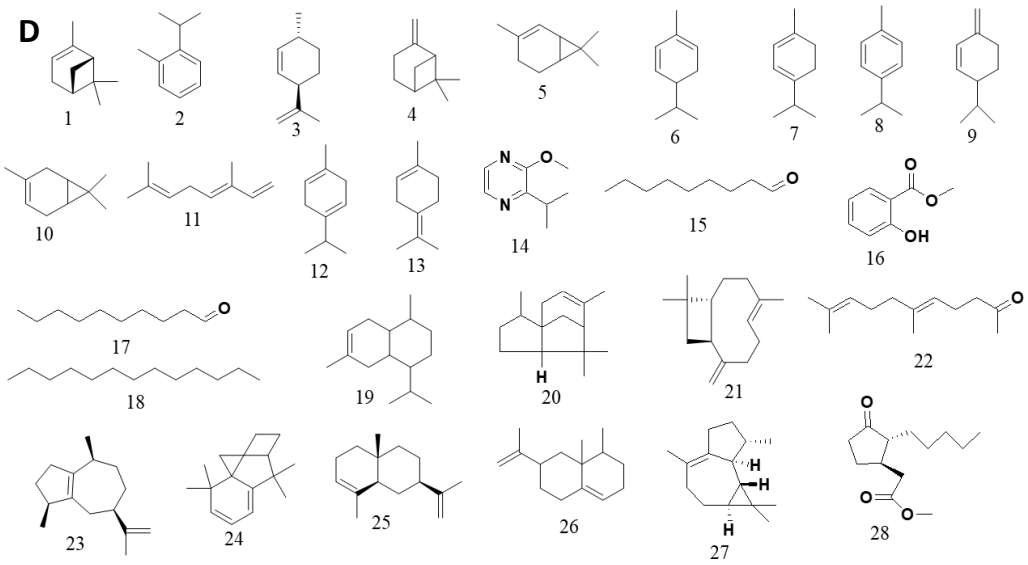



**Figure 2**

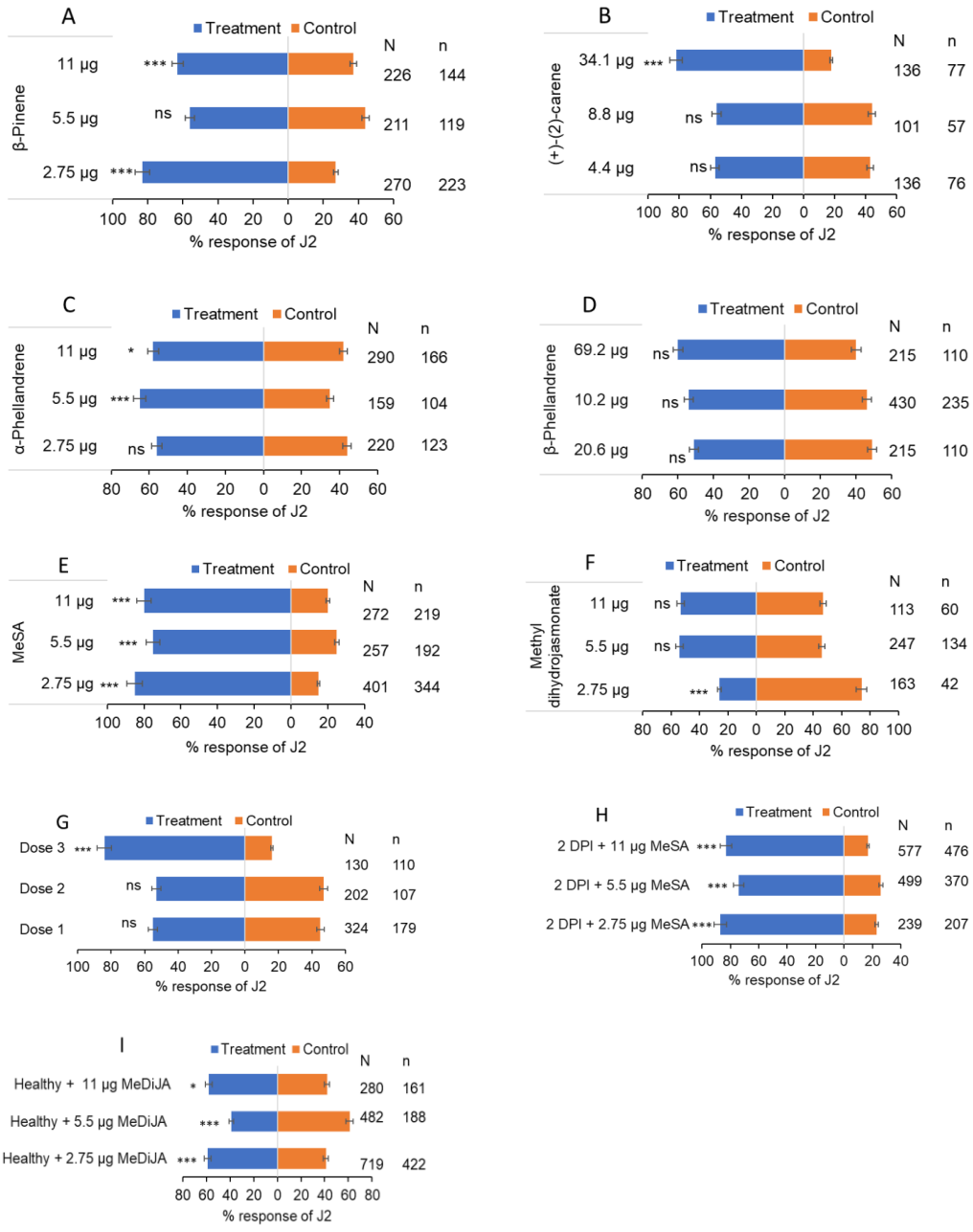
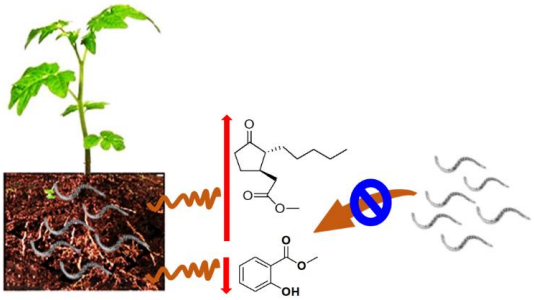

A
Treatment
Control
β-Pinene
11 µg
5.5 µg
2.75 µg
***
ns
***
N
n
226
144
211
119
270
223
100 80 60 40 20 0 20 40 60
% response of J2
B
Treatment
Control
(+)-(2)-carene
34.1 µg
8.8 µg
4.4 µg
***
ns
ns
N
n
136
77
101
57
136
76
100 80 60 40 20 0 20 40 60
% response of J2
C
Treatment
Control
α-Phellandrene
11 µg
5.5 µg
2.75 µg
*
***
ns
N
n
290
166
159
104
220
123
80 60 40 20 0 20 40 60
% response of J2
D
Treatment
Control
β-Phellandrene
69.2 µg
10.2 µg
20.6 µg
ns
ns
ns
N
n
215
110
430
235
215
110
80 60 40 20 0 20 40 60
% response of J2
E
Treatment
Control
MeSA
11 µg
5.5 µg
2.75 µg
***
***
***
N
n
272
219
257
192
401
344
100 80 60 40 20 0 20 40
% response of J2
F
Treatment
Control
Methyl dihydrojasmonate
11 µg
5.5 µg
2.75 µg
ns
ns
***
N
n
113
60
247
134
163
42
80 60 40 20 0 20 40 60 80 100
% response of J2
G
Treatment
Control
Dose 3
Dose 2
Dose 1
***
ns
ns
N
n
130
110
202
107
324
179
100 80 60 40 20 0 20 40 60
% response of J2
H
Treatment
Control
2 DPI + 11 µg MeSA
2 DPI + 5.5 µg MeSA
2 DPI + 2.75 µg MeSA
***
***
***
N
n
577
476
499
370
239
207
100 80 60 40 20 0 20 40
% response of J2
I
Treatment
Control
Healthy + 11 µg MeDiJA
Healthy + 5.5 µg MeDiJA
Healthy + 2.75 µg MeDiJA
*
***
***
N
n
280
161
482
188
719
422
80 60 40 20 0 20 40 60 80
% response of J2

**Figure 3**

**TOC graphic**



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