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- (71) **Applicant** (for all designated States except US): **UNIVERSITY OF FLORIDA RESEARCH FOUNDATION, INC.** [US/US]; 223 Grinter Hall, Gainesville, FL 32611 (US).
- (72) **Inventors; and**
- (75) **Inventors/Applicants** (for US only): **STELINSKI, Lukasz, L.** [US/US]; 289 James Circle, Lake Alfred, FL 33850 (US). **ALI, Jared, G.** [US/US]; 107 Judd Falls Road, Ithaca, NY 14850 (US). **ALBORN, Hans, T.** [US/US]; 4513 NW 130th Street, Gainesville, FL 32606 (US).
- (74) **Agents:** **VAN DYKE, Timothy, H.** et al.; Beusse Wolter Sanks Mora & Maire, 390 N. Orange Avenue, Ste 2500, Orlando, FL 32801 (US).

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(54) **Title:** PROTECTIVE NEMATODE ATTRACTANTS AND METHODS FOR USING THE SAME

(57) **Abstract:** The present invention is directed to methods and compositions for the indirect defense of plants. In one embodiment, there is provided a method for inhibiting infestation of a plant by root feeding pests. The method includes applying an amount of a nematode attractant lure effective to attract a plurality of protective nematodes to the plant that will kill the pests of the plant.



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PROTECTIVE NEMATODE ATTRACTANTS AND METHODS FOR USING THE SAME

CROSS REFERENCE TO RELATED APPLICATIONS

This application is related to U.S. Provisional Application No. 61/440,498 filed
5 February 8, 2011, to which priority is claimed under 35 USC 119, and whose teachings
are incorporated herein in their entirety by this reference.

STATEMENT REGARDING FEDERALLY SPONSORED DEVELOPMENT

Development for this invention was supported in part by USDA-CSREES grant
10 number 2005-34429-16432. Accordingly, the United States Government has rights in
this invention.

FIELD OF THE INVENTION

The present invention relates to nematode attractant lures, and more particularly
15 to nematode attractant lures and articles of manufacture comprising nematode
attractant lures, and to methods for attracting protective nematodes to a predetermined
site for killing pests of plants using such nematode attractant lures.

BACKGROUND OF THE INVENTION

20 Plants produce an array of signals with diverse roles, providing them with
responses necessary to survive in their dynamic environment. Examples of plants
luring organisms to facilitate their reproductive requirements are ubiquitous and often
taken for granted (Pichersky and Gershenzon 2002). Less acknowledged is the ability
of a plant to manipulate the behavior of organisms to serve defensive roles (Turlings
25 and Wäckers 2004) against particular pests. *Diaprepes abbreviatus* (L.) is a significant
belowground pest of plant roots on more than 290 plant species including citrus,
sugarcane, vegetables, potatoes, strawberries, woody field-grown ornamentals, sweet
potatoes, papaya, guava, mahogany, containerized ornamentals, and non-cultivated
wild plants (Simpson et al. 2000). *D. abbreviatus* was first introduced into Florida in
30 1964 (Beavers and Selhime 1975). Over the past 40 years it has significantly
contributed to the spread of disease and damage to citrus, ornamental plants, and other
crops causing approximately \$70 million in damage annually (Weissling et al. 2002). *D.*
abbreviatus damage the vegetative portion of plants by notching young leaves (Fennah

1940). Mature adults lay eggs between older leaves and emerging first instar larvae drop to the soil where they develop and feed on roots causing the most severe damage to plants (Schroeder 1992; Fennah 1940).

5 BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic diagram of sand column assay unit. Glass jar (17 ml) with samples at base (A), connecting tube (3 cm) with hole for nematode application (B), extracts placed on filter paper (C), arena was filled with heat sterilized sand at 10% moisture for all assays.

10 **Figure 2** shows a mean number of *S. diaprepesi* attracted to chambers containing weevil-infested plants versus non-infested control plants (A), weevil-infested plants versus larvae alone (B), weevil-infested plants, mechanically damaged plants or sand control (C). Each panel represents a separate experiment (n=10) conducted in a 6-arm olfactometer.

15 **Figure 3** provides exemplary chromatograms showing volatile profiles of *D. abbreviatus*-infested plants, non-infested plants and larvae alone. Volatile profile of infested *Citrus paradise* × *Poncirus trifoliata* rootstock (A) Volatile profile of non-infested *Citrus paradise* × *Poncirus trifoliata* rootstock (B) Volatile profile of *D. abbreviatus* alone in sand (C). All samples were collected for a 24 h. Geijerene (3), Pregeijerene (4), α –
20 Santalene (5), α-Z-Bergamotene (6). (Compound numbers correspond to Table 1).

Figure 4 shows the mean number of nematodes attracted to volatiles from *D. abbreviatus*-infested roots compared with volatiles from undamaged roots.

Figure 5 is a schematic diagram of a simultaneous above- and belowground volatile collection apparatus (ARS, Gainesville, FL, USA). The guillotine volatile
25 collection chambers used for aboveground collections received a constant flow of charcoal-purified and humidified air, which was suctioned at a rate of 300 ml / min through a trap containing 50 mg of Super Q adsorbent (Alltech Assoc., Deerfield, Illinois). Root-zone collection chambers used to collect belowground volatiles were filled with heat sterilized sand standardized at 10 % saturation.

30 **Figure 6** shows various nematode species when presented with: A) with volatiles from roots of *P. trifoliata* infested with *Diaprepes abbreviatus* larvae versus volatiles from undamaged *P. trifoliata* roots or B) volatiles from roots of *Citrus paradisi* ×

Poncirus trifoliata (Swingle hybrid) infested with *Diaprepes abbreviatus* larvae versus volatiles from undamaged *Citrus paradisi* × *Poncirus trifoliata* roots in two-choice olfactometer.

Figure 7 shows exemplary chromatograms depicting volatile profiles from simultaneous collections of root and shoot volatiles of Swingle (*Citrus paradise* × *Poncirus trifoliata*) in response to A) belowground and B) aboveground herbivory by *Diaprepes abbreviatus* larvae and adults, respectively. All samples were collected for 24hr.

Figure 8 shows an exemplary chromatogram showing volatile profiles from roots of A) *Poncirus trifoliata* or B) Sour orange (*Citrus aurantium*) in response to *Diaprepes abbreviatus* herbivory upon roots or undamaged controls. All samples were collected for 24hr.

Figure 9. Time course of pregeijerene (1, 5-dimethylcyclodeca-1,5,7-triene) release following initiation of weevil (*Diaprepes abbreviatus*) feeding on citrus roots.

Insert in the upper right displays chromatogram of volatile abundance at each interval.

Figure 10. Optimal dosage of pregeijerene (1, 5-dimethylcyclodeca-1,5,7-triene) for attracting entomopathogenic nematodes (*Steinernema riobrave* and *Heterorhabditis indica*) based on the log scale dilution of purified compound. Picture in upper left displays sand filled two-choice olfactometers used for nematode bioassays.

Figure 11. Effect of pregeijerene on *Diaprepes abbreviatus* larvae mortality and associated attraction of entomopathogenic nematode infective juveniles (IJs) (all species combined). **A)** Average mortality of larvae buried with purified pregeijerene compared with the solvent control ($N = 10$, $t = 4.01$, $P = 0.0008$) **B)** Mean number of IJs recovered from cages containing purified pregeijerene compared with cages containing the solvent control ($N = 10$, $t = 5.33$, $P = 0.00005$). **C)** Mean number of IJs recovered from soil samples surrounding cages containing pregeijerene compared with the solvent control ($N = 10$, $t = 5.67$, $P = 0.00003$).

Figure 12. Cylindrical mesh cages containing a single *D. abbreviatus* larva in autoclaved sandy soil (30) were treated with: (i) volatiles collected from weevil-infested roots, or (ii) a blank solvent control. Larval mortality was $74 \pm 6.9\%$ in the presence of volatiles from infested roots, but only $41 \pm 7.5\%$ in the solvent alone treatment ($N = 10$, $df = 18$, $t = 2.75$, $P = 0.013$).

Figure 13 Conversion of pregeijerene (A) to geijerene (B).

Figure 14 Chromatograms showing the initial crude extract prior to purification and final purified pregeijerene.

Figure 15 Soil probe design used to sample volatiles belowground. Probe is inserted into soil and connected to a vacuum pump.

Figure 16 Chromatograms of volatiles taken from intact citrus roots in the field at 1 and 10 m distances from the trunk of the tree.

Figure 17 Schematic diagram of the deployment and sampling procedure for field experiments in which sentinel traps with root weevils were deployed with or without HIPVs. One treatment replicate is depicted.

DETAILED DESCRIPTION OF THE INVENTION

It has been unexpectedly found that specific nematode recruitment signals are released by citrus species. These signals can be released constantly or only in response to herbivore damage by pests, such as weevil larvae, including but not limited to *Diaprepes abbreviates*. The nematode species attracted to these signals may include entomopathogens. As set forth in the examples below, it has been surprisingly found that the nematode recruitment signals (species) useful for attracting protective nematodes to kill weevil larvae feeding on citrus plants in a desired area may comprise one or more terpene compounds, such as pregeijerene and geijerene. In accordance with aspects of the present invention, there are provided compositions, articles of manufacture, and methods that utilize one or more of such terpene compounds to attract a plurality of protective nematodes to a desired location to kill a plurality of pests.

As used herein, by "effective amount," "amount effective," or the like, it is meant an amount effective at dosages and for periods of time necessary to achieve the desired result, e.g., attracting a greater amount of nematodes to a particular location to

kill a plurality of pests of plants in a desired area than without the particular nematode attractant lure.

By "killing" as used herein, it is meant the biological control agents, e.g., protective nematodes, attracted by the nematode attractant lure kill the pest and/or inhibits or reduces the growth of the pest. The growth of a pest has been inhibited if there has been a relative reduction in the number of pests in a desired area. The growth of a pest may also be said to have been inhibited if the normal growth pattern of the pest has been modified so as to have a negative effect on the individual pest. The number of pests has been reduced by an action if there are fewer pests in a desired area than there would have been without the action.

In accordance with one aspect of the invention, there is provided a method for inhibiting infestation of a plant. The method comprises releasing an amount of a nematode attractant lure and an agriculturally acceptable carrier to or adjacent to a plant effective to attract a plurality of protective nematodes to the plant that will kill the pests of the plant. In one embodiment, the nematode attractant comprises a terpene compound, such as geijerene or pregeijerene. In a particular embodiment, the terpene compound comprises pregeijerene. The term "adjacent" is intended to include any of area application about the plant where the desired effect (attracting nematodes to kill a plurality of pests at a desired location) is produceable. In a specific embodiment, the term "adjacent" means within 10 meters, 9 meters, 8, meters, 7 meters, 6 meters, 5 meters, 4 meters, 3 meters, 2 meters, 1 meter, 0.5 meters away from the plant, either above ground or belowground, or both.

In accordance with another aspect of the present invention, there is provided a nematode attractant lure. The nematode attractant lure comprises an amount of a terpene compound effective to attract a plurality of protective nematodes to a plant that will kill pests of the plant and an agriculturally acceptable carrier.

In accordance with another aspect of the present invention, there is provided an article of manufacture. The article of manufacture comprises an amount of a terpene compound effective to attract a plurality of protective nematodes to a plant that will kill pests of the plant. In some embodiments, the article of manufacture may further comprise an agriculturally acceptable carrier for the terpene compound and a container comprising the amount of the terpene compound and optionally the agriculturally

acceptable carrier. Further, the article of manufacture comprises a structure, such as a sprayer, for applying at least the terpene compound from the container to a target area about the plant, e.g., an area at or adjacent to the plant.

The nematode attractant lure in the embodiments described herein may
5 comprise a terpene compound. In a particular embodiment, the nematode attract lure comprise pregeijerene. Generally, a terpene compound refers to one or more compounds from a class of hydrocarbons empirically regarded as built up from isoprene, a hydrocarbon consisting of five carbon atoms attached to eight hydrogen atoms (C₅H₈). The term "terpene compound" as used herein may also include
10 terpenoids, which are oxygenated derivatives of these hydrocarbons. In a particular embodiment, the terpene compound may be pinene, limonene, geijerene, pregeijerene, or combinations thereof. The pinene may be alpha-pinene or beta-pinene. As set forth in the examples below, the present inventors have surprisingly found that these terpene compounds, e.g., pregeijerene, may act as particular attractants for protective
15 nematodes that will kill pests of citrus plants, such as *Diaprepes abbreviatus* (L.), as well as pests of other plants.

The amount of the terpene compound provided may be any particular concentration suitable for the particular application as would be readily determinable by one skilled in the art. In one embodiment, the terpene compound may be provided in a
20 concentration of from 0.004µg/ml to 4µg/ml. In any of the embodiments described herein, the terpene compound can be made synthetically by known methods, may be purchased from a suitable commercial source, or may be extracted from suitable plants as set forth herein.

The nematode attractant lure as described herein may be effective to attract
25 protective nematodes to any desired plant to kill a population of pests of the plant. In one embodiment, the plant may be any tree or plant that is infected or may be infected with insect pests, such as, but not limited to weevil larvae (e.g., *Diaprepes abbreviatus*) which are pests to over 290 plant species. Exemplary plants include, but are not limited to citrus, sugarcane, vegetables, potatoes, strawberries, woody field-
30 grown ornamentals, sweet potatoes, papaya, guava, grapes, mahogany, containerized ornamentals, and non-cultivated wild plants. In a particular embodiment, the plant may be any citrus plant, such as any plant derived from a citrus rootstock, hybridized or non-

hybridized. In one embodiment, the targeted plant as described herein is a non-hybridized rootstock, such as *P. trifoliata* rootstock or *Citrus aurantium*. In another embodiment, the targeted plant is derived from a hybrid rootstock, such as *Citrus paradise* Macf. x *P. trifoliata* L. Raf., rootstock.

5 The nematodes attracted by the nematode attractant lure described herein include any species, e.g., entomopathogenic nematodes, that will kill a plurality of pests in a desired location as described herein. Exemplary entomopathogenic nematodes include *S. diaprepesi*, *S. carpocapsae*, *S. riobrave*, and *H. indicia*. Entomopathogenic nematodes may employ any type of typical foraging strategy, such as ambush or
10 cruising foraging strategies.

 The targeted pests to kill may be any pest which is reduced when a quantity of protective nematodes are intentionally led or attracted to a desired location by the nematode attractant lures as described herein. As mentioned above, the present inventors have found that particularly terpene compounds, e.g., pregeijerene, are
15 suitable for attracting destroyers (nematodes) of *Diaprepes abbreviatus*. It is understood that the present invention, however, is not limited to killing a particular pest and that the compositions described herein may be utilized to kill a number of other pests if the compositions are able to attract a quantity of species, e.g., protective nematodes, that will destroy the targeted pest.

20 The nematode attractant lure of the present invention may be formulated as desired and incorporated into any suitable apparatus for application onto or within the vicinity of the targeted crops. In a more specific embodiment, the apparatus is designed for extended release of the nematode attractant. The apparatus may be configured for extended release above-ground or belowground, or both. For example, the terpene
25 compound may be prepared under pressure in a metering device, that may be inserted into the ground with a port for release of attractant aboveground, a port for release belowground, or two ports for release aboveground and belowground.

 In addition, the nematode attractant lure may be applied to the subject plants by spraying the nematode attractant lure on the plants, and in one embodiment, by the
30 controlled release of the nematode attractant lure. Alternatively, any other method of applying the nematode attractant lures may be used. Typically, it is desirable to apply the nematode attractant lure to the top and underside of the leaves of the plants, as well

as an area around the trunk and root system of the plant. In a particular embodiment, the nematode attractant lure may be applied to a subterranean area about the plant, e.g., below the soil adjacent to a location of the plant. In other embodiments, plants may be genetically engineered to release the nematode attractant lure in greater
5 quantities. It is appreciated that the amount of nematode attractant lure applied in any particular situation will vary depending upon a number of factors such as the nature of the crop, the level of pest infestation etc.

In addition, the nematode attractant lures described herein may be used either alone or in conjunction with other insecticides known in the art. In the latter case, the
10 nematode attractant lures of the present invention can lead to an improvement in performance of the other insecticide, and thus the nematode attractant lure produces an adjuvant effect. Further, the nematode attractant lure of the present invention may reduce application rate and application frequency.

The nematode attractant lures of the present invention are generally formed into
15 formulations suitable for use according to a normal method for formulating agricultural/horticultural pesticides. Namely, a terpene compound as described herein may be mixed with an appropriate agriculturally acceptable carrier, and if required, an auxiliary at a proper proportion, and the resultant mixture is subjected to dissolution, separation, suspension, mixing, impregnation, adsorption or adhesion and can be
20 formulated into any desired forms for practical use, such as soluble concentrates, emulsifiable concentrates, wettable powders, water soluble powders, water dispersible granules, water soluble granules, suspension concentrates, concentrated emulsions, suspoemulsions, microemulsions, dustable powders, granules, tablets and emulsifiable gels. By "agriculturally acceptable carrier," it is meant an agent that does not have a
25 substantial detrimental effect on the activity of the active ingredients (e.g., terpene compound(s)) described herein as well as the target crops.

The agriculturally acceptable carrier may be a solid, liquid, or gas. Examples of a material usable as a solid carrier include soybean flour, grain flour, wood flour, bark
flour, sawing flour, tobacco stalk flour, walnut shell flour, bran, cellulose powder, a
30 residue after plant extraction, a synthetic polymer such as a synthetic resin powder, clay (e.g., kaoline, bentonite, or acid white clay), talc (e.g., talc or pyrophyllite), silica (for example, diatomite, silica powder, mica, activated carbon, sulfur powder, pumice,

calcined diatomite, brick powder, fly ash, sand, inorganic mineral powders such as calcium carbonate and calcium phosphate, chemical fertilizers such as ammonium sulfate, ammonium phosphate, ammonium nitrate, urea, and ammonium chloride, and compost.

5 A suitable liquid carrier may be one having a solvent ability or a material having no solvent ability, but having an ability to assist in the dispersion of the active ingredient compound. Exemplary liquid carriers include water, alcohols (e.g., methanol, ethanol, isopropanol, butanol, and ethylene glycol); ketones (e.g., acetone, methylethyl ketone, methyl isobutyl ketone, diisobutyl ketone, and cyclohexanone); ethers (e.g., diethyl
10 ether, dioxane, cellosolve, diisopropyl ether, and tetrahydrofuran); aliphatic hydrocarbons (e.g., kerosine and mineral oil); aromatic hydrocarbons (e.g. benzene, toluene, xylene, solvent naphtha, and alkylnaphthalene); halogenated hydrocarbons (e.g., dichloromethane, chloroform, carbon tetrachloride, and chlorobenzene); esters (e.g., ethyl acetate, butyl acetate, ethyl propionate, diisobutyl phthalate, dibutyl
15 phthalate, and dioctyl phthalate); amides (e.g., dimethylformamide, diethylformamide, and dimethylacetamide); and nitriles (e.g., acetonitrile). In one particular embodiment, the agriculturally acceptable carrier comprises an agriculturally acceptable carrier oil, including but not limited to, mineral oil or a vegetable oil such as canola oil, sunflower oil, cottonseed oil, palm oil, soybean oil, and the like. In one further particular
20 embodiment, mineral oil is provided as the agriculturally acceptable carrier.

When the composition or nematode attractant lure will be used as an aerosol, a propellant may be added such as propane, butane, isobutane, dimethyl ether, carbon dioxide, nitrous oxide, nitrogen, and combinations thereof. Further, it is understood that the compositions of the present invention may additionally include any suitable
25 surfactant, penetrating agent, spreading agent, thickener, anti-freezing agent, binder, anti-caking agent, disintegrating agent, anti-foaming agent, preservative, stabilizer, and the like.

The following examples are intended for the purpose of illustration of the present invention. However, the scope of the present invention should be defined as the claims
30 appended hereto, and the following examples should not be construed as in any way limiting the scope of the present invention.

EXAMPLE 1

The following example describes the behavior of the entomopathogenic nematode, *Steinernema diaprepesi* Nguyen & Duncan, in response to citrus plants damaged by larval *D. abbreviatus*. The results show that entomopathogenic nematodes (EPNs) are attracted to weevil-damaged roots, but not so to mechanically damaged roots, undamaged roots or larvae alone. Also identified were volatile compounds induced by weevil feeding and show that EPN response is specifically mediated by solvent extracts of infested roots. Identification of the signals that mediate interactions between *D. abbreviatus*-infested plants and the associated EPNs could advance biological control of *D. abbreviatus* by selectively increasing the functional and/or numerical response of its natural enemies.

Materials and Methods

Insects: *D. abbreviatus* larvae were obtained from a culture at University of Florida's Citrus Research and Education Center (CREC) in Lake Alfred, FL. This culture was periodically supplemented from a large culture maintained at the Division of Plant Industry Sterile Fly Facility in Gainesville, FL. Larvae are reared on an artificial diet developed by Beavers (1982) using procedures described by Lapointe and Shapiro (1999). Larvae used in experiments were 3rd to 6th instars.

Nematodes: *S. diaprepesi* were isolated from *D. abbreviatus* larvae buried in a commercial citrus orchard in Florida. The nematodes were then reared in last-instar greater wax moth larvae, *Galleria mellonella* (L.) (Lepidoptera: Pyralidae), at approximately 25°C according to procedures described in Kaya and Stock (1997). Infective juveniles (IJs) that emerged from insect cadavers into White traps (White 1927) were stored in shallow water in transfer flasks at 15°C for up to 2 weeks prior to use.

Plants: 'Swingle citrumelo' (*Citrus paradisi* Macf. × *Poncirus trifoliata* L. Raf.) rootstock is very prominent in commercial citrus production. The prevalence of this genotype is due to its tolerance to blight, citrus tristeza virus, plant parasitic nematodes and *Phytophthora* spp., as well as cold tolerance (Stover and Castle 2002). The extensive use of this rootstock in commercial citrus production justified its use in this investigation. All plants were grown and maintained at the CREC in Lake Alfred, FL, USA in a greenhouse at 26°C, and 60-80% RH.

Olfactometer: EPN response to *D. abbreviatus*-infested roots was tested with a root zone olfactometer (Analytical Research Systems, Gainesville, FL, USA) according to the design described in Rasmann et al. (2005). The olfactometer consisted of a central glass chamber (8 cm in diameter and 11 cm deep) attached by 6 side arms to 6 glass pots (5 cm in diameter and 11 cm deep) in which various plants/treatments were tested. The side arms were joined to the 6 treatments pots with Teflon connectors fitted with a fine mesh filter impervious to nematodes (2300 mesh, Smallparts, Inc., Miramar, FL). For all tests, the olfactometer was filled with sand that had been autoclaved for 1 h at 250°C and then adjusted to 10% moisture (dry wt. sand:water volume; W/V). In tests involving plants, seedlings were given three days to adjust to their sand filled olfactometer for each experiment in this example.

In the first experiment of this example, nematode response to weevil-infested plants was tested versus non-infested controls. Infested plants were subjected to three days of feeding by 3rd-6th instar weevil larvae. Non-infested plants were not exposed to weevils. Three of the arms of the olfactometer were randomly assigned to a weevil-infested plant while the remaining three received the non-infested control. 1J nematodes (2500) were released into the central olfactometer chamber. Twenty-four hours after nematode release, the olfactometer was disassembled and nematodes from each connecting arm were recovered from soil using Baermann extractors; extracted nematodes were collected and counted with a dissection scope. The tests were replicated with ten nematode releases for each treatment.

In the second experiment of this example, the response of EPNs to weevil-infested plants was compared with larvae alone in sand. The bioassay consisted of three chambers with plants infested with six larvae each (as above) and three chambers containing six larvae in sand only. The experimental protocol and sampling procedures were otherwise identical to experiment one of this example.

In a third experiment of this example, EPN response was assayed to weevil-infested plants (as above) versus mechanically damaged roots. The treatments compared consisted of two mechanically damaged plants, two infested plants, and two sand only control arms. Treatments were randomly assigned to chambers. Plant roots were mechanically damaged by stabbing roots five times daily with a metal corkborer for 3 days prior nematode release (7 mm in diameter). This damage procedure was

used because it visually resembled the type of damage inflicted by feeding *D. abbreviatus* larvae after 72 h. All other experimental and sampling procedures were identical to those described for experiment one of this example.

Volatile collections: The objective of this experiment was to identify volatiles emitted by citrus roots damaged by weevil larvae. Volatiles were collected from 1) sand alone (negative control), 2) larvae alone in sand, 3) non-infested plant roots, and 4) weevil-infested roots. Each treatment was prepared within a chamber and connecting arm of the 6-chambered olfactometer and filled with the same 10% moistened sand as in the bioassays. Larvae, non-infested plants, and infested plants were maintained for three days before sampling. All plants were maintained in the olfactometer chambers for three days prior to weevil infestation. Thereafter, each chamber of the olfactometer containing a treatment was connected to a vacuum pump (ARS, Gainesville, FL, USA) for 24 h with a suction flow of 0.8 ml/min. Compounds emitted from chambers were collected on adsorbent traps filled with 50 mg Super-Q, 800-1000 mesh (Alltech Deerfield, IL, USA) held in glass fittings between the chamber and vacuum pump. Thereafter, Super-Q traps were rinsed with 150 μ L of dichloromethane into individual 2.0 ml clear glass vials (Varian, Palo Alto, CA, USA, part number: 392611549 equipped with 500 μ L glass inserts).

GC-MS analysis: A 1 μ L aliquot of each dichloromethane extract was injected onto a GC-MS gas chromatograph (HP 6890) equipped with 30 m $\times$ 0.25-mm-ID, 0.25 μ m film thickness DB-5 capillary column (Quadrex, New Haven, CT, USA), interfaced to a 5973 Mass Selective Detector (Agilent, Palo Alto, CA, USA), in both electron impact and chemical ionization modes. The column was held at 40°C for 1 min after injection and then programmed at 10 $\pm$ °C/min to 260°C. The carrier gas used was helium at a flow average velocity of 30 cm/sec. Isobutane was used as the reagent gas for chemical ionization, and the ion source temperature was set at 250°C in CI and 220°C in EI. EI Spectra library search was performed using a floral scent database compiled at the Department of Chemical Ecology, Göteborg Sweden, the Adams2 terpenoid/natural product library (Allured Corporation, Adams 1995) and the NIST05 library. When available, mass spectra and retention times were compared to that of authentic standards.

EPN response to root extracts: To compare EPN response to solvent extracts of citrus roots before and after weevil feeding, citrus plants were placed individually into chambers of the 6-arm olfactometer for three days as previously described. Thereafter, volatiles were collected from chambers for 24 h as described above in the volatile collections procedure. Six larvae were then placed into each chamber containing a plant and allowed to feed for 3 d. Thereafter, volatiles were collected a second time from the intact feeding system for 24 h. The adsorbent Super-Q traps from both treatments (before and after feeding) were extracted by rinsing with 150 μ L of dichloromethane directly after their 24 h collections as described above.

To quantify EPN response to the root extracts collected, a two choice sand-filled olfactometer was used (Fig. 1). The olfactometer consists of three detachable sections: two opposing glass jars (A) (16 ml BTL, sample type 111, CLR, SNAPC, Wheaton, Millville, NJ), which contained treatments and a central connecting tube 3cm in length (Blue Maxtm 50 ml polypropylene conical tube 30x115 mm, Becton Dickinson Labware, Becton Dickinson Company, Franklin Lakes, NJ, USA), with an apical hole into which nematodes were applied (B). Extracts were placed on filter paper, which was allowed to dry 30 s for solvent evaporation. Thereafter, filter papers were placed on the bottom of each glass jar (C) which were subsequently filled with 10% saturated, sterilized sand as described above. The central chamber connecting the two jars (arms of the olfactometer) was also filled with sterilized and moistened sand. The entire olfactometer was 8 cm in length when assembled with two possible extract treatments at opposite ends of the nematode release point. Nematodes (200 IJs) were applied into the central orifice of the connecting tube and given 8 h to respond. Thereafter, the column was disassembled and the contents of the two collection pots were sampled using Baermann extractors; extracted nematodes were collected and counted. The experiment was replicated ten times.

Statistical Analysis: Paired t-tests were used to compare nematode response in experiments testing root extracts in the two-choice olfactometers ($df=9$). Data from experiments using the six-arm olfactometer were analyzed with a log-linear model.

Given that these data did not conform to simple variance assumptions implied in using the multinomial distribution, quasi-likelihood functions were used to compensate for the over dispersion of nematodes within the olfactometer (Turlings et al. 2004). The model

was fitted by maximum quasi-likelihood estimation in the software package R (R Development Core Team 2004).

Results

Olfactometer Bioassays: Significantly more EPNs were found attracted to *D. abbreviatus*-infested roots than non-infested control roots ($F=12.76$, $df=1$, 58 , $P<0.001$) (Fig. 2A). Infested roots attracted significantly more EPNs per arm than those containing larvae alone ($F=13.78$, $df=1$, 58 , $P<0.001$) (Fig. 2B). Significantly more EPNs were attracted to *D. abbreviatus*-infested roots than to either mechanically damaged roots or the sand control ($F=12.34$, $df=2$, 57 , $P<0.001$) (Fig. 2C). There was no significant attraction to mechanically damaged roots as compared with the sand control ($P=0.34$) (Fig. 2C).

GC-MS analysis: Both α -pinene and β -pinene were identified in non-infested and infested plant roots by GC-MS (Table 1). *D. abbreviatus*-infested roots released four additional unique compounds that were not present in non-infested roots (Table 1). Two sesquiterpenes were the most abundant and were consistently present in infested roots. These were geijerene and its precursor pregeijerene (Fig 3). On-column GC/MS analyses showed significantly less geijerene and a comparable increase of pregeijerene strongly suggesting a thermal degradation of geijerene to pregeijerene during GC analyses with splitless injection. It is therefore an open question how much geijerene might actually be released by the infested roots. The above six compounds were absent from pots containing larvae alone (Table 1).

Table 1. GC-MS identification of volatiles from Swingle citrumelo rootstock (*Citrus paradise* $\times$ *Poncirus trifoliata*)

Peak #	RT	Name	CAS#	Infested root	Non-infested root	Larvae only
				Presence		
1	7.50	α -pinene ^{1,2}	000080-56-8	+	+	-
2	8.08	β -pinene ^{1,2}	000127-91-3	+	+	-
3	10.81	Geijerene ²	006902-73-4	+	-	-
4	12.93	Pregeijerene ²	020082-17-1	+	-	-
5	14.75	α -Santalene ²	000512-61-8	+	-	-
6	14.93	α -Z-Bergamotene ²	018252-46-5	+	-	-

¹Synthetic standard comparison. ²Identification was based on comparisons of retention times with standard and spectral data from Adams, EPA, and Nist05 Libraries.

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EPN response to root extracts Significantly more EPNs were found in arms containing solvent extracts of *D. abbreviatus*-infested roots than non-infested roots ($P=0.03$) (Fig. 4).

The above results indicate that Swingle citrumelo rootstock releases herbivore induced volatiles that recruit EPNs. In addition, the current results indicate that a commercially used citrus rootstock emits induced volatile chemicals in response to herbivore feeding that attract beneficial nematodes. One application of these finding is to apply EPN attractant compounds to plants infested by root weevil by the methods and apparatuses described above. Another application of the above findings is to engineer plants for increased release of terpenes (Schnee et al. 2006).

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EXAMPLE 2

In the below example, it was determined whether: 1) nematode foraging strategy (cruiser vs. ambusher), and trophic level (plant parasitic vs. entomopathogenic) affects nematode response to herbivore-induced plant volatiles (HIPVs); 2) plant release of nematode recruitment signals varies between various citrus species including a cultivated hybrid line of citrus and one of its parental rootstocks; and 3) aboveground

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feeding by adult *Diaprepes* weevils induces release of subterranean HIPVs analogous to those released following root damage by larvae and *vice versa*.

Materials and methods:

Insects: *D. abbreviatus* larvae were obtained from a culture maintained at University of Florida's Citrus Research and Education Center (CREC) in Lake Alfred, FL, USA. This culture was periodically supplemented from a larger culture maintained at the Division of Plant Industry Sterile Fly Facility in Gainesville, FL, USA. Larvae were reared on an artificial diet developed by Beavers (1982) using procedures described by Lapointe and Shapiro (1999). Larvae used in experiments were third to sixth instars.

Female adults were used two weeks after emergence.

Nematodes: Nematode foraging strategy and trophic level status is summarized in Table 2 below. The entomopathogenic nematodes, *Steinernema diaprepesi* Nguyen & Duncan (Sd), *S. riobrave* (Sr), *S. carpocapsae* (Sc), and *Heterorhabditis indica* (Hi) were isolated from *D. abbreviatus* larvae buried in commercial citrus orchards in Florida.

S. riobrave and *S. carpocapsae* isolates were descendants of commercial formulations intended for field application to manage *Diaprepes* weevils. All entomopathogenic nematode (EPN) species were cultured in last instar larvae of the greater wax moth, *Galleria mellonella* (L.), at approximately 25°C according to procedures described in Kaya and Stock (1997). Infective juveniles (IJs) that emerged from insect cadavers into White traps (White 1927) were stored in shallow water in transfer flasks at 15°C for up to 2 wk prior to use.

Table 2. Trophic level, foraging strategy, and ecological background of nematodes tested

Nematode spp.	Trophic Level	Foraging Strategy	Ecological Status
<i>S. diaprepesi</i>	Entomopathogen	Intermediate	Indigenous to FL
<i>S. carpocapsae</i>	Entomopathogen	Ambush	Commercially introduced

<i>S. riobrave</i>	Entomopathogen	Intermediate	Commercially introduced
<i>H. indica</i>	Entomopathogen	Cruiser	Commercially applied indigenous to FL
<i>T. semipenetrans</i>	Plant parasite	Sedentary root endoparasite	Agricultural pest citrus parasite

Entomopathogenic nematode species can be categorized according to their foraging behaviour. “Ambush” (sit-and-wait) versus “cruiser” (active wide search radius) strategies are generally considered as dipoles of a continuum of salutatory search tactics (Lewis *et al.* 1992, 1993; Grewal *et al.* 1996; Campbell & Gaugler 1997).
 5 Cruisers allocate more of their time scanning for resource-associated cues as they move through their environment, exhibiting only brief pauses, and are therefore more effective at finding sedentary and cryptic hosts (Campbell & Gaugler 1997). In contrast, ambush foragers scan during long pauses and allocate less time to active movement
 10 through their environment (Campbell & Gaugler 1997). They are thought to wait for resources to come to them, increasing effectiveness of finding highly mobile prey. *S. carpocapsae* is a representative ambushing type EPN, while *H. indica* (non-nictating) is a typical cruising type EPN (Lewis 2002). *S. diaprepesi* is a recently discovered species indigenous to Florida's central ridge and specializes on *Diaprepes* root weevils
 15 and is considered intermediate on the spectrum between ambushers and cruisers (Nguyen & Duncan 2002). Finally, *S. riobrave* was discovered in Texas and is currently commercially formulated for control *D. abbreviates* in citriculture; it is also considered intermediate with respect to foraging strategy (Cabanillas *et al.* 1994).

The citrus nematode, *T. semipenetrans* (Ts), is one of the most significant
 20 parasites of plants worldwide affecting 8-12 % of all citrus species. In Florida, it is estimated to affect 53-89 % of described plant species (Noling 1993). The life cycle of *T. semipenetrans* consist of an egg and four larval stages followed by a sexually reproducing adult stage. Second stage larvae are the infective juveniles that infest citrus roots. This larval stage penetrates deeply into feeder root cortical tissues, where they

become immobile, establishing permanent, specialized feeding sites within the root (Munn & Munn 2002). Second stage larvae molt three times, increasing in size with each molt to form large, posteriorly swollen females capable of depositing ca. 75,500 eggs per female (Munn & Munn 2002). *T. semipenetrans* were obtained from infected field grown citrus. Infected roots and surrounding soil were soaked and IJ nematodes were subsequently extracted via sieving and centrifugation-flotation (Southey 1986).

Plants: All plants were grown and maintained at the CREC in Lake Alfred, FL, USA in a greenhouse at 26 °C, and 60–80% RH. *P. trifoliata* is a common rootstock for commercial production of oranges, grapefruit, most mandarins and lemons. Its prevalence is based on advantages such as resistance to *Phytophthora* fungi, citrus nematode, citrus tristeza virus, as well as cold tolerance and high fruit quality (Stover & Castle 2002). A major drawback is its slow growth (Stover & Castle 2002). It is typically hybridized to blend its desirable qualities with the faster growth of other varieties (Gardner & Horanic 1967). Swingle citrumelo, *Citrus paradisi* Macf. × *P. trifoliata* L. Raf., rootstock is one of these hybrids and is very prominent in commercial citrus production (Hutchinson 1974; Stover & Castle 2002). Sour orange, *Citrus aurantium*, is one of the oldest and most widely used rootstocks used for commercially grown citrus (Stover & Castle 2002). However, its susceptibility to tristeza virus and citrus nematode has decreased its prevalence in the past decade (Stover & Castle 2002). These three rootstocks were chosen in an effort to determine the breadth of nematode recruitment signaling among diverse citrus varieties with and without hybridization for commercial plant breeding.

Nematode Behaviour: The behavioural responses of nematodes to collected root samples were quantified in a two choice sand-filled olfactometer described thoroughly by Ali *et al.* (2010). The olfactometer consisted of three detachable sections: two opposing 16 ml glass jars which contained treatments and a central connecting tube 3 cm in length with an apical hole into which nematodes were applied (Ali *et al.* 2010). Extracts from each plant species were collected according to the methods described by Ali *et al.* (2010). Extracts from infested and non-infested roots were placed on filter paper, which was allowed to dry 30 sec for solvent evaporation. Thereafter, filter papers were placed on the bottoms of each glass jar, which were subsequently filled with 10% saturated, sterilized sand (Ali *et al.* 2010). The central

chamber connecting the two arms of the olfactometer was also filled with sterilized and moistened sand. Nematodes (*ca.* 200) were applied into the central orifice of the connecting tube and given 8 h to respond. Thereafter, the column was disassembled and the contents of the two collection pots were sampled using Baermann extractors; extracted nematodes were collected and counted. The experiment was replicated ten times for each nematode species and plant rootstock combination. The control treatment for each nematode species consisted of solvent blanks placed in each arm of the olfactometer. This double blank treatment produced identical results for each nematode species (no response), and thus a mean for all nematode species examined is reported for this treatment.

Above Versus Belowground Volatile Collections: Volatile samples were collected to examine whether adult feeding on plant shoots induces a nematode recruitment response by plants analogous to that observed in response to direct root damage by larvae. Complementary sampling of shoot volatiles was conducted to determine if aboveground release of similar signals occurs in response to root damage by larvae. Volatiles were sampled simultaneously from the roots and shoots of Swingle plants using a headspace guillotine chamber coupled with a root-zone collection chamber (Fig. 5). Plants were initially placed in glass root-zone chambers (ARS, Gainesville, FL, USA) filled with sand that had been autoclaved for one h at 250°C and then adjusted to 10% moisture (dry wt. sand: water volume; W/V) as described in Ali et al. (2010). The chambers and plants were placed below a platform on which a Teflon guillotine was attached (FIG. 5). The shoots of the plant passed through the guillotine opening and Teflon slides were positioned at the base to seal off the upper portions of the plant from the root zone. A glass chamber was then placed on the Teflon platform containing all upper portions of the exposed plant. Charcoal purified and humidified air was drawn over plants and pulled out at a rate of 300 ml / min through a trap containing 50 mg of Super Q adsorbent (Alltech Assoc., Deerfield, Illinois). Volatiles were collected for 24 h after which Super-Q traps were rinsed with 150 µl of dichloromethane into individual 2.0 ml clear glass vials as described above.

Volatiles from both roots and shoots of plants were initially sampled three days after preparation to determine baseline volatile production. On day four, plants were infested with either six larvae at the rootzone or six female adults were placed on leaves

aboveground. The below- and aboveground chambers of each infestation type were sampled for three subsequent days after infestation. Beetle feeding was easily noticeable in damaged leaves aboveground and was visually confirmed on roots after the feeding interval (Ali et al. 2010). Each infestation treatment was replicated 5 times.

5 **Volatile Collection From Infested Versus Non-infested Plants:** The objective of this experiment was to compare volatile release by roots of *P. trifoliata* and Sour orange (*Citrus aurantium*) that were damaged by *D. abbreviatus* feeding or left undamaged. Plants were potted in sand-filled glass root-zone chambers as described above. Seedlings were given three d to adjust to their sand filled chambers. Infested
10 plants were subjected to an additional three d of feeding by third to sixth instar weevil larvae. Non-infested plants were not exposed to weevils during this period. Thereafter, each root-zone chamber was connected to a vacuum pump (ARS, Gainesville, FL, USA) for 24 hr with a suction flow of 80 ml / min (Ali et al. 2010). Compounds emitted from chambers were collected on adsorbent traps filled with 50 mg Super-Q, (800–1000
15 mesh, Alltech Deerfield, IL, USA) held in glass fittings between the chamber and vacuum pump (Ali et al. 2010). Thereafter, Super-Q traps were rinsed with 150 µl of dichloromethane into individual 2.0 ml clear glass vials (Varian, Palo Alto, CA, USA, part number: 392611549 equipped with 500 µl glass inserts) (Ali et al. 2010).

GC-MS Analysis: All samples were injected as 1 µl aliquots of dichloromethane
20 extracts onto a gas chromatograph (HP 6890) equipped with 30 m×0.25-mm-ID, 0.25 µm film thickness DB-5 capillary column (Quadrex, New Haven, CT, USA), interfaced to a 5973 Mass Selective Detector (Agilent, Palo Alto, CA, USA), in both electron impact and chemical ionization modes. The column was held at 40°C for 1 min after injection and then programmed at 10 ±° C / min to 260°C. The carrier gas used was helium at
25 an average flow velocity of 30 cm / sec. Isobutane was used as the reagent gas for chemical ionization, and the ion source temperature was set at 250°C in CI and 220°C in EI. EI Spectra library search was performed using a floral scent database compiled at the Department of Chemical Ecology, Göteborg Sweden, the Adams2 terpenoid/natural product library (Allured Corporation, Adams 1995) and the
30 NIST05library. When available, mass spectra and retention times were compared to those of authentic standards.

STATISTICAL ANALYSIS: Nematode response investigated in the two-choice bioassay chambers was analyzed with a two-factor analysis of variance (ANOVA) with root extract treatment and nematode species comprising the two factors. Where ANOVA showed significant differences, Tukey's HSD tests ($\alpha < 0.05$) were conducted to discriminate among means in the software package R (R Development Core Team 2004). Given that a lack of response to the double blank control occurred consistently for each nematode species tested, the responses of each species were pooled for this treatment.

Results

Nematode Behavior: Nematodes of each species tested responded equally to *Diaprepes*-infested versus uninfested (negative control) *P. trifoliata* roots ($F = 3.0$, $df = 2,72$, $P = 0.087$) (Fig. 6A). However, significantly more nematodes moved in response to extracts of both *Diaprepes*-infested as well as uninfested *P. trifoliata* roots than in response to a double-blank negative control ($F=35.66$, $df = 2,129$, $P<0.001$) for each species except *S. carpocapsae* (ambush forager type, $P = 0.134$) (Fig. 2A). Nematode attraction, for all species tested, was greater to Swingle plants infested with *Diaprepes* larvae than paired uninfested controls ($P < 0.001$) (Fig. 6B). Movement of *S. diaprepesi* in response to *Diaprepes*-infested Swingle rootstocks was significantly greater than that observed for the other nematode species tested ($P < 0.001$) (Fig. 6B).

Effect of Below versus Above Ground Herbivory on Release of Nematode

Attractants: Feeding by *Diaprepes* larvae on citrus roots induced production of geijerene and pregeijerene in the subterranean root zone; however, release of these nematode attractants did not occur from aboveground shoots in response to larval feeding (Fig. 7A). Conversion of pregeijerene to geijerene is an artifact of heat exposure in the GC injector and thus the total production of pregeijerene in response to herbivory is likely a combination of the proportions of both observed pregeijerene and geijerene peaks (Fig. 7A). These C_{12} terpenes are thought to elicit nematode recruitment (Ali *et al.* 2010). Analogous sampling of volatile production in response to adult beetle feeding on aboveground shoots did not induce production of these two belowground signals from roots (Fig. 7B). Production of volatiles in response to aboveground herbivory by *Diaprepes* beetles adults was similar above- and

belowground, except that production of limonene occurred in shoots but not roots (Fig. 7B).

Subteranean Release of Volatiles By Various Plant Species Pregeijerene

was released from roots of *P. trifoliata* constantly and was not further affected by larval *D. abbreviatus* feeding (Fig. 8A). Release of this volatile from *P. trifoliata* roots does not require the trigger of herbivory (Fig. 8A). In contrast, pregeijerene was released by Swingle roots (Table 2 and Ali *et al.* 2010) and Sour Orange rootstocks (Fig. 8B) only in response to *D. abbreviatus* larval feeding, but was never produced by non-infested roots of this species (Fig. 8B, Table 2 below).

Table 2. GC-MS Identification of Volatiles From various Citrus Rootstocks

RT	Names	CAS#	Swingle (<i>C. paradisi</i> × <i>P.</i> <i>trifoliata</i>)		Poncirus (<i>P. trifoliata</i>)		Sour Orange <i>C. aurantium</i>	
			Infested	Non- infested	Infested	Non- infested	Infested	Non- infested
7.25	α-pinene ^{a,b}	000080- 56-8	+	+	+	+	-	-
7.90	β-pinene ^{a,b}	000127- 91-3	+	+	+	+	-	-
8.69	Limonene ^{a,b}	000138- 86-3	-	-	-	-	-	-
12.94	Geijerene ^b	006902- 73-4	+	-	+	+	+	-
10.81	Pregeijerene ^b	020082- 17-1	+	-	+	+	+	-

^a Synthetic standard comparison. ^b Identification was based on comparisons of retention times with standard and spectral data from Adams, EPA, and Nist05 Libraries

By the above results, it was determined whether *P. trifoliata*, the parental line of the Swingle hybrid, as well as another common non-hybridized species, sour orange

(*Citrus aurantium*), also produce these specific nematode recruitment signals in response to herbivory. Surprisingly, it was found that the parent of the Swingle hybrid, *P. trifoliata*, released the nematode recruitment chemical, pregeijerene, constantly and that this release did not depend on insect herbivory as was observed with the commercialized hybrid. In contrast, release of the nematode recruitment chemical from the non-hybridized sour orange species occurred only in response to herbivory as was originally observed with the Swingle hybrid (Ali et al. 2010). While not wishing to be bound by theory, it appears that production of this nematode attractant occurs broadly among diverse citrus varieties. Although it is released constantly by roots of at least one species (*P. trifoliata*), its release is herbivore-induced in another (*C. aurantium*). Furthermore, it appears that modern plant breeding to develop the cultivable hybrid *P. trifoliata* x *C. paradisi* may have silenced genes responsible for constant signaling observed in one its parents, creating an herbivore-induced response similar to that observed with the non-hybridized sour orange (*C. aurantium*) species. Current efforts are being focused on microarray analysis to resolve gene regulation in response to herbivory among these different citrus varieties. Although *D. abbreviatus* is the main root weevil species affecting commercial citriculture, a complex of related species also attack citrus roots, and thus nematode recruitment is expected to have broad significance for citrus pest management.

In addition to determining the extent of nematode recruitment signaling among various citrus varieties, the breadth of responsiveness among several nematode species was investigated. Importantly, the entomopathogenic species tested herein can be categorized according to foraging strategy. Furthermore, we included a plant parasitic species as a trophic level out-group. Foraging behaviour of EPNs can be broadly divided into two major categories: 1) 'cruisers' that are highly mobile with a wide searching circumference and 2) 'ambushers' that exhibit low mobility and hunt by sitting and waiting for mobile prey (Lewis et al. 1993). The above results indicate that each nematode species tested exhibited attraction to herbivore-induced volatiles irrespective of their foraging strategy (Fig. 7). Specifically, the 'ambusher' *S. carpacapse* (Lewis 2002), the cruiser *H. indica* (Lewis 2002), as well as the two species thought to exhibit an intermediate behavioural foraging strategy (Cabanillas et al. 1994) were each attracted to *Diaprepes*-damaged roots of the Swingle rootstock. The specific release of

pregeijerene in response to herbivore damage is thought to mediate this response. Analogously, the Swingle parent line, *P. trifoliata*, also attracted nematodes of each species (except for *S. carpocapse*); however, this recruitment occurred constantly and did not require herbivore feeding (Fig. 6A). Furthermore, attraction of nematodes to *P.*
5 *trifoliata* roots was correlated with a constant production of pregeijerene. Of the nematode species investigated, *S. diaprepesi* exhibited the greatest behavioural response even though this species is thought to be intermediate on the spectrum between pure 'ambusher' versus 'cruiser'. *S. diaprepesi* is a host specialist attacking *Diaprepes* weevils (Nguyen & Duncan 2002). It thus appears that host specialization
10 rather than foraging strategy may better explain EPN use of HIPVs for host location.

Furthermore, the above results suggest that plant parasitic nematodes are attracted to specific roots volatiles, whose production is in some cases enhanced by herbivore damage. These root-specific volatiles may facilitate host finding among opportunistic plant parasitic nematodes that likely use a multitude of cues to locate
15 feeding sites.

With respect to herbivore-induced plant defense, the above results point to two interesting contradictions. First, constant production and release of attractants for beneficial nematodes by plant roots is likely physiologically costly. Therefore, evolution of an herbivore-induced signaling response may have been selected for in order to
20 channel resources toward production of 'cries for help' only when necessary. It is puzzling that the parental *P. trifoliata* line of the commercial Swingle rootstock produces these signals constantly. From the perspective of commercial citriculture, use of resources for constant volatile production may reduce overall tree vigor and yield as compared with plants that do not constantly emit such signals. Therefore, it is less
25 surprising that the cultivable and faster growing Swingle hybrid only released this signal upon herbivory.

S. carpocapsae (ambusher) is a less effective entomopathogen of *D. abbreviatus* (Schroeder 1994; Bullock *et al.* 1999) than *S. riobrave* (intermediate between ambusher and cruiser) (Cabanillas *et al.* 1994). It is thought that active movement in search of
30 sedentary hosts as opposed to the 'sit-and wait' strategy may explain this difference in efficacy (Lewis *et al.* 1995; Campbell & Gaugler 1997). Nematode response to citrus-produced recruitment chemicals in the current investigation appeared to differ based on

foraging strategy. The lone 'pure' ambushing species investigated did not move when the pregeijerene signal was present in both arms of the olfactometer (Fig. 6); however, it did respond when this signal was present in only one of the two arms versus a blank control (Fig. 7). Cruising and intermediate foraging strategy species always responded to these signals; whether they were in one or both arms of the 2-choice test chamber (Figs. 6,7). The above results appear congruent with the proposed foraging strategy behaviours of the nematode species tested. The ambushing or 'sit-and-wait' forager did not move when signal was ubiquitous and coming from each possible direction of movement. This may indicate that *S. carpocapsae* waited for a potential mobile host when detecting the HIPV from all possible directions. In contrast, each of the other species tested, known to be active searchers (or at least intermediate on the continuum), always moved in response to the signal whether it occurred in one or both arms of the 2-choice olfactometer.

Though distinct, the shoots and roots of plants act synergistically using primary resources from both above- and belowground plant organs to produce organic matter. These ecologically valuable plant products are constantly threatened by primary consumers. Plants have thus developed numerous strategies to withstand the impacts of herbivores, pathogens and parasites. For several decades there has been an emphasis on the aboveground mechanisms of plant defense (Zangerl 2003; Howe & Jander 2008). However, the synergy between below- and aboveground organs associated with plant growth is likely paralleled by interactions that contribute to plant defense (Erb et al. 2010; Bezemer & van Dam 2010). Roots synthesize a number of secondary metabolites that are known leaf defenses, including furocoumarins, alkaloids, terpenoids aldehydes, and nicotine (Erb et al. 2009). Until recently, pregeijerene had only been detected in herbivore-damaged roots of Swingle citrus (Ali et al. 2010). In the current investigation, volatiles from the above- and belowground appendages of plants were simultaneously sampled while they were actively damaged at the root or shoot zone by different stages of the same holometabolous insect herbivore. Pregeijerene was only released by roots in response to belowground herbivory by *Diaprepes* larvae (Fig. 7A). Neither roots nor foliage released this putative nematode attractant upon aboveground herbivory by adult beetles (Fig. 7B). Although our results indicate that the major constituent of nematode attraction is unique to the belowground portions of the

plant, it remains possible that correlations exist between aboveground and belowground herbivory in this system. In the current investigation, we did not address recruitment of aboveground natural enemies of *Diaprepes* adults in response to belowground or aboveground herbivory. However, our results suggest an aboveground HIPV release in response to adult beetle feeding (i.e. increased levels of limonene from leaves (Fig. 7B), which deserves further investigation.

With respect of the influence of aboveground herbivory on belowground plant defense, we hypothesized that adult beetle feeding may induce production of an EPN recruitment signal as a form of “priming.” Given that adults lay eggs on leaves and first instar larvae drop and burrow into the soil, we postulated that it would be advantageous for the plant to recruit a community of entomopathogens as herbivore larvae are dropping to the soil and before they have established active feeding sites on roots. Our results provide no evidence in support of this priming hypothesis as the nematode recruitment signals were only induced by belowground herbivory.

Nematode recruitment signals may be released by a diversity of citrus species. These signals can be released constantly or only in response to herbivore damage. Modern plant breeding to develop a cultivable citrus hybrid may have silenced constant production of such attractions, congruent with the notion that constant production of such volatiles may impact plant vigor and yield. A diversity of nematode species was attracted to these signals including entomopathogens or entomopathogenic nematodes. It appears that these nematode recruitment signals have less effect on ‘sit-and wait’ strategists than ‘ambushers, but nematode-host specialization appeared to play a more important role than foraging strategy in terms of efficiency of chemotaxis in response to these signals. The surprisingly similar response of a plant parasitic species to that of several entomopathogens suggests that these signals cannot be easily categorized as either kairomones or allomones.

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Example 3

Introduction

Natural enemies of herbivorous pests use flexible foraging strategies that often incorporate environmental cues emitted by the herbivore's host-plant. While the role of herbivore-induced volatiles in plant-herbivore-natural enemy interactions has been studied aboveground (e.g. (1-5), some evidence suggests that induced root volatiles

may protect plants by attracting entomopathogenic nematodes (EPNs) (6-12). However, to date, only one root-induced attractant has been described and shown to enhance the effectiveness of natural enemies: (*E*)- β -caryophyllene from the roots of maize (*Zea Mays* L.) (11, 13). The disparity in the number of aboveground investigations versus
5 analogous belowground research on indirect defense is largely due to technical limitations rather than a lack of ecological or agricultural relevance (9, 14). No previous studies have detected a belowground herbivore-induced volatile from intact plants in the field or measured the effectiveness of belowground attractants for recruiting populations of naturally occurring EPNs in the soil. Depending on the specificity of interactions, the
10 identification and manipulation of a root signal in the field could well enhance biological control of diverse root pests in agroecosystems.

As described above, Larvae of the weevil *Diaprepes abbreviatus* (L.), introduced into Florida in 1964 (15), feed on the roots of more than 290 plant species including citrus, sugarcane, potatoes, strawberries, sweet potatoes, papaya, and non-cultivated
15 wild plants (16). Over the past 40 years, the weevil has significantly contributed to the damage and spread of disease in agricultural plants (17). Because pesticides are expensive, environmentally hazardous and often ineffective (18, 19), currently the most effective alternative method of control is the application of EPNs from the genera *Heterorhabditis* and *Steinernema* (20). EPNs are obligate parasites that kill their host
20 with the aid of a symbiotic bacterium (21, 22). Over its 20 years of use, the efficacy of mass release of EPNs as a biopesticide for *D. abbreviatus* has been reported as varying and unpredictable, ranging anywhere between 0 to >90% (23). Promoting plant attractiveness to natural enemies is a novel alternative to traditional broad-spectrum pesticides which indiscriminately kill predators and parasitoids and often lead to
25 subsequent pest resurgence (24-27). Deploying herbivore induced plant volatiles (HIPVs) above ground by controlled release dispensers has been shown to increase recruitment and retention of beneficial natural enemies to plants (27-29). In an analogous belowground investigation, EPN infection of western corn rootworm (*Diabrotica virgifera virgifera* LeConte) larvae was increased by spiking soil surrounding
30 maize roots with the HIPV, (*E*)- β -caryophyllene (11).

It has recently been shown that a citrus root stock (*Citrus paradisi* Macf. $\times$ *Poncirus trifoliata* L. Raf.) releases HIPVs in response to larval feeding by the weevil, *D.*

abbreviatus, and that these HIPVs attract EPN species in lab bioassays (7, 8). As discussed herein, the specific HIPV attractant has been identified as 1, 5-dimethylcyclodeca-1,5,7-triene (pregeijerene) and its real-time release in response to herbivory is shown. It is also demonstrated herein that field application of this volatile increases mortality of belowground root feeding weevils by attracting naturally occurring nematodes. qPCR primers and probes were developed to detect and enumerate cryptic species of EPNs allowing for species-specific quantification of nematode response to attractants belowground. Given the broad effect of pregeijerene on EPN species, its efficacy in an alternative non-citrus agroecosystem was tested. The use of plant produced signals, such as the damage-induced release of pregeijerene, along with conservation biological control strategies could extend the usefulness of EPNs in crops damaged by belowground herbivores.

Results

Volatiles were non-destructively sampled every 3 h from the root zone of citrus seedlings in glass chambers (Analytical Research Systems, Gainesville, FL, USA) with sandy soil (8). GC-MS revealed 1,5-dimethylcyclodeca-1, 5, 7-triene (pregeijerene) as the dominating volatile, reaching a maximum release between 9 and 12 h after initiation of larval feeding (Fig.9). There was no appreciable increase of any additional volatiles. A stainless steel probe (Fig. 15) was designed to collect volatiles in the field from the soil beds surrounding citrus trees in an unmanaged orchard. Here, GC-MS again revealed pregeijerene in the root zone, as the most abundant volatile at 1 m away from the trunks of trees and still at detectable levels at a 10 m from trees (Fig. 16).

We next conducted field tests to determine whether application of volatiles collected from infested roots would impact EPN-inflicted mortality of sentinel *D. abbreviatus* larvae. Commercially available EPN had been applied to the test orchard at numerous occasions; however, their persistence was not monitored. Cylindrical mesh cages containing a single *D. abbreviatus* larva in autoclaved sandy soil (30) were treated with: (i) volatiles collected from weevil-infested roots, or (ii) a blank solvent control (Fig. 12). Larval mortality was $74 \pm 6.9\%$ in the presence of volatiles from infested roots, but only $41 \pm 7.5\%$ in the solvent alone treatment ($N = 10$, $df = 18$, $t = 2.75$, $P = 0.013$).

A second experiment tested whether pregeijerene alone would increase mortality of larvae by attracting EPNs. For these experiments, a sufficient amount of pregeijerene was first extracted and purified from the roots of Common Rue (*Ruta graveolens* L.). To test for the attractiveness of pregeijerene, we first used serial dilutions of purified compound in dichloromethane in two-choice sand-filled olfactometers (7, 8, 31); 8ng/ μ L (in 30 μ L aliquots) was found to be the optimally attractive dosage to EPNs (*S. riobrave* and *H. indica*) (Fig. 10). We used real-time qPCR to quantify the attraction of naturally occurring EPNs in the field and identified them to species. Our approach was to use species-specific primers and probes to identify EPN species known to either naturally occur in Florida (*S. diaprepesi*, *H. indica*, *H. zealandica*, and *Steinernema* sp. LWD1 (an undescribed species in the *S. glaseri*-group); those which were applied to citrus orchards in the form of commercial biopesticides (*S. riobrave*); or those which might be introduced from natural long-distance spread from pastures and golf courses to manage mole crickets (*S. scapteriscus*) (32, 33). Mortality of larvae buried with purified pregeijerene was > 3-fold higher than that of larvae buried with the solvent control (Fig. 11A). The number of EPNs detected within (Fig. 11B) and around (Fig. 11C) cages containing the purified compound was significantly higher than that from cages with the solvent control. Tukey HSD test indicated *H. indica* and *H. zealandica* were more abundant than *Steinernema* sp. LWD1 and *S. diaprepesi* ($P < 0.0001$ in all comparisons); however, there were no differences in the relative representation of species between treated and control samples. Neither *S. riobrave*, nor *S. scapterisci*, were detected in any of the samples.

Finally, we tested the generality of pregeijerene as an EPN attractant by applying the compound in a geographically distant, non-citrus agricultural system: Commercial highbush blueberry, *Vaccinium corymbosum* L. in Chatsworth, NJ, U.S.A. No, pregeijerene was detected in volatiles collected from soil surrounding blueberry roots (data not shown). Cages (described above) containing either a third-instar oriental beetle, *Anomala orientalis*, a scarab blueberry root pest or a late instar greater wax moth, *Galleria mellonella* L., larva (a widely used EPN sentinel) were deployed in blueberry fields. As described above, cages were treated with either blank solvent or pregeijerene. EPN-inflicted larval mortality (combined *A. orientalis* and *G. mellonella*) was nearly 2-fold greater in treatments with pregeijerene (55%) than those with solvent

alone (30%) ($P = 0.009$). The increase was highly significant (from 40% to 80%) for *G. mellonella* ($P = 0.003$) but not statistically significant (from 20% to 30%) for *A. orientalis* ($P = 0.552$). Emerging EPNs were identified as *S. glaseri* with real-time PCR. On average, there were more *S. glaseri* nematodes surrounding the treatment (Mean $\pm$ SE, 7.96 $\pm$ 2.91) than in the control (4.43 $\pm$ 2.56); however, this difference was not significant ($P = 0.38$, $t = 0.909$, $df = 18$).

Discussion

The obstacles of investigating belowground chemically mediated interactions between plants and animals are being overcome gradually, opening opportunities for manipulating these interactions for enhanced biological control (34-36). At least half of all plant biomass is attacked by underground herbivores and pathogens, living in a complex ecological foodweb in the soil (37). Although induced plant responses were originally postulated as a potential novel approach to pest management in agricultural systems (38) and insect herbivore population regulation (39), few studies (40-43) of induced responses (particularly volatiles) have addressed their practical application beyond fundamental concepts in ecology and evolutionary biology (35, 37, 43), with particularly few studies for belowground systems. HIPVs are likely important mediators of tritrophic interactions that afford indirect plant defense within the root zone. The data shown in this Example 3, not only shows this approach in the field, but provides the first description of an ecological role for the C₁₂ terpene, pregeijerene.

To evaluate applied volatiles for the attraction of belowground natural enemies in the field, studies usually quantify mortality of a target pest by trapping adults emerging from soil (11). This technique frequently results in low recovery and also gives no confirmation of the specific cause of mortality. In addition, it can be difficult to quantify populations of naturally occurring EPNs, which may be abundant in soil, but remain cryptic. We used real-time qPCR as an efficient method for describing EPN diversity and quantifying their abundance (32, 44-46). Moreover, it is shown herein that pregeijerene was directly responsible for attracting five species of native EPN in the soil so as to enhance pest mortality. Given the efficacy of this compound, there may be little need for exogenous application of non-native EPNs in systems with a rich fauna of endemic EPNs. In orchards with established EPN populations, large-scale introduction of non-native species may temporarily reduce native populations due to trophic

cascades that increase predatory fungi that attenuate net efficacy of biological control (19, 47). Although it is known that artificially reared and commercially formulated EPNs can persist, it is possible that natives have advantages associated with habitat acclimation and response to HIPVs (9); thus, further investigation of enhancing conservation biological control of belowground pests in concert with behavioral modification via HIPVs is warranted.

The results of the experiment conducted in blueberries, an agricultural setting vastly different from citrus, demonstrate the potential broad applicability of pregeijerene on diverse species of EPN. Timing application of pregeijerene to target the most susceptible instar of *A. orientalis* should optimize its efficacy (depending on EPN species, final-instar *A. orientalis* may be less susceptible to EPN infection than earlier larval instars (48)).

Previous research suggests that volatile production in response to herbivore feeding differs between citrus species (8). Thus, the current findings could have broad impacts not only for rootstock selection in commercial agriculture, but also for use of attractants in alternative agroecosystems as demonstrated in blueberry fields. Here, it is identified that an additional naturally occurring species of EPN responsive to pregeijerene that was not found in Florida. Pregeijerene may thus have extensive application for enhancing native biological control of root feeding insects, including those which attack a wide range of crops.

Where most aboveground studies have identified blends of volatiles as being responsible for the attraction of natural enemies (49), analogous belowground studies that identify an attractant in an agricultural system have demonstrated that a single compound can elicit natural enemy responses. This certainly makes application potentially less complex, but also points to an interesting potential property of belowground cues and natural enemy response. Future work should evaluate the complexity of belowground cues and the range of volatiles that cause belowground natural enemies, like EPNs, to respond. Only recently have new methodologies been employed to investigate belowground induced plant volatiles (50) and much more progress is necessary to fully understand these relationships.

Material and Methods

Insect Larvae

D. abbreviates larvae were obtained from a culture maintained at University of Florida's Citrus Research and Education Center (CREC) in Lake Alfred, FL, U.S.A. This culture was periodically supplemented from a larger culture maintained at the Division of Plant Industry Sterile Fly Facility in Gainesville, FL, U.S.A. Larvae were reared on a commercially prepared diet (Bio-Serv, Inc., Frenchtown, NJ) using procedures described by Lapointe and Shapiro (51). Larvae used in experiments were from third to sixth instars.

Third-instar *A. orientalis* were collected from untreated turf areas at the Rutgers University Horticultural Research Farm (North Brunswick, NJ, U.S.A.) in late April. The larvae were stored individually in the cells of 24-well plates in sandy loam at 10°C for 2 weeks and returned to room temperatures (21–24°C) for 24 h before use in experiments. Late instar *G. mellonella* larvae were obtained from Big Apple Herpetological (Hauppauge, NY).

Plants

'Swingle citrumelo' (*C. paradisi* Macf. × *P. trifoliata* L. Raf.) rootstock is very prominent in commercial citrus production (52). The extensive use of this rootstock in commercial citrus production justified its use in this investigation. All plants were grown and maintained at the CREC in Lake Alfred, FL, U.S.A. in a greenhouse at 26 ± 3°C, and 60-80% RH. *R. graveolens* was purchased as full grown plants 46-61cm in height. The plants were immediately bare rooted and rinsed to remove as much soil material as possible; only roots were placed into vials containing dichloromethane for further extractions and purification.

Nematodes used for laboratory qPCR and bioassays

The entomopathogenic nematodes, *S. diaprepesi* HK31, *S. riobrave* Btw1, *Steinernema* sp. (LWD1), and *H. indica* Ker1 and *H. zealandica* Btw1 were isolated from *D. abbreviatus* larvae buried in commercial citrus orchards in Florida. *S. riobrave* and *S. carpocapsae* isolates were descendants of commercial formulations intended for field application to manage *D. abbreviatus*. Other EPN species included in this study were *S. scapteriscus* (provided by Dr. J.H. Frank, University of Florida, FL, U.S.A.). All EPN species were cultured in last instar larvae of the greater wax moth, *G. mellonella* larvae, at approximately 25°C according to procedures described in Kaya and Stock

(53). Infective juveniles (IJs) that emerged from insect cadavers into emergence traps were stored in shallow water in tissue culture flasks at 15 °C for up to two wk prior to use.

***In situ* Volatile Collection from Infested Roots in the Greenhouse.**

Six 'Swingle citrumelo' plants were initially placed in glass root-zone chambers (Analytical Research Systems) filled with sand that had been autoclaved for one h at 121 °C and then adjusted to 10% moisture as described in (7, 8, 31). All seedlings were given three d to adjust to their sand filled chambers. Three of the plants were subjected to feeding by weevil larvae for three d, the remaining three served as undamaged controls. During this period, each of the six root-zone chambers were connected to a vacuum pump (Analytical Research Systems) with a suction flow of 80 mL / min.(7). Compounds emitted from chambers were collected on adsorbent traps filled with 50 mg Super-Q (800–1000 mesh, Alltech Deerfield, IL, USA) held in glass fittings between the chamber and vacuum pump (7). Super-Q traps were replaced every 3 h for a 72 h period to track the time course of volatile release. The removed Super-Q traps were subsequently eluted with 150 µL of dichloromethane into individual 2.0 mL clear glass vials (Varian: part number: 39261 1549 equipped with 500 µL glass inserts) (7).

It was a challenge to remove sufficient pregeijerene from infested roots for bioassays and field-testing. However, it was previously established (54) that a hydrodistillate of common rue (*Ruta graveolens*) roots contained the related terpene, geijerene, as a major constituent (67% of the total volatile compounds). Pregeijerene easily converts to geijerene at temperatures exceeding 120°C (55) (Fig. 13). On-column gas chromatography-mass spectrometry (GC-MS) analyses confirmed pregeijerene as the main naturally occurring terpene in roots of common rue that could be easily extracted and purified from crushed roots using a series of solid phase extractions (Fig. 14).

***In situ* volatile collection from infested roots in the field.**

Volatiles were collected from the soil beds surrounding citrus trees in the field. A soil probe (Fig. 15) was used to sample soil volatiles at a depth of 20 cm, and at distances of 1 and 10 m from the trunks of citrus trees. A vacuum pump was used to pull air at a rate of 200 mL/min for a total of 30 min. Compounds were collected on

adsorbent traps filled with 50 mg of Super-Q attached to the top of the soil probe (Fig. 15). The Super-Q traps were subsequently eluted as described in the previous section.

Identification of pregeijerene.

Pregeijerene isolated from common rue and that from citrus roots after herbivore feeding was identical by electron impact (EI) and chemical ionization (CI) GC-MS analyses on DB1, DB5 and DB35 GC columns. Although the EI mass spectra matched pregeijerene in the Adams 2 library, the lack of a standard made it necessary to confirm the structure by NMR (described in SI).

Two-choice bioassay to determine optimal dosage to attract EPNs

The behavioral responses of EPNs to collected pregeijerene were quantified in a two choice sand-filled olfactometer (7, 31). Briefly, the olfactometer consists of three detachable sections: two opposing 16 mL glass jars which contained treatments and a central connecting tube three cm in length with an apical hole into which EPN were applied. Dilutions from the purified *R. graveolens* root extract were placed on filter paper, which was allowed to dry for 30 s to allow solvent evaporation. Thereafter, filter papers were placed on the bottoms of each glass jar, which were then filled with moist (10% w/v) sterilized sand. The central chamber connecting the two arms of the olfactometer was also filled with sterilized and moistened sand. EPNs (*ca.* 200 IJs) were applied into the central orifice of the connecting tube and given eight h to respond. Following the incubation period, the column was disassembled and the IJs from the two collection jars were extracted using Baermann funnels. The experiment was replicated five times for each dilution and separately tested with two EPN species: *S. riobrave* Btw1 and *H. indica* Ker1.

A student's *t*-test was used to compare nematode response in the two-choice olfactometer. Since responses of both species to pregeijerene versus the solvent controls were identical, data for both species were combined prior to analysis (*df*= 18). The dosage at which a significant proportion of EPNs were attracted to the treatment arm was selected for our field trial.

Application of HIPVs in the Field

An experiment was conducted in a sandy soil (97:2:1, sand:silt:clay; pH 7.1; 0.1% OM) citrus orchard at the CREC (28 07 26.84 N, 81 42 55.31 W). The experiment

was placed within a section of mature orange trees spaced (without beds) 4.5 m within and 8.1 m between rows that were irrigated with microsprinklers. A randomized design was used to place treatments between trees in eight adjacent rows. Cylindrical wire-mesh cages containing autoclaved sandy soil (10% moisture) and a single *D.*

5 *abbreviatus* larva (reared on artificial diet for 3 to 5 weeks) were buried 20 cm deep in the soil beneath the tree canopies. Cages were made of 225-mesh stainless steel cylinders (7 length × 3-cm diam.) secured at each end with polypropylene snap-on caps. A replicate consisted of six cages placed equidistantly from one another in a circle pattern (48cm diam.) for each treatment. All cages contained a single *D. abbreviatus*
10 larva and were baited with one of two treatments: (i) volatiles from roots fed upon by a *D. abbreviatus* larva, or (ii) blank solvent control. There were 10 replicates per treatment. Treatments were applied as 30 µL aliquots to 3-cm diameter filter paper discs (Whatman). Solvent was allowed to evaporate for 30 s prior to insertion of filter papers at the base of each cage. The cages were left buried for 72 h. Eight soil core
15 samples (2.5 cm diam. × 30 cm deep) were taken from soil surrounding the treatment arena before the cages were removed. Recovered larvae were rinsed and placed on moistened filter paper in individual Petri dishes for observation. Mortality of the larvae was recorded from 0 to 72 h after removal from soil.

The effect of isolated pregeijerene on larval mortality was investigated in two
20 additional experiments (one of which was conducted in a blueberry planting in Chatsworth, NJ, USA, using *A. orientalis* and *G. mellonella*). The methods for these experiments were similar to those described above, except that the soil remaining within the six cages from each replication was placed in a container and homogenized for later nematode DNA extraction (n=10). Soil cores taken from the surrounding treatment
25 arenas were also combined and stored for nematode DNA extraction (n=10). Fisher's exact test was used to compare larval mortality between the treatment and control. Only soil samples from the citrus experiment were analyzed for DNA quantification.

Detection, identification and quantification of entomopathogenic nematodes using real time qPCR

30 Real time qPCR was used to quantify attraction of naturally occurring EPN species to volatiles applied in the field and to identify nematodes to species. This technique targeted 11 EPN species (32, 33, 44). In the citrus experiment, we surveyed

the natural occurrence of six species (*S. diaprepesi*, *Steinernema* sp. *glaseri*-group, *S. riobare*, *S. scapterisci*, *H. indica* and *H. zealandica*); in the blueberry experiment, nine species were surveyed (*S. carpocapsae*, *S. feltiae*, *S. glaseri*, *S. kraussei*, *S. scapterisci*, *Steinernema* sp. *glaseri*-group, *H. indica*, *H. zealandica* and *H.*

5 *bacteriophora*). Briefly, species-specific primers and TaqMan® probes were designed from the ITS rDNA region using sequences of the target species as well as closely related species recovered from the NCBI database or generated by the authors in that study. Multiple alignments of the corresponding sequences were performed (56) to select areas of variability in the ITS region. The designed primers and probes provided
10 no non-specific amplification when they were tested using other EPN species. Standard curve points were obtained from DNA dilution. Four independent DNA extractions were performed from Eppendorf tubes containing 300 IJs in 100 µL of the corresponding nematode species (Ultra Clean Soil™ DNA kit, MO BIO) to generate a standard curve (32, 44). Dilutions corresponding to 100, 30, 10, 3 and 1 IJs were prepared using serial
15 dilution of the appropriate DNA.

Nematodes from soil samples were extracted by sucrose centrifugation (57) from aliquots of 500 cm³ from the mixed composite sample. Each nematode community was concentrated in a 1.5 mL Eppendorf tube. DNA was processed using the UltraClean™ soil DNA extraction Kit and quantification was performed for each DNA extraction using
20 the nanodrop system with the control program (ND-1000 v3.3.0). All DNA samples were adjusted to 0.2 ng/µL that is required for nematode quantification (32). The resulting real values were analyzed with analysis of variance (ANOVA) for the EPN species recovered ($F = 41$, $df = 5$, 204). Where ANOVA showed significant differences, Tukey's HSD test ($\alpha < 0.05$) was conducted to separate means in the software R (R
25 Development Core Team 2004).

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Supplemental information for Example 3

10 GC-MS Analysis

All samples were injected as 1 µl aliquots of dichloromethane extract onto a gas chromatograph (HP 6890) equipped with 30 m×0.25-mm-ID, 0.25 µm film thickness DB-1 or DB35 capillary column (Agilent, Palo Alto, CA, U.S.A.), interfaced to a 5973 or 5975 Mass Selective Detector (Agilent), in both electron impact and chemical ionization
15 modes. Samples were introduced using either splitless injection at 220 °C or by cold on column injection. In the second case, a 1 m fused silica deactivated retention gap was added between injector and analytical column and the injector was programmed to follow the oven temperature. The column was held at 35 °C for 1 min after injection and then programmed to change at 10 °C/min to 260 °C. The carrier gas used was helium at
20 an average flow velocity of 30 cm/s. Isobutane was used as the reagent gas for chemical ionization, and the ion source temperature was set at 250 °C in CI and 220 °C in EI. EI Spectra library search was performed using a floral scent database compiled at the Department of Chemical Ecology, Göteborg Sweden, the Adams2 terpenoid/natural product library (Allured Corporation, 72) and the NIST05 library. When available, mass
25 spectra and retention times were compared to those of authentic standards in addition to internal standard [nonyl-acetate (4 µg/µl)].

Isolation and Purification of Pregeijerene

Although pregeijerene (1,5-dimethylcyclodeca-1,5,7-triene) was collected from citrus
30 roots damaged by *D. abbreviatus*, it was necessary to find an alternative source richer in the pure compound for laboratory bioassay and field testing. Hydrodistilled common rue (*Ruta graveolens*) essential oil contains geijerene as a major constituent (67% of

the total volatile compounds) (2). However, at temperatures exceeding 120°C [(3), Fig. 13] the macrocyclic pregeijerene will rearrange to geijerene; thus, by on-column analyses of common rue root extracts we found, as anticipated, large quantities of pregeijerene rather than geijerene. For isolation of pregeijerene, rue roots were crushed in dichloromethane. GC-MS analyses revealed that pregeijerene constituted approximately 95% of the terpene content, in addition to large quantities of more polar compounds, mostly furanocoumarins. To remove the furanocoumarins, the dichloromethane extract was first eliminated by gently evaporating the sample to a small volume (0.5ml) and was re-suspended in 4 ml of pentane. After centrifugation, the supernatant was again gently concentrated and re-suspended in 4 ml of pentane and again centrifuged to remove solids. An attempt to use a silica column resulted in a partial conversion of pregeijerene to co-geijerene. The yellow solution was therefore slowly passed through a diol column, successfully removing the cyanocoumarins while maintaining intact pregeijerene (Fig. 14). The two remaining impurities were removed by first repeatedly partitioning the hexane extract with methanol followed by a slow filtering through a quaternaryamin ion exchange column. The final hexane solution was analyzed by GC-MS for purity and by GC-FID with nonyl acetate as an internal standard for quantification (Fig 14). Serial dilutions were made from this extract providing five concentrations of pregeijerene (8.0, 0.80, 0.08, 0.008, and 0.0008 µg/µl).

NMR analysis of Pregeijerene

Pregeijerene was purified for NMR using preparative GC, as a mixture of pregeijere and geijerene 70:30 ratio. The pregeijerene and geijerene mixture (~60 ug) in ~150 µL of C₆D₆ (Cambridge Isotope Laboratories Inc.) was placed in a 2.5 mm NMR tube (Norell). One-dimentional ¹H and nuclear overhauser enhancement (NOE) difference experiments and two-dimensional NMR spectroscopy, including gradient correlation spectroscopy, heteronuclear single-quantum coherence, heteronuclear multiple-bond correlation and NOE spectroscopy were used to characterize pregeijerene. All 2D NMR spectra were acquired at 24 °C and an additional 1D NOE difference experiment was conducted at 10 °C using a 5-mm TXI CryoProbe and a Bruker Avance II 600 console (600 MHz for ¹H, 151 MHz for ¹³C). Residual C₆D₆ was used to reference chemical shifts to δ(C₆H₆) = 7.16 ppm for ¹H and δ(C₆H₆) = 128.2 ppm for ¹³C (4). NMR spectra

were processed using Bruker Topspin 2.1 and MestreLabsMestReNova software packages. Numbering is based on Jones and Southerland (5). The ^1H and ^{13}C NMR data in C_6D_6 are presented for pregeijerene and geijerene in Tables S1 and S2 because the original NMR data was obtained in carbon tetrachloride solution.

5

The ^1H NMR data (Table S1) for pregeijerene with reported proton chemical shifts and J-couplings for pregeijerene A (5) are consistent, but not with pregeijerene B (Cool & Adams 2003#42). Jones and Southerland (5) did not report ^{13}C NMR data, thus we compared the ^{13}C NMR data with Germacrene C containing a cyclodecadiene ring like pregeijerene with the exception of an isopropyl substitution at C8 position. Both ^1H and ^{13}C NMR data agreed with germacrene C (6) except for carbons adjacent to C8 as expected. The two-dimensional NOESY experiment at room temperature (24 °C) resulted in two very weak NOE. The flexible cyclodecadiene ring was found to exist in three different conformational isomers for germecrine A at or lower than 25 °C (7).

10

15

Therefore, NOE difference experiments were conducted on the two methyl groups at C1 and C5 at 10 °C, above freezing temperature, and 30 °C in C_6D_6 . Overall NOEs were small, but signal intensity was better at 10 °C for NOE difference experiments. The protons of methyl group at C5 had NOEs to proton 6.52 of C7, 2.08 of C4 and 1.94 of C3/1.97 of C9. The protons of the methyl group at C1 had NOEs to 1.73 of C10 and 1.97 of C9/1.94 of C3. The NOE results agree with pregeijerene and flexible cyclodecadiene ring structures(5-7). In addition, we found that chemical shifts of protons at C2, C7 and C8 are sensitive to temperature changes.

20

25

Supplemental Tables:

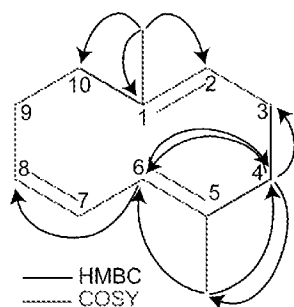
- 5 S Table 1. ^1H (600 MHz), ^{13}C (151 MHz), HMBC and NOESY NMR spectroscopic data for pregeijerene in C_6D_6 . ^{13}C was also detected directly (126 MHz) using a 5 mm Cryoprobe. Chemical shifts referenced to residual proton signal in C_6D_6 benzene $\delta(^1\text{H}) = 7.16$ ppm for ^1H and $\delta(\text{C}_6\text{D}_6\text{H}) = 128.2$ ppm for ^{13}C .

Position	$\delta^{13}\text{C}$ [ppm]	$\delta^1\text{H}$ [ppm]	J coupling constants [Hz]	HMBC correlations (C.No)	NOE peaks
1	140.6				
2	125.2	1H 4.83 [#] 2H 2.06,	ddt J = 11.5, 4.9, 1.4 2.06, 1H, m		2.45*
3	27.6	1.94 2H 2.08,	1.94, 1H, m 2.08, 1H, dt J = 11.5, 3.4	1.67-C6, C3 (weak), CH3 of C5	1.94-1.19*
4	39.8	1.67	1.67, 1H, dt J = 4.4, 12.0		
5	\$				
6	128.9	1H 5.39	br d J = 9.7	C4, C8	*2.28, *1.67
7	130.0	1H 6.52 [#]	t J = 10		1.49*
8	127.5	1H 5.53 [#] 2H 2.28,	~dt J = 10.0, 8 2.28, 1H, m		
9	29.5	1.97 2H 1.73,	1.97, 1H, m 1.73, 1H, dt J = 4.6, 12.8 2.45, 1H, ~ddd J = 12.8,		1.97-1.19*
10	39.1	2.45	6.0, 1.9		1.73-1.19* **1.73,
CH3-C1	20.6	3H 1.19	d J = 1.1	C1, C2, C10	**1.96/1.97 **6.52, **2.08,
CH3-C5	16.2	3H 1.49	s	C6, C4	**1.94/1.97

* weak NOEs observed with 2D NOESY experiment at 24C, **observed from 1D NOE

- 10 difference experiments at 10C. [#]Chemical shifts are temperature sensitive. ^{\$}We think

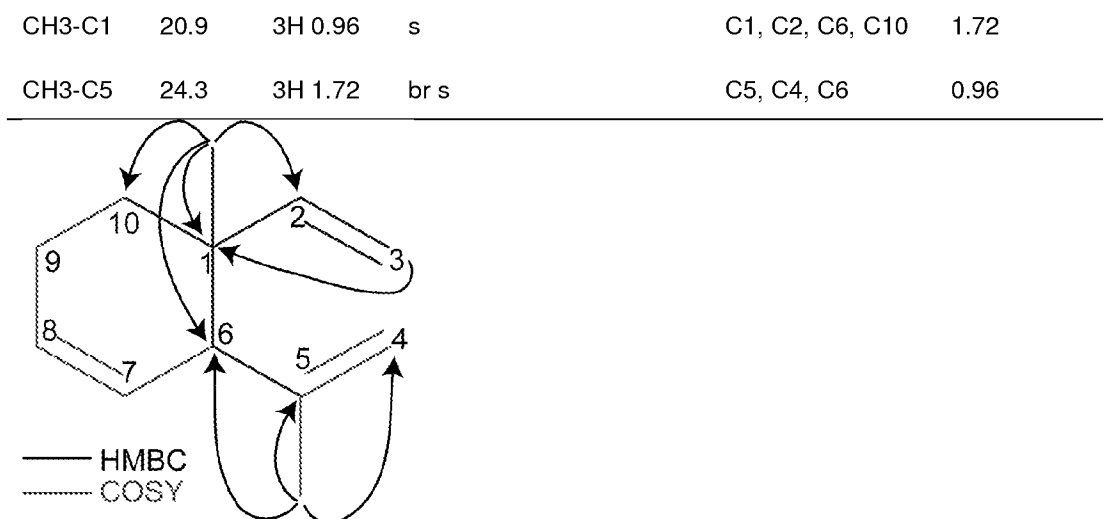
carbon chemical shifts of C5 and C6 overlap. Carbons numbered based on Jones and Sutherland (1968).



- 5 STable 2. ^1H (600 MHz), ^{13}C (151 MHz), HMBC and NOESY NMR spectroscopic data for geijerene in C_6D_6 . ^{13}C was also detected directly (126 MHz) using a 5 mm Cryoprobe. Chemical shifts referenced to residual proton signal in C_6D_6 benzene $\delta(^1\text{H}) = 7.16$ ppm for ^1H and $\delta(\text{C}_6\text{D}_6\text{H}) = 128.2$ ppm for ^{13}C . For convenience, the pregeijerene numbering is retained after cope rearrangement to geijerene.

10

Position	$\delta^{13}\text{C}$ [ppm]	$\delta^1\text{H}$ [ppm]	J coupling constants [Hz]	HMBC correlations (C.No)	Unique NOESY peaks
1	38.0				
2	149.0	1H 5.86 2H 4.99,	dd J = 17.5, 10.8 4.99, 1H, dd J=17.5, 1.3	4.99 - C1	
3	110.6	4.94 2H 4.82,	4.94, 1H, dd J = 10.8, 1.3 4.82, 1H, br s	4.95 - C1	4.99 - 0.96
4	114.2	4.97	4.97, 1H, m		
5	146.7				
6	51.5	1H 2.7	quintet J = 2.7 dddd J = 10.1, 2.2, 3.5,		
7	126.2	1H 5.66	3.5 dddd J = 10.1, 3.2, 2.1,		
8	129.9	1H 5.59	2.1		
9	22.6	2H 1.91	m		0.96
10	33.4	2H 1.43	m		



Reference for Supplement information for Example 3

1. Castle W, Stover E (2002) Citrus Rootstock Usage, Characteristics, and Selection in the Florida Indian River Region. *Horttech* 12:143–147.
- 10 2. Kuzovkina, I, Szarka, S, Héthelyi, É (2009) Composition of essential oil in genetically transformed roots of *Ruta graveolens*. *Russ J Plant Physiol* 56:846-851.
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12. Jenkins, W (1964) A rapid centrifugal-flotation technique for separating nematodes from soil, *Plant disease reporter*. 48: 692.

While various embodiments of the present invention have been shown and described herein, it will be obvious that such embodiments are provided by way of example only. Numerous variations, changes and substitutions may be made without departing from the invention herein. Accordingly, it is intended that the invention be limited only by the spirit and scope of the appended claims.

CLAIMS

The invention claimed is:

1. A method for inhibiting infestation of a plant by pests, the method comprising:
5 applying an amount of a nematode attractant lure effective to attract a plurality of protective nematodes to the plant that will kill the pests of the plant.
2. The method of claim 1, wherein the plant is a citrus plant and the pest is
10 *Diaprepes abbreviatus*.
3. The method of claim 1, wherein the plurality of protective nematodes comprise entomopathogenic nematodes.
4. The method of claim 1, further comprising applying the nematode attractant lure with
15 an agriculturally acceptable carrier to or adjacent to the plant.
5. The method of claim 1, wherein the at least one terpene compound comprises at least one of geijerene, pregeijerene, limonene, alpha-pinene, and beta-pinene.
- 20 6. The method of claim 5, wherein the at least one terpene compound comprises pregeijerene.
7. The method of claim 1, wherein the nematode attractant lure is applied to a subterranean area about the plant.
- 25 8. A nematode attractant lure comprising:
 an amount of a terpene compound effective to attract a plurality of protective nematodes to a plant that will kill pests of the plant; and
 an agriculturally acceptable carrier.

9. The nematode attractant lure of claim 8, wherein the terpene compound comprises at least one of geijerene, pregeijerene, limonene, alpha-pinene, and beta-pinene.

5 10. The nematode attractant lure of claim 8, wherein the at least one volatile terpene compound comprises pregeijerene.

11. The nematode attractant lure of claim 8, wherein the terpene compound comprise geigerene, pregeijerene, or combinations thereof.

10

12. An article of manufacture comprising:
an amount of a terpene compound effective to attract a plurality of protective nematodes to a citrus that will kill pests of a plant;

15 a container comprising the amount of the terpene compound; and
means for applying the terpene compound from the container to a target area about the plant.

13. The article of manufacture of claim 12, wherein the terpene compound comprises geigerene, pregeijerene, or combinations thereof.

20

14. The article of manufacture of claim 12, wherein the terpene compound comprises pregeijerene.

15. The article of manufacture of claim 12, further comprising an agriculturally
25 acceptable carrier for the terpene compound.

16. The method of claim 1, wherein the plant is a berry.

17. The method of claim 1, wherein the plant is blueberry, strawberry, blackberry,
30 raspberry, sugarcane, vegetables, potatoes, strawberries, woody field-grown
ornamentals, sweet potatoes, papaya, guava, mahogany or grapes.

18. The method of claim 1, wherein the plant is blueberry.

19. The method of claim 17, wherein the pest is *Galleria mellonella* and/or *Anomala orientalis*.

5

20. The method of claim 17, wherein the pest is *Otiorhynchus cribricollis*, *Nemocestes incomptus*, *Otiorhynchus sulcatus* and/or *Pantomorus cervinus*.

21. An article of manufacture comprising:

10 an amount of a terpene compound effective to attract a plurality of protective nematodes to a citrus that will kill pests of a plant;

 a container comprising the amount of the terpene compound, said container being adapted for extended, subterranean release of said terpene compound.

15

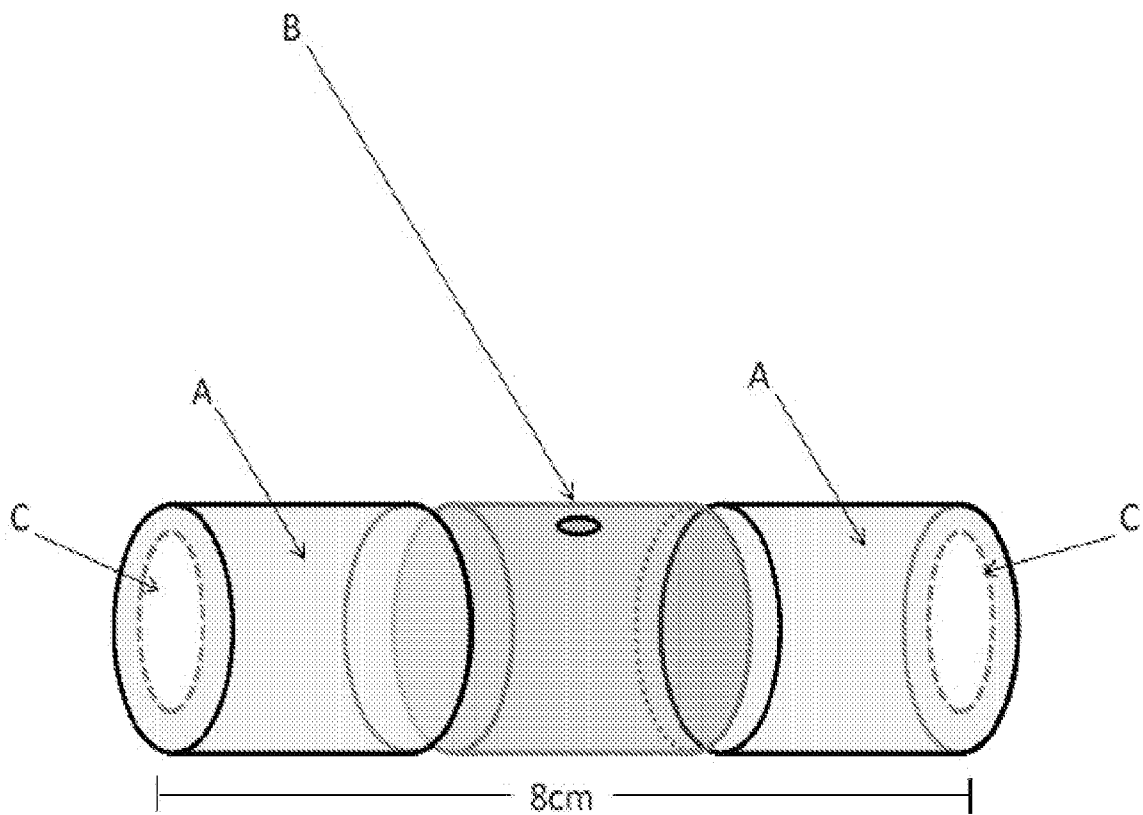


FIG. 1

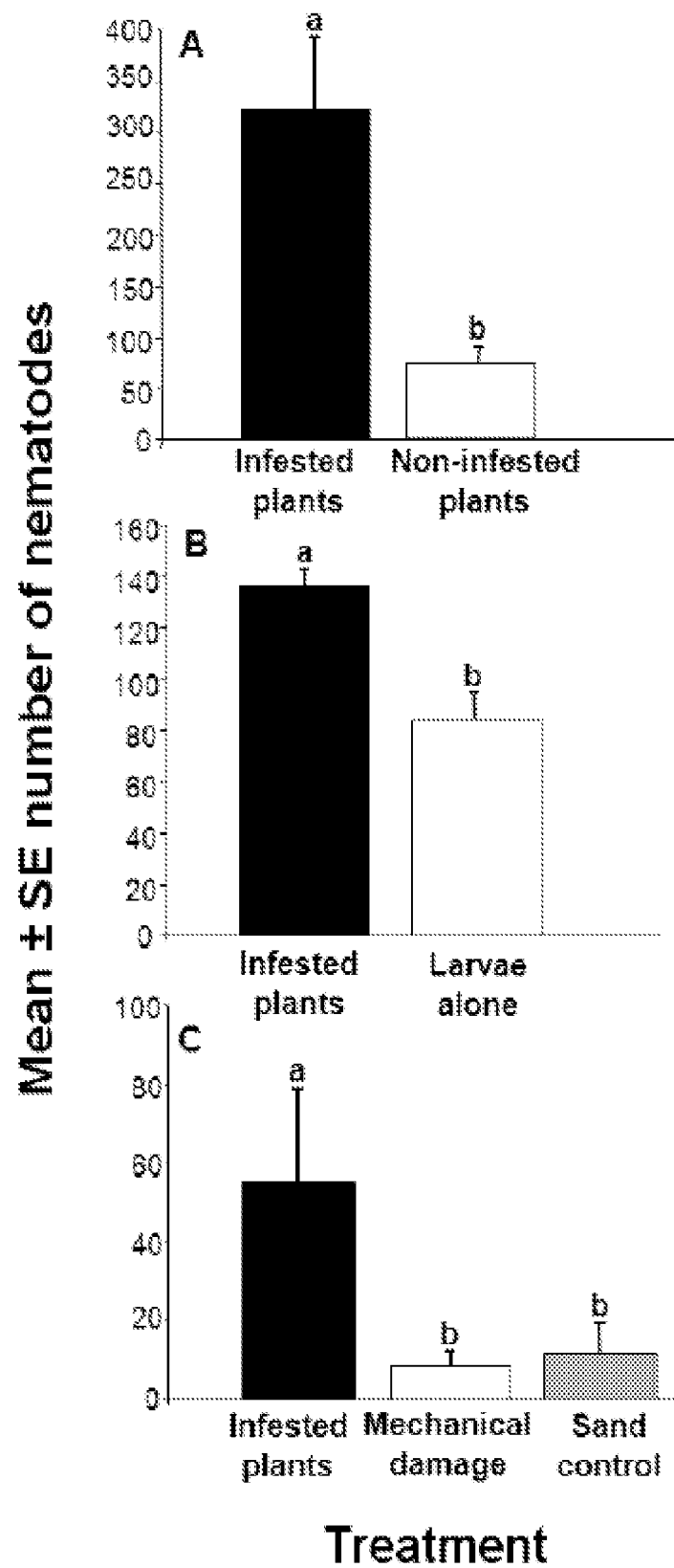


FIG. 2

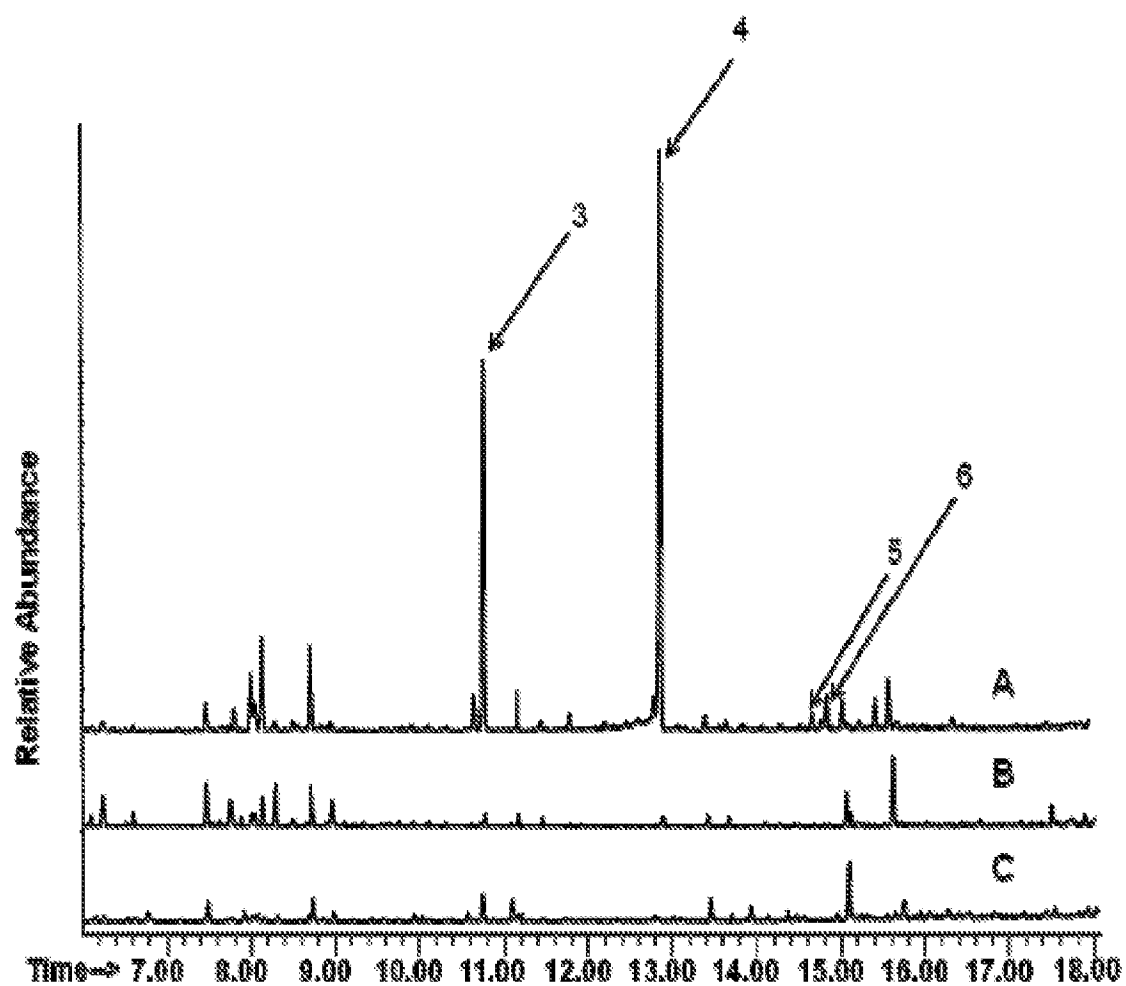


FIG. 3

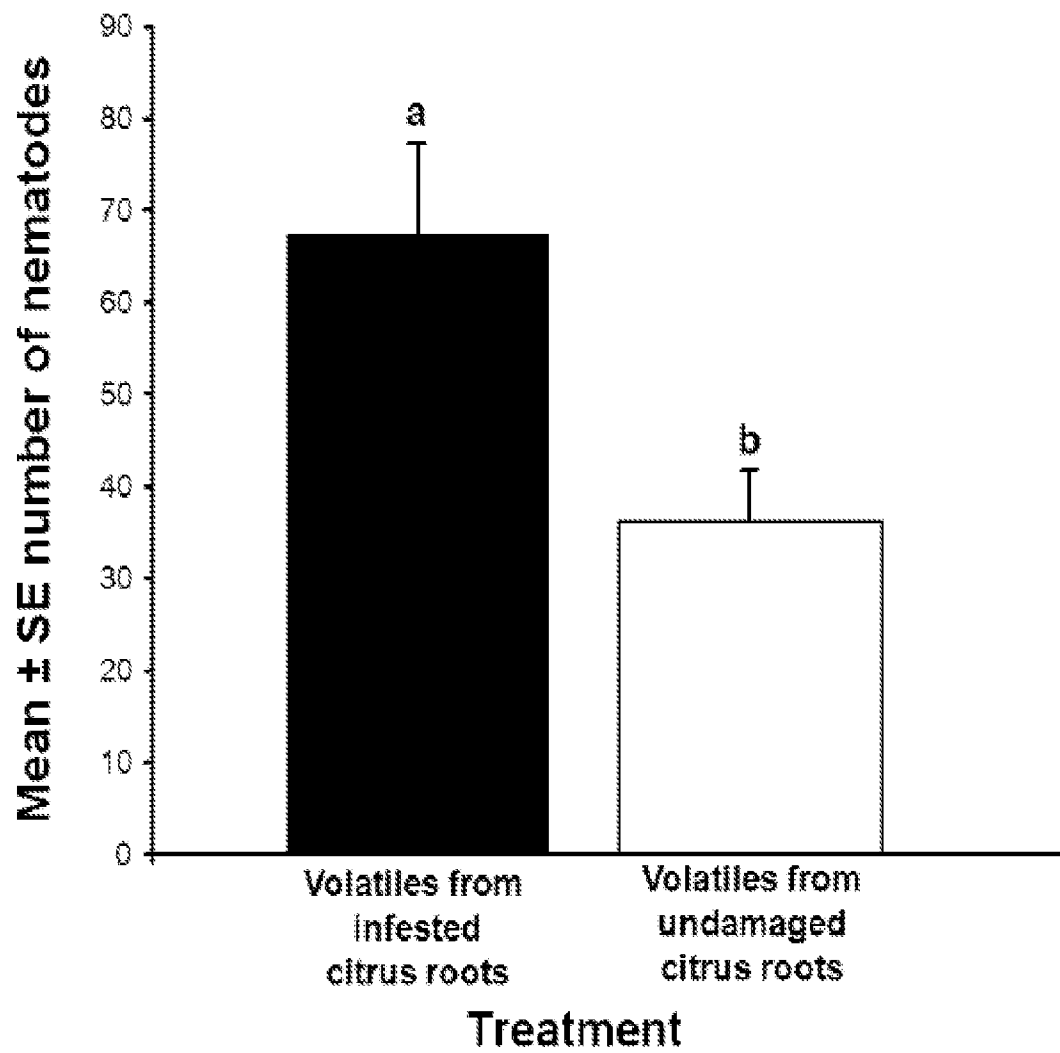


FIG. 4

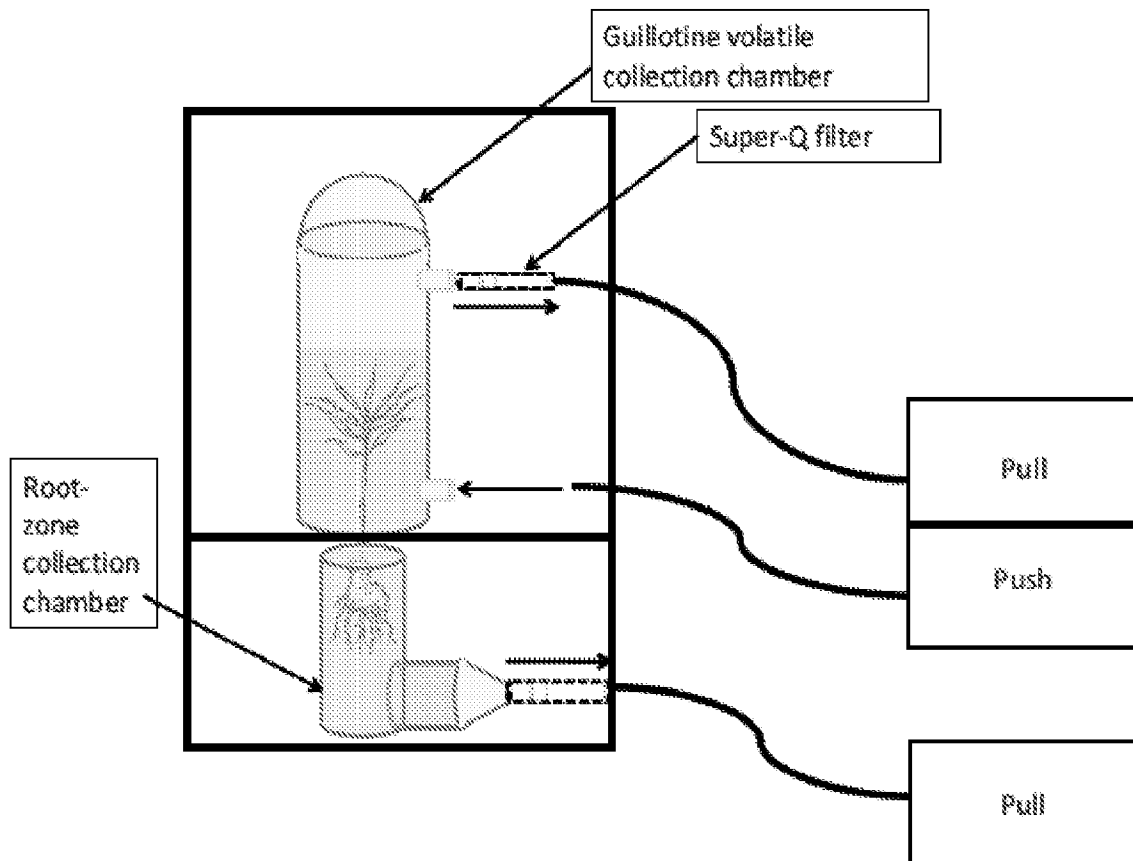
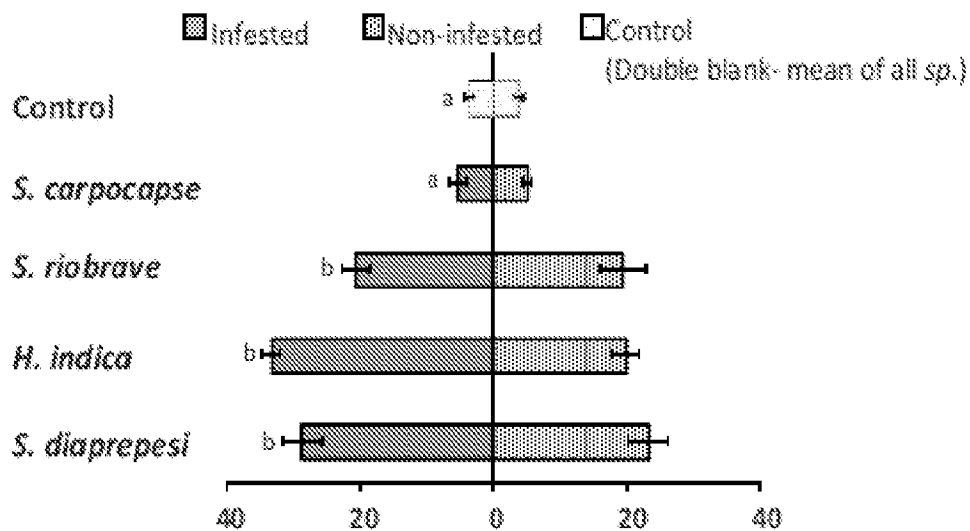
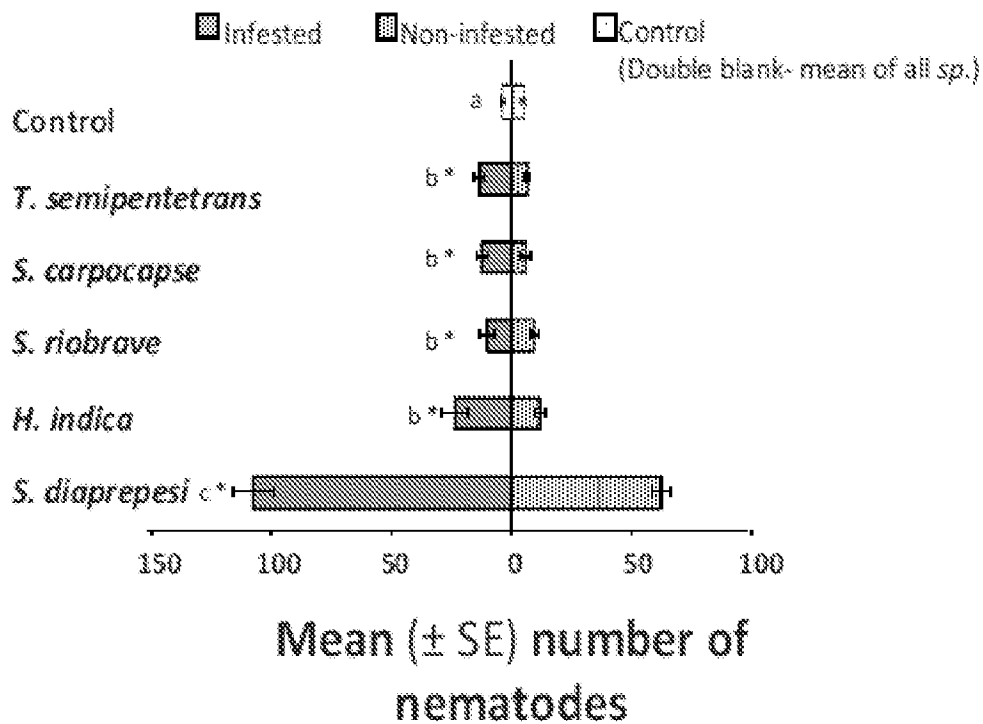
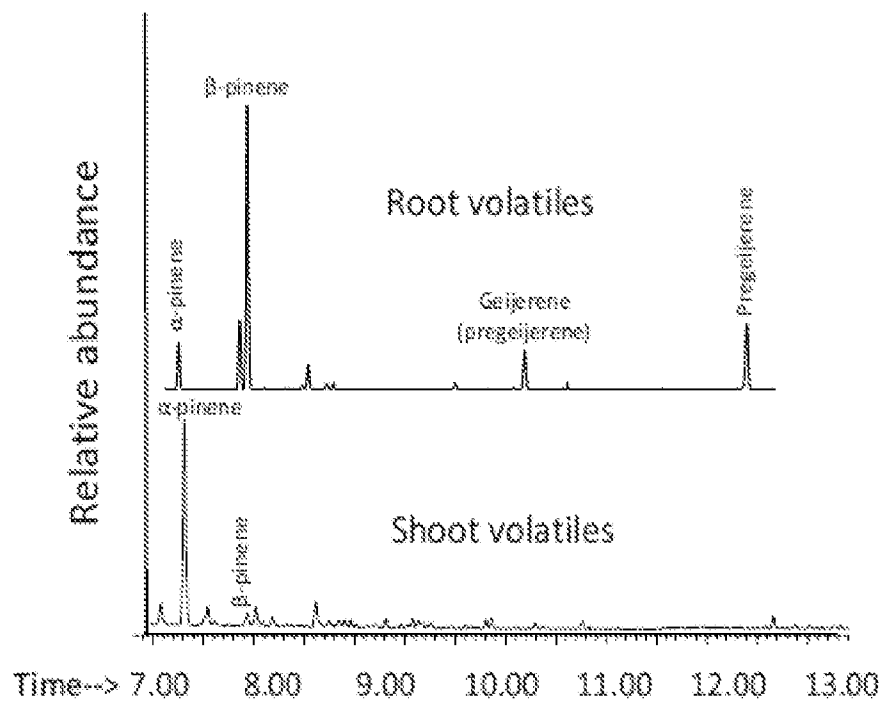


FIG. 5

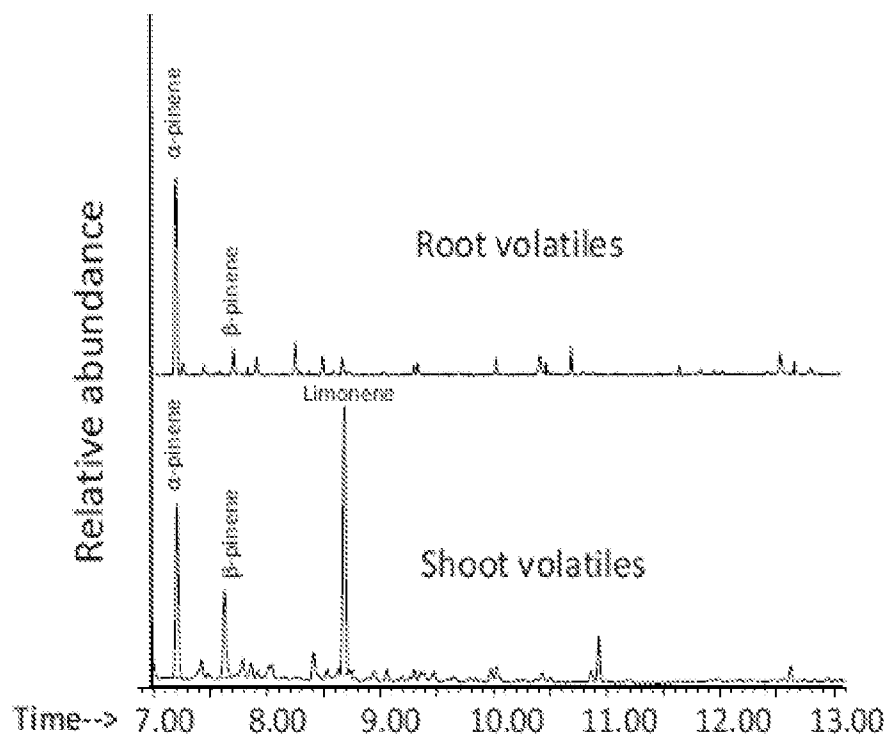
A. *P. trifoliata*B. *C. paradisi* x *P. trifoliata* 'Swingle citromelo'

FIGS. 6A - 6B

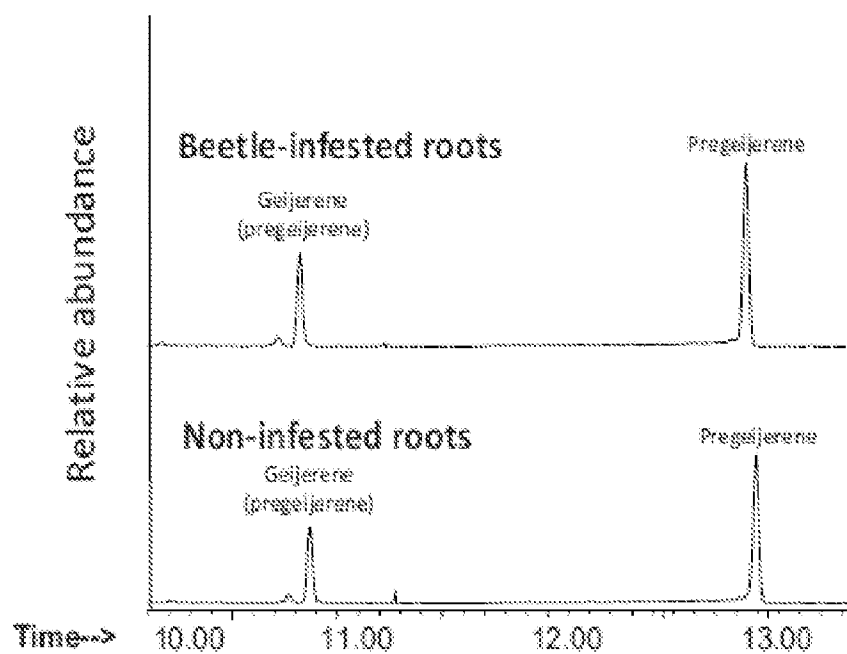
A. Belowground herbivory



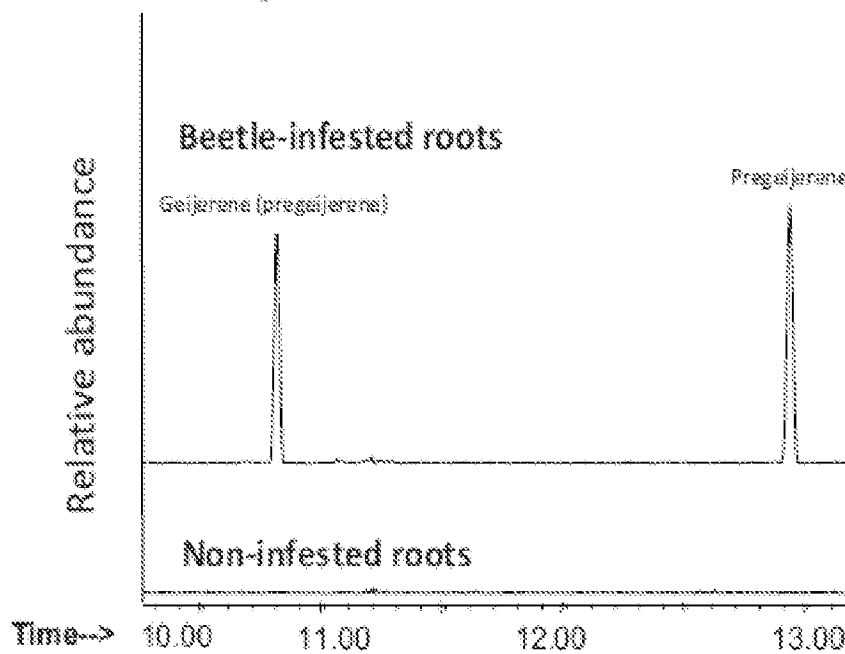
B. Aboveground herbivory



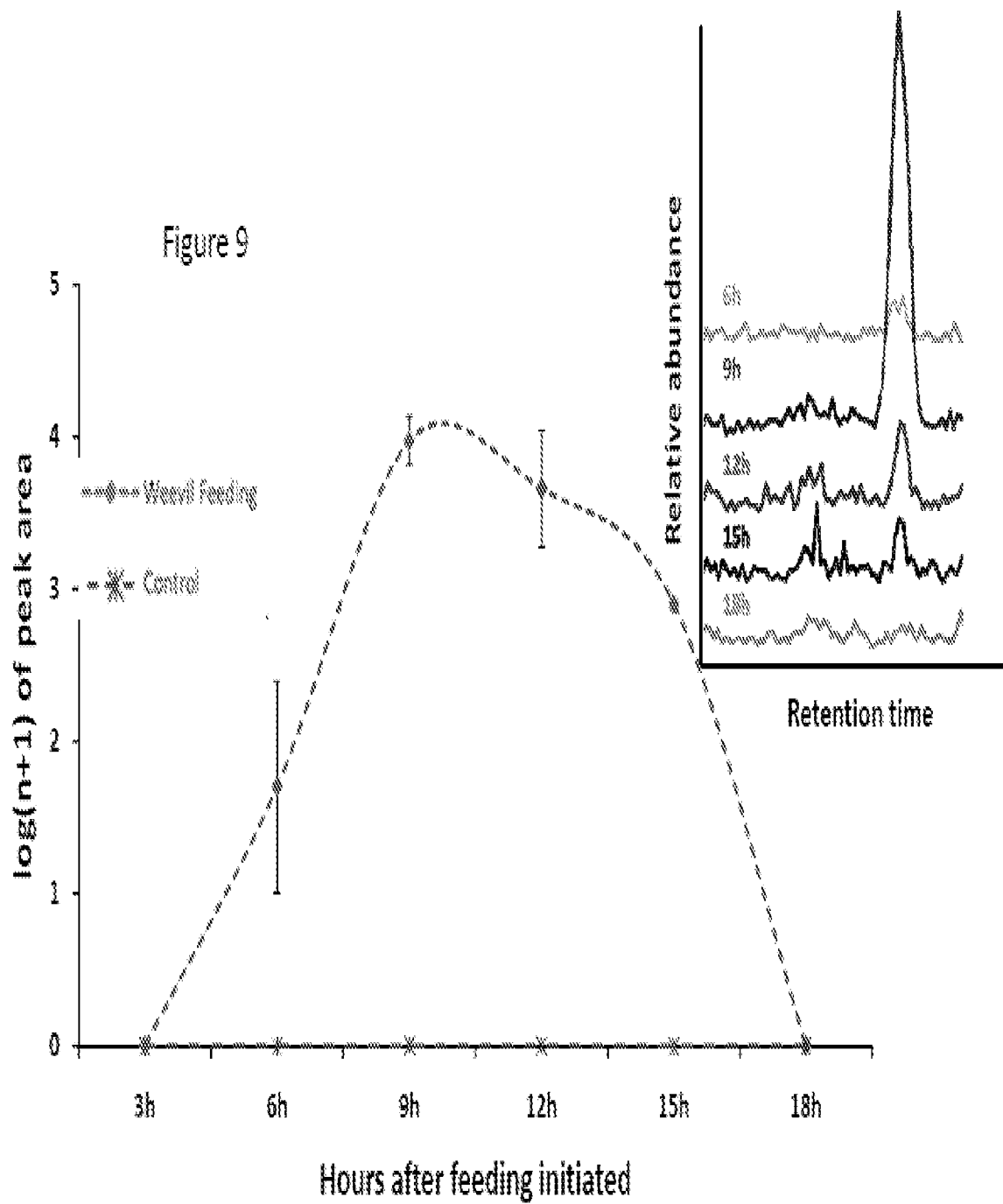
FIGS 7A - 7B

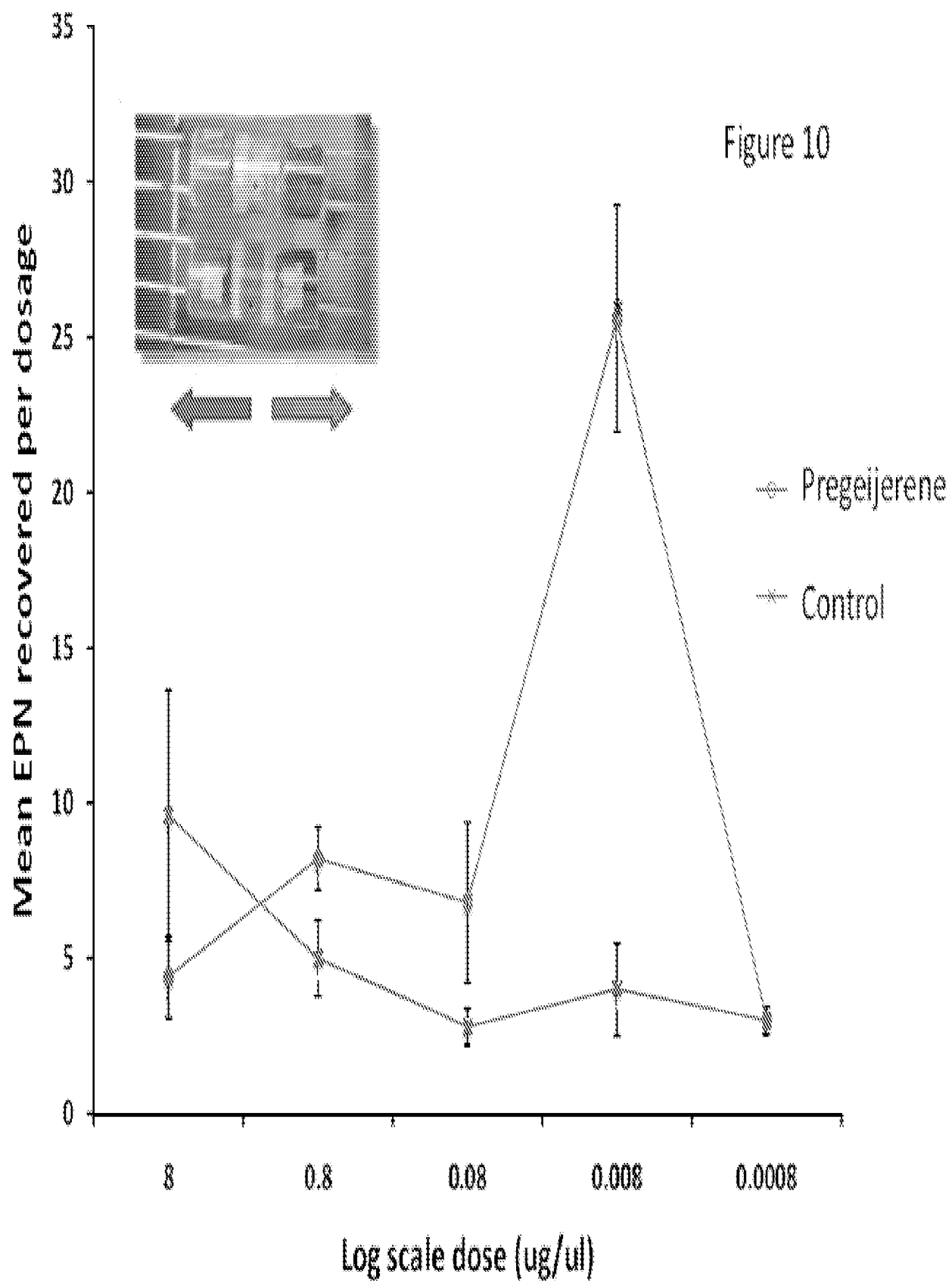
A. *Poncirus*

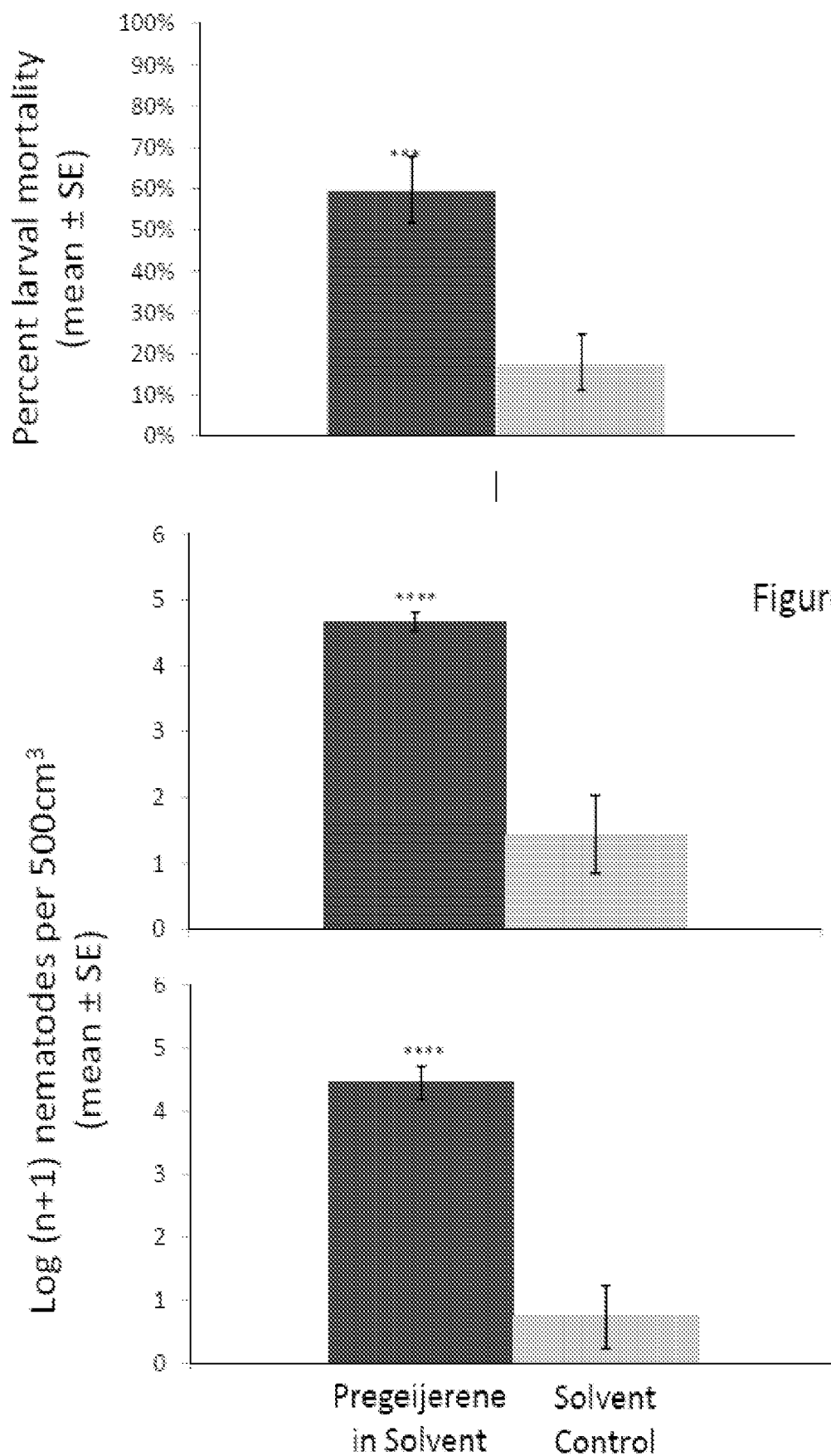
B. Sour orange

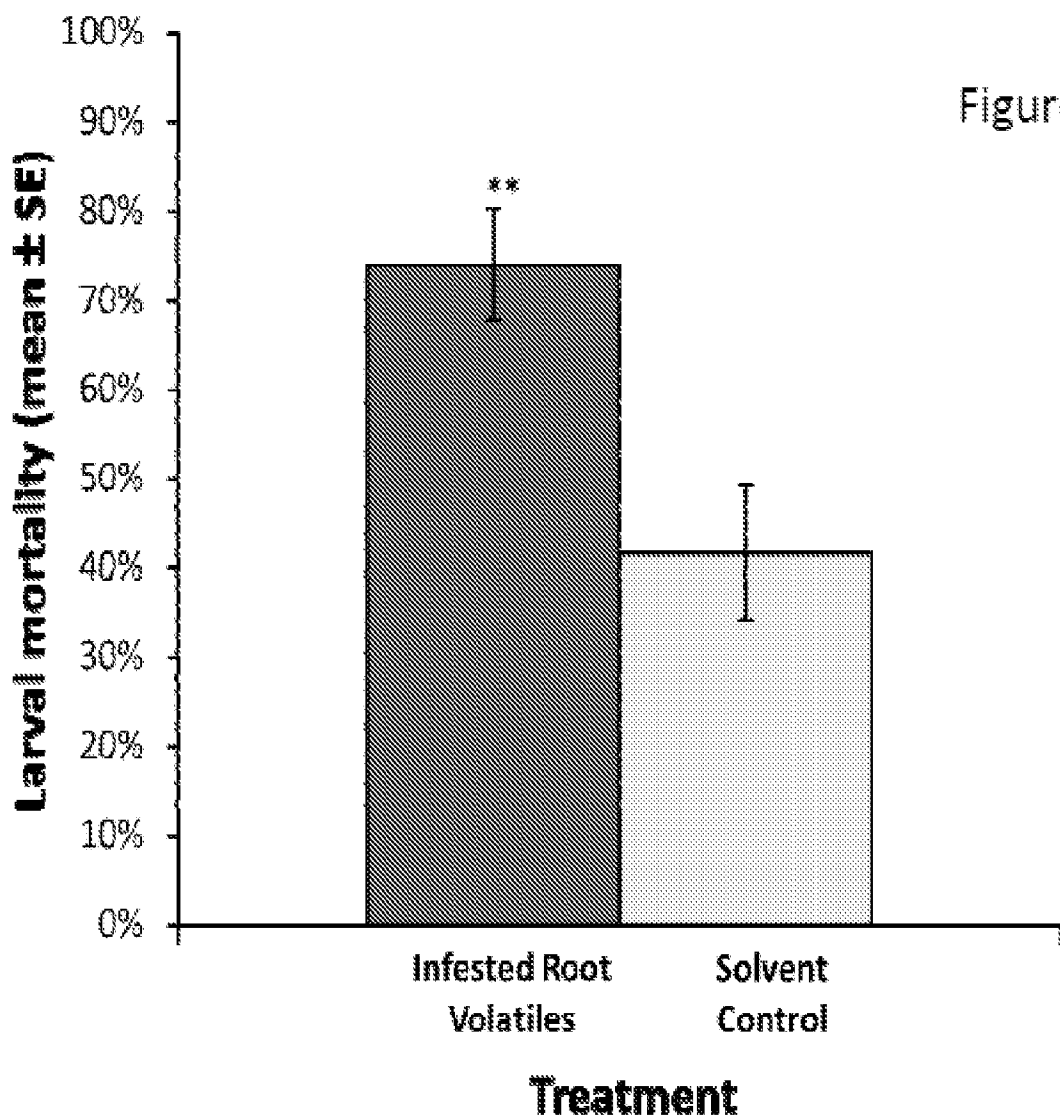


FIGS 8A - 8B









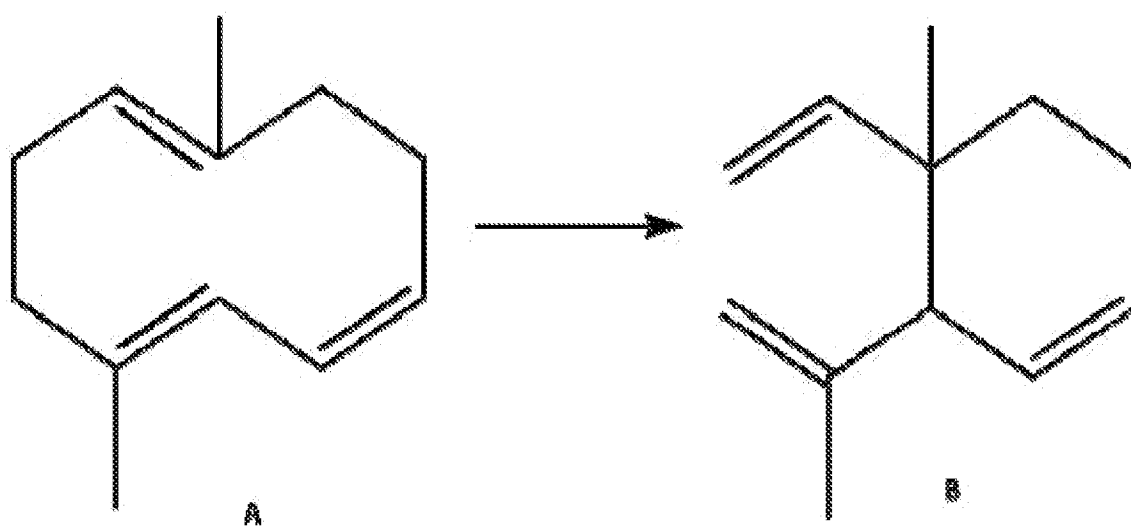


Figure 13

Figure 14

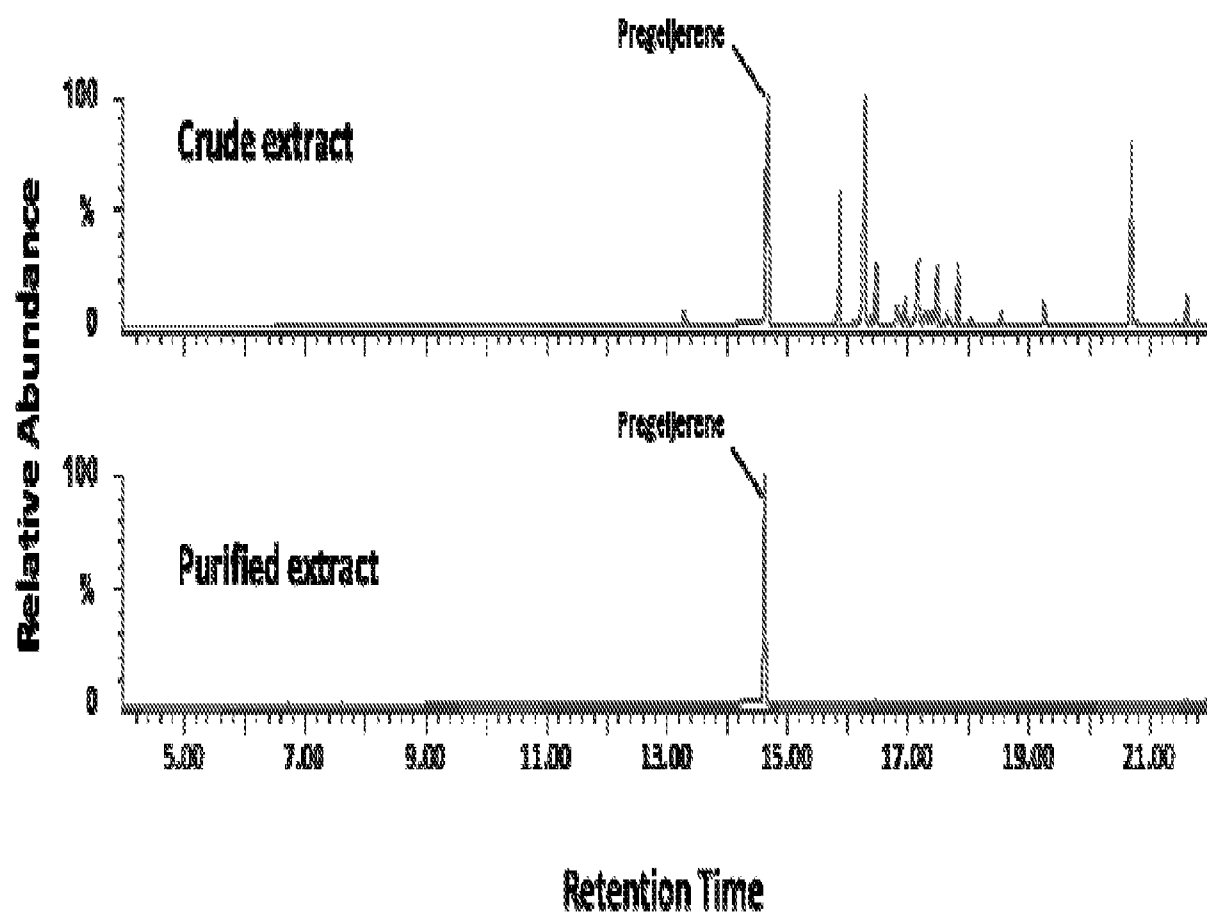


Figure 15

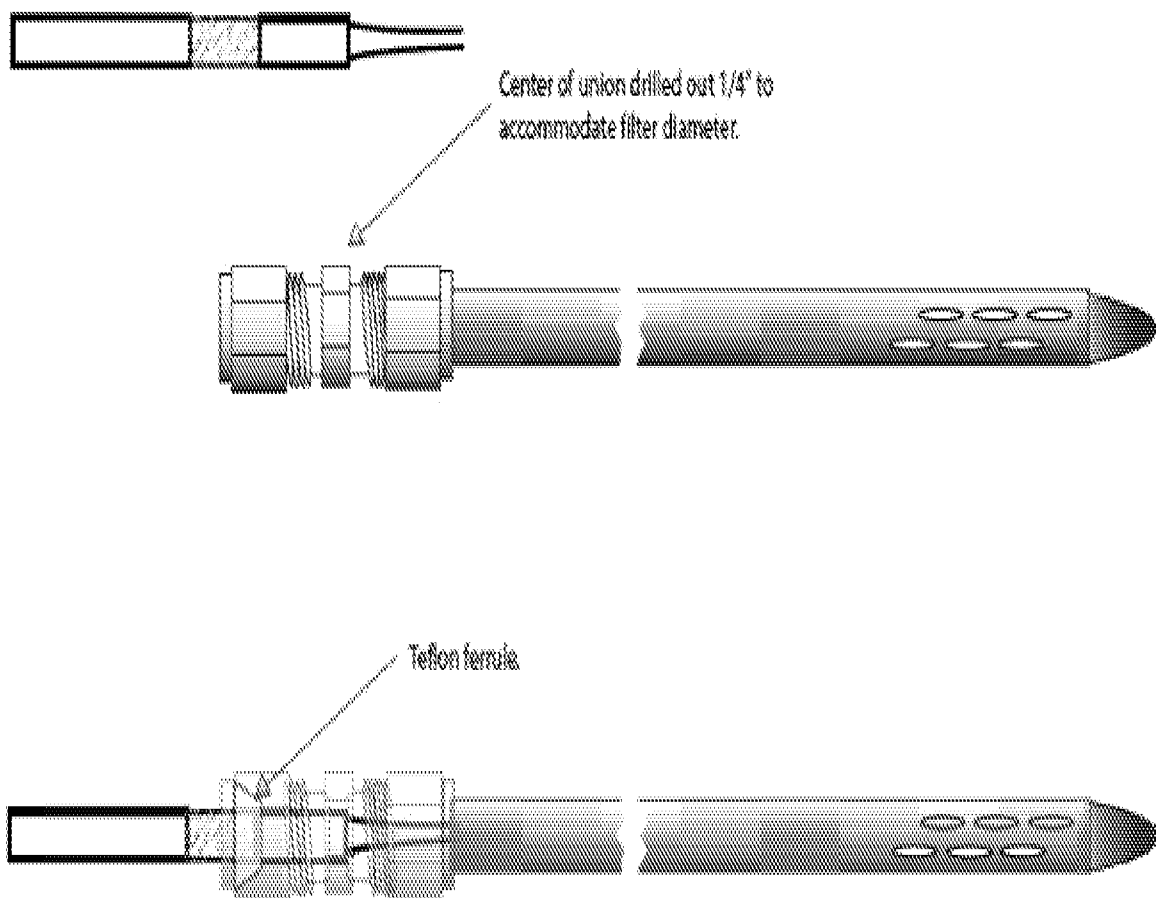


Figure 16

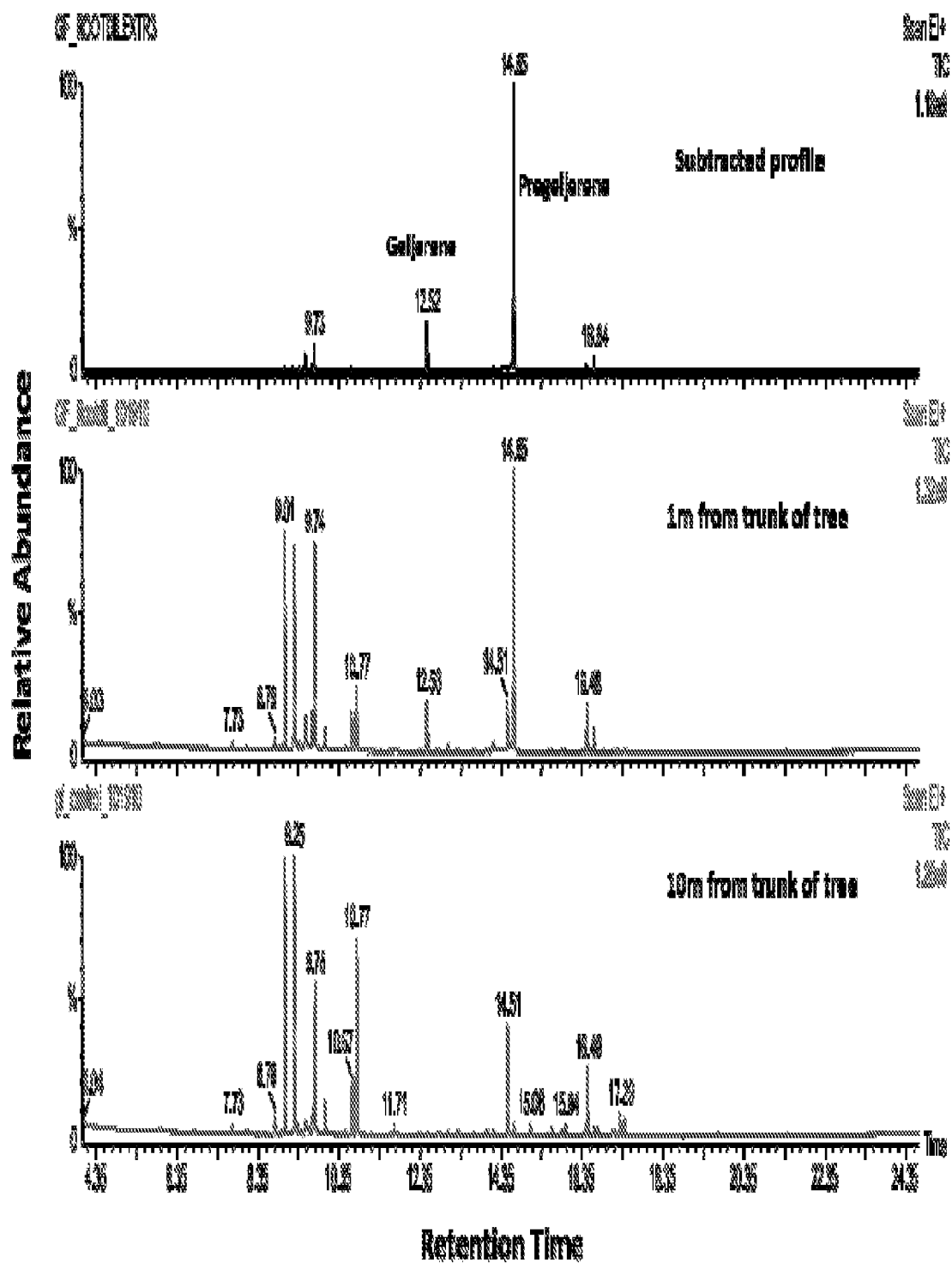


Figure 17

