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Subterranean Herbivore-induced Volatiles Released by Citrus Roots upon Feeding by *Diaprepes abbreviatus* Recruit Entomopathogenic Nematodes

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Abstract Herbivore-induced volatile emissions benefit plant hosts by recruiting natural enemies of herbivorous insects. Such tritrophic interactions have been examined thoroughly in the above-ground terrestrial environment. Recently, similar signals have also been described in the subterranean environment, which may be of equal importance for indirect plant defense. The larvae of the root weevil, *Diaprepes abbreviates*, are a serious pest of citrus. Infestations can be controlled by the use of entomopathogenic nematodes, yet the interactions between the plant, insect and nematode are poorly understood and remain unpredictable. In bioassays that used a root zone six-arm olfactometer, citrus roots ('Swingle citrumelo' rootstock) recruited significantly more entomopathogenic nematodes (*Steinernema diaprepesi*) when infested with root weevil larvae than non-infested roots. Infested plants were more attractive to nematodes than larvae alone. Roots damaged by weevil larvae attracted more nematodes than mechanically damaged roots and sand controls. By dynamic *in situ* collection and GC-MS analysis of volatiles from soil, we determined that four major terpene compounds were produced by infested plant roots that were not found in samples from non-infested roots or soil that contained only larvae. Solvent extracts of weevil-infested roots attracted more nematodes than extracts of non-infested roots in a two choice sand-column bioassay. These findings suggest that Swingle citrus roots release induced volatiles as an indirect defense in response to herbivore feeding, and that some of these induced volatiles function as attractants for entomopathogenic nematodes.

Key Words Entomopathogenic nematodes · *Diaprepes abbreviatus* · Herbivore induced volatiles · Below-ground tritrophic interactions · *Steinernema diaprepesi*

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Introduction

Plants produce an array of signals with diverse roles, thus providing them with responses necessary to survive in their dynamic environment. Examples of plants luring organisms and thereby 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 and Wäckers 2004). However, examples of such tritrophic interactions, among plants, herbivores, and natural enemies are common (Agrawal and Rutter 1998; Agrawal and Karban 1999; Baldwin and Preston 1999; Dicke et al. 2003).

Herbivore feeding on plants results in release of volatile compounds, which may attract arthropod predators and/or parasitoids. For instance, lima bean plants (*Phaseolus lunatus*), release volatiles when infested with spider mites (*Tetranychus urticae*), which attract the predatory mite *Phytoseiulus persimilis* (Takabayashi and Dicke 1996). Oviposition can also stimulate plant exudates that are attractive to egg parasitoids; the legume, *Vicia faba* emits volatiles that attract the egg parasitoid, *Trissolcus basalis* after oviposition by the Pentatomid, *Nezara viridula*

(Colazza et al. 2004). Specific compounds from both the plant and salivary elicitors from the herbivore have been shown to mediate these interactions (Alborn et al. 1997). For example, methyl salicylate attracts herbivore predators (e.g., De Boer and Dicke 2004). Volicitin, found in oral secretions of caterpillars (*Spodoptera exigua*), has been well characterized and induces volatile production in maize (Alborn et al. 2000; Turlings et al. 2000). As the details of above-ground tritrophic interactions have become substantially resolved (Vet et al. 1991; Vet and Dicke 1992), recent attention has focused on analogous communication systems in the subterranean environment.

Volatile signaling by plant roots can contribute to belowground defense by acting as antimicrobial or anti-herbivore substances (Tumlinson et al. 1992, 1999; Neveu et al. 2002; Bais et al. 2006). Plants also can benefit by releasing herbivore-induced volatile emissions that recruit natural enemies of subterranean herbivores, as shown by van Tol and Sommen (2001), Aratchige et al. (2004), and Rasmann et al. (2005). The pressure from belowground pests of plants is significant and likely imparts selection pressure for evolution of induced plant responses.

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). *Diaprepes abbreviatus* was first introduced into Florida in 1964 (Beavers and Selhime 1975). Over the past 40 years, it has contributed significantly 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). *Diaprepes abbreviatus* damage the vegetative portion of plants by notching young leaves (Fennah 1940). Mature adults lay eggs between older leaves and emerging first instars drop to the soil where they develop and feed on roots causing the most severe damage to plants (Fennah 1940; Schroeder 1992). Currently, the most effective method for controlling the larval stage is with entomopathogenic nematodes (EPN), from the genera *Heterorhabditis* or *Steinernema* (Downing et al. 1991; Schroeder 1992).

EPNs are obligate parasites that kill their host with the aid of a symbiotic bacterium (Poinar 1990). Mass-produced EPNs have been used for control of *D. abbreviatus* by citrus growers for over 20 years (Duncan et al. 1999). Mass release of EPNs can effectively reduce larval populations of *D. abbreviatus* (Downing et al. 1991; Schroeder 1992; Bullock et al. 1999). However, the reported efficacy of EPNs against *D. abbreviatus* ranges from 0% to >90% suppression (Adair 1994; Bullock et al. 1999; McCoy et al. 2000), and thus improved consistency of this tactic is desired.

One approach to enhance the effectiveness of EPNs against *D. abbreviatus* may be to exploit plants' naturally produced chemical defenses. Recent work has shown EPNs (*Heterorhabditis megidis*) are attracted to exudates of Thuja plants (*Thuja occidentalis*) infested with larvae of the vine weevil (*Otiorhynchus sulcatus*) (van Tol and Sommen 2001). Furthermore, maize roots infested with larvae of the western corn rootworm (*Diabrotica virgifera*) release terpenoids, typically (*E*)-β-caryophyllene, which attracts EPNs (*Heterorhabditis megidis*) (Rasmann et al. 2005).

In this investigation, we quantified the behavior of the entomopathogenic nematode, *Steinernema diaprepesi* Nguyen & Duncan, in response to citrus plants damaged by larval *D. abbreviatus*. We show that EPNs are attracted to weevil-damaged roots, but not so to mechanically damaged roots, undamaged roots or larvae alone. We also identified volatile compounds induced by weevil feeding and showed that EPN response is specifically mediated by solvent extracts of infested roots.

Methods and Materials

Insects *Diaprepes abbreviatus* larvae were obtained from a culture at University of Florida's Citrus Research and Education Center (CREC) in Lake Alfred, FL, USA. This culture was periodically supplemented from a large 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 3rd to 6th instars.

Nematodes *Steinernema 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 wk prior to use.

Plants 'Swingle citrumelo' (*Citrus paradisi* Macf. × *Poncirus trifoliata* L. Raf.) rootstock is 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 consists of a central glass chamber (8 cm diam and 11 cm deep) attached by six side arms to six glass pots (5 cm diam and 11 cm deep) in which various plants/treatments were tested. The side arms are joined to the six treatments pots with Teflon connectors fitted with a fine mesh filter impervious to nematodes (2300 mesh, Smallparts, Inc., Miramar, FL, USA). For all tests, the olfactometer was filled with sand that had been autoclaved for 1 hr at 250°C and then adjusted to 10% moisture (dry wt. sand:water volume; W/V). In tests involving plants, seedlings were given 3 d to adjust to their sand filled olfactometer for each experiment.

In the first experiment, we tested nematode response to weevil-infested plants vs. non-infested controls. Infested plants were subjected to 3 d of feeding by 3rd–6th instar weevil larvae. Non-infested plants were not exposed to weevils. Three of the arms of the olfactometer were assigned randomly to a weevil-infested plant, while the remaining three received the non-infested control. IJ nematodes (2,500) 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, we compared the response of EPNs to weevil-infested plants 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 1.

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

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 3 d before sampling. All plants were maintained in the olfactometer chambers for 3 d 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 hr 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 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).

GC-MS Analysis A 1 µl aliquot of each dichloromethane extract was injected onto a GC-MS 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 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 those of authentic standards.

EPN Response to Root Extracts The objective of this experiment was 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 3 d as previously described. Thereafter, volatiles were collected from chambers for 24 hr as described above in the volatile collections procedure. Six larvae were 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 hr. The adsorbent Super-Q traps from both treatments (before and after feeding) were extracted by rinsing with 150 µl of dichloromethane directly after their 24 hr collections as described above.

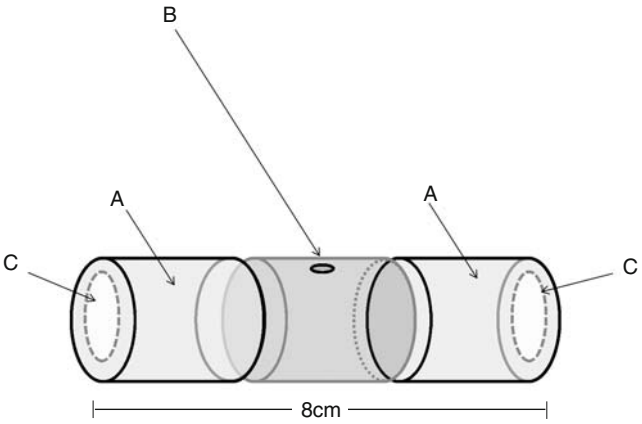

Fig. 1 Schematic diagram of sand column assay unit. Glass jar (17 ml) with samples at base (**a**), connecting tube (3 cm) with hole for nematode application (**b**), extracts placed on filter paper (**c**), arena was filled with heat sterilized sand at 10% moisture for all assays

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 (Fig. 1a) (16 ml BTL, sample type 111, CLR, SNAPC, Wheaton, Millville, NJ, USA), which contained treatments and a central connecting tube 3 cm in length (Blue Max™ 50 ml polypropylene conical tube 30×115 mm, Becton Dickinson Labware, Becton Dickinson Company, Franklin Lakes, NJ, USA), with an apical hole into which nematodes were applied (Fig. 1b). Extracts 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 (Fig. 1c), which subsequently were filled with 10% saturated, sterilized sand as described above. The central chamber connecting the two jars (arms of the olfactometer) also was 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 hr 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 more EPNs per arm than those containing larvae alone (F=13.78, df=1, 58, P<0.001) (Fig. 2b). 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).

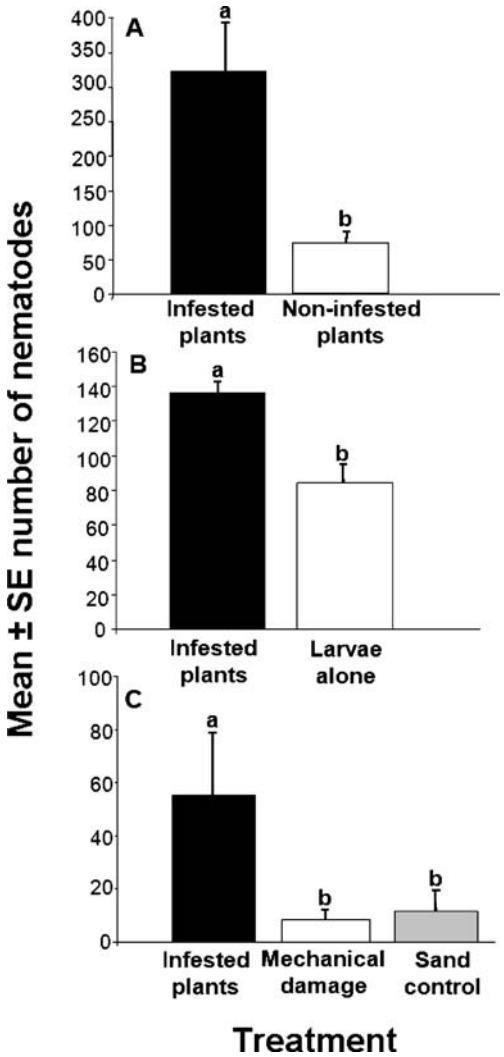

Fig. 2 Mean number of *Steinernema diaprepesi* attracted to chambers containing weevil-infested plants vs. non-infested control plants (**a**), weevil-infested plants versus larvae alone (**b**), weevil-infested plants, mechanically damaged plants or sand control (**c**). Each panel represents a separate experiment (N=10) conducted in a 6-arm olfactometer. Different letters indicate statistical significance at P<0.001

Table 1 GC-MS identification of volatiles from Swingle citrumelo rootstock (*Citrus paradise* × *Poncirus trifoliate*)

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

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

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). *Diaprepes abbreviatus*-infested roots released four additional unique compounds that were not present in non-infested roots (Table 1). Two C_{12} terpenes were the most abundant, and they 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 pregeijerene to geijerene 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).

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).

Discussion

Interactions between EPNs and their host insects, competitors, and natural enemies are well documented, but the degree to which herbivore-induced plant signals alter EPN orientation is largely unknown (Jaffee and Strong 2005; Duncan et al. 2007). Carbon dioxide has long been known

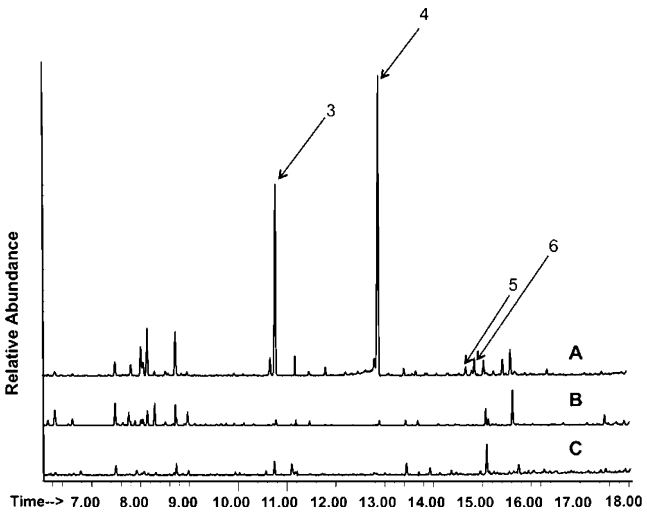

Fig. 3 Example chromatograms showing volatile profiles of *Diaprepes abbreviatus*-infested plants, non-infested plants and larvae alone. Volatile profile of infested *Citrus paradise* × *Poncirus trifoliate* rootstock (**a**) Volatile profile of non-infested *Citrus paradise* × *Poncirus trifoliate* rootstock (**b**) Volatile profile of *D. abbreviatus* alone in sand (**c**). All samples were collected for a 24 hr. Geijerene (3), Pregeijerene (4), α-Santalene (5), α-Z-Bergamotene (6). (Compound numbers correspond to Table 1)

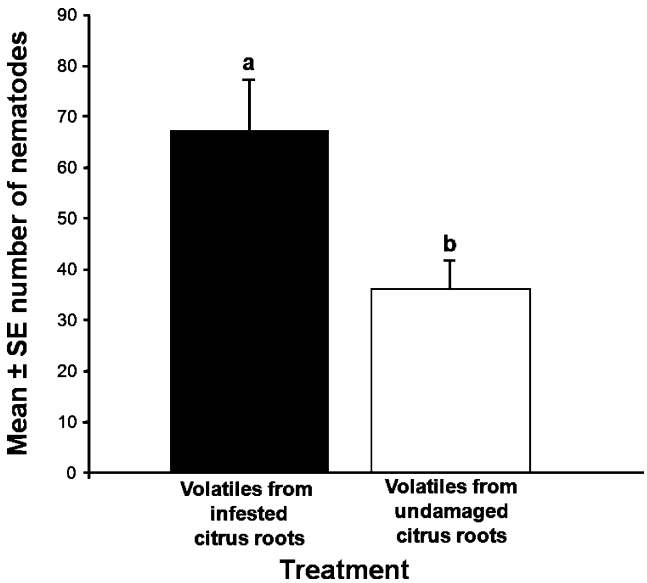

Fig. 4 Mean number of nematodes attracted to volatiles from *Diaprepes abbreviatus*-infested roots compared with volatiles from undamaged roots. Different letters indicate statistical significance at P=0.03

to attract nematodes to plant roots (Gaugler et al. 1980; Prot and Van Gundy 1981). However, functioning alone, such an ambiguous signal might not allow efficient host location by EPNs. van Tol and Sommen (2001) postulated that plants produce induced compounds that attract EPNs; this hypothesis has been confirmed in two systems (Boff et al. 2002; Rasmann et al. 2005). Furthermore, (*E*)-β-caryophyllene has been identified as the specific EPN recruitment signal emitted by maize roots damaged by corn rootworms (Rasmann et al. 2005).

The current results indicate that Swingle citrumelo rootstock releases herbivore induced volatiles that recruit EPNs. Our results also suggest that 'geijerenes' mediate this response. These C_{12} terpenes have not been described for citrus previously; however, they are known for insecticidal, antifeedant, and oviposition deterrent effects in leaves of other rutaceous plant species (Kiran et al. 2006; Kiran and Devi 2007). Geijerenes also have been described in hairy root cultures of *Pimpinella anisum* (Santos et al. 1998). Although these compounds were consistently present in infested root samples and are presumed candidate attractants for *S. diaprepesi*, we have yet to confirm the behavioral activity of the individual compounds. Solvent extracts of infested roots attracted *S. diaprepesi* suggesting that one or a blend of these compounds may be active. Fractionation studies of the induced compounds via preparative gas chromatography in concert with two choice bioassays of the partitioned profile may enable us to resolve the role of individual compounds on EPN behavior.

Recent identification of an EPN recruitment chemical is in the initial stages of application for crop protection and has been promising (Degenhardt et al. 2003, 2009; Turlings and Ton 2006). Direct application of (*E*)-β-caryophyllene to soil has been shown to reduce rootworm damage through enhanced action of their EPNs (Rasmann et al. 2005). Furthermore, recent advances in biochemistry/molecular genetics have made it possible to engineer cultivated maize that releases (*E*)-β-caryophyllene and recruits EPNs, thus protecting roots from herbivore damage (Degenhardt et al. 2003, 2009; Hiltpold et al. 2010). The currently investigated citrus rootstock system is different from the annual maize cropping system for which EPN recruitment is already being developed for corn rootworm management. Perennial systems characterized by fewer disturbances are believed to support more effective biological control than annually disturbed crops (Southwood and Comins 1976). Thus, augmenting the impact of *S. diaprepesi* in a perennial tree fruit system by application of recruitment chemicals may prove even more effective than in annual crops.

It also will be informative to investigate the parent lines of the Swingle rootstock, *Citrus paradisi* and *Poncirus trifoliata* to determine if either or both lines exhibit the herbivore-induced EPN recruitment seen in the hybrid. We plan also to investigate if other non-citrus hosts of *D. abbreviatus* release induced recruitment signals. Given the wide host range of *D. abbreviatus,* it will be important to determine the breath of this EPN recruitment response among its diverse host plants.

Several nematode species attack *D. abbreviatus*. *Steinernema glaseri*, *S. carpocapsae*, and *Heterorhabditis bacteriophora* were investigated initially as possible control agents (Downing et al. 1991; Schroeder 1992). Of the species evaluated in laboratory bioassays and greenhouse trails, *S. riobrave* and a Florida isolate of *H. indica* were the most effective (Shapiro-Ilan and McCoy 2000). Currently, *S. riobrave* and *H. indica* are formulated for commercial application against *D. abbreviatus* in Florida citrus. These two EPN species, in addition to *S. diaprepesi*, will be evaluated and compared in similar future studies to determine whether the tentatively identified EPN recruitment signals are specific to the natively occurring EPN associated with the weevil or whether these signals function more broadly for other EPN species.

We report here for the first time an *in situ* method for sampling subterranean herbivore-induced volatiles during real time insect feeding. Previously used methods involve freeze-drying and crushing root samples (Rasmann et al. 2005), which will affect and misrepresent volatile production from intact roots. The currently described method allows identification of belowground volatiles as they are released over time without disturbance to the system.

The current results indicate that a commercially used citrus rootstock emits induced volatile chemicals in response to herbivore feeding that attract beneficial nematodes. Identification of the specific active compounds may lead to the development of an augmentive EPN recruitment tactic that improves biological control of *D. abbreviatus*. Also, such identification would be the first step towards development of genetically-engineered citrus rootstocks for enhanced recruitment of EPNs. Alternatively, it is possible that engineering plants for increased release of terpenes in general may prove effective (Schnee et al. 2006).

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