

## SPECIAL FEATURE

## PLANT-MEDIATED INTERACTIONS BETWEEN ABOVE- AND BELOW-GROUND COMMUNITIES

# Constitutive and induced subterranean plant volatiles attract both entomopathogenic and plant parasitic nematodes

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## Summary

1. Indirect plant defences are well documented for the above-ground constituents of plants. Although less investigated to date, below-ground defences that mediate multitrophic interactions are equally important. Entomopathogenic nematodes (*Steinernema diaprepesi*) are attracted to herbivore-induced volatiles from Swingle var. (*Citrus paradisi* × *Poncirus trifoliata*) when fed upon by root weevil, *Diaprepes abbreviatus*.

2. We examined the extent to which below-ground volatiles modify behaviour of nematode species representing various foraging strategies (cruisers versus ambushers) and trophic levels (plant parasites versus entomopathogens). We compared attraction to volatiles of weevil-infested and non-infested roots from Swingle citrus rootstock and a parent line of the Swingle hybrid, *Poncirus trifoliata* (Pt).

3. Swingle weevil-infested roots attracted more nematodes than non-infested roots irrespective of nematode foraging strategy and trophic status. The parental line, Pt, attracted all nematode species irrespective of insect herbivory.

4. Dynamic *in situ* collection and GC–MS analysis of volatiles from soil revealed that Pt roots release attracting cues constitutively. A different non-hybrid citrus species (sour orange, *Citrus aurantium*) released nematode attracting cues only in response to larval feeding, similar to responses found in Swingle. Volatile collections from above- and below-ground portions of citrus plants revealed that above-ground feeding by weevils does not induce production of nematode attracting cues analogous to that induced by root damage, nor does damage by larvae below-ground induce a similar volatile above ground.

5. *Synthesis*. Our results suggest that release of nematode attractants by citrus roots occurs broadly and can be constant or herbivore-induced. The major constituent of this indirect defence is produced by roots and not shoots and in response to below-ground, but not above-ground herbivory. Our findings suggest that this cue acts on nematode species broadly, attracting entomopathogenic nematodes that exhibit various foraging strategies. Unexpectedly, we also found that this cue attracts a plant parasitic nematode species. It appears, thus, that release of nematode attracting cues by citrus plants can cause ecological costs. The plants, however, appear to counteract against these costs, because constitutive release was found only in a cultivar that is resistant to phytopathogenic nematodes, while herbivore-induced release occurred in lines susceptible to pathogenic nematode species.

**Key-words:** above-ground–below-ground interactions, biological control, chemical ecology, *Diaprepes abbreviatus*, entomopathogenic nematodes, herbivore-induced plant volatiles, plant–herbivore interactions, pregeijerene

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## Introduction

Plant–insect–predator (parasite) interactions are often described in the above-ground terrestrial environment. However, analogous below-ground plant–herbivore interactions should also be considered (van Dam 2009). General understanding of plant communication has greatly improved since early insights into plant–insect mutualisms, which arise as plants meet their reproductive requirements (Ehrlich & Raven 1964). Our understanding of the impact that herbivore-induced plant volatiles (HIPVs) have on the tertiary trophic level continues to increase and is expanding beyond the general understanding that HIPVs attract predators (Turlings, Tumlinson & Lewis 1990; Heil 2008; Dicke & Baldwin 2010). Above-ground plant defence by HIPV signalling is now considered a common and broadly understood phenomenon (Agrawal & Rutter 1998; Agrawal & Karban 1999; Baldwin & Preston 1999; Dicke *et al.* 2003; Turlings & Wäckers 2004).

Herbivore-induced plant volatiles are often only released after herbivore feeding. For instance, lima bean plants (*Phaseolus lunatus*) release volatiles when infested with spider mites (*Tetranychus urticae*), which attract the predatory mite *Phytoseiulus persimilis* (Dicke & Sabelis 1988). It is known that compounds associated with the feeding insect can mediate such plant response (Alborn *et al.* 1997). Most known are volicitin and other fatty acid amides, found in oral secretions of herbivores, which induce volatile production in plants (Alborn *et al.* 2000; Turlings *et al.* 2000).

Over the past decade the role of subterranean release of HIPVs and their indirect impact on plant defence has become increasingly evident (van Tol *et al.* 2001; Aratchige, Lessna & Sabelis 2004; Rasmann *et al.* 2005; Ali, Alborn & Stelinski 2010). Below-ground herbivory likely imparts significant selection pressure for evolution of induced plant responses (Blossey & Hunt-Joshi 2003). In plant–herbivore systems in which the life cycle of the herbivore is partitioned between above- and below-ground plant zones, a unique opportunity exists for investigating plant defences in both above-ground and subterranean environments in response to damage from a single herbivore species. Furthermore, investigations of HIPV release with perennial, cultivated plant species allow insights into the evolution of responses in both naturally occurring and artificially selected genotypes (Köllner *et al.* 2008; Degenhardt *et al.* 2009).

Larvae of the weevil *Diaprepes abbreviatus* feed on the roots of more than 290 plant species including citrus, sugarcane, potatoes, strawberries, woody field-grown ornamentals, sweet potatoes, papaya, guava, mahogany, ornamentals, and non-cultivated wild plants (Simpson *et al.* 1996). *Diaprepes abbreviatus* was first introduced to Florida in 1964 (Woodruff 1964). Over the past 40 years, it has contributed significantly to the spread of disease and damage (Weissling *et al.* 2002). Above-ground, *D. abbreviatus* damages 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). Ento-

mopathogenic nematodes (EPNs) from the genera *Heterorhabditis* or *Steinernema* (Downing, Erickson & Kraus 1991; Schroeder 1992) are known to infect this insect (McCoy *et al.* 2000). EPNs are parasitoids that kill their host with the aid of a symbiotic bacterium (Poinar 1990).

Recently, we showed that citrus roots ('Swingle citrumelo' rootstock *Citrus paradisi* × *Poncirus trifoliata*) fed upon by *D. abbreviatus* attract EPNs (*S. diaprepesi*) (Ali, Alborn & Stelinski 2010). We found that weevil-infested roots release volatile compounds not found in undamaged roots and suggested this to be an indirect defence associated with attraction of beneficial nematodes. Of the four main compounds released by damaged roots, the C<sub>12</sub> terpenes pregeijerene and its breakdown product, geijerene, were the main two volatiles potentially associated with attraction of beneficial nematodes, and preliminary research supports the hypothesis that in this system the geijerenes are the major nematode attractants (unpublished). The above experiment investigated only 'Swingle citrumelo', a hybrid rootstock that is commonly used due to its resistance to diseases, plant parasitic nematodes and adverse environmental conditions (Stover & Castle 2002). The question therefore arose how broadly release of nematode-attracting cues occurs among various citrus varieties.

*Diaprepes abbreviatus* is the main root weevil species affecting citrus and thus the major interest of our present research. However, a complex of related insect species also attack citrus roots (Duncan *et al.* 1999), thus nematode attraction may have broad significance for citrus defence. Therefore, in addition to determining the extent of nematode attraction among various citrus varieties, we also investigated the breadth of responsiveness among several EPN species. EPNs 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, Gaugler & Harrison 1992, 1993; Grewal *et al.* 1994). 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 (Lewis, Grewal & Gaugler 1995; Lewis *et al.* 2006). In contrast, ambush foragers scan during long pauses and allocate less time to active movement through their environment (Campbell & Gaugler 1993). They are thought to wait for resources to come to them, increasing effectiveness of finding highly mobile prey. *Steinernema carpocapsae* is a representative ambush-type EPN, while *H. indica* (non-nictating) is a typical cruise-type EPN (Lewis 2002). *Steinernema diaprepesi* is a recently discovered species indigenous to Florida's central ridge and flatwoods that specializes on *D. abbreviatus* and is considered intermediate on the spectrum between ambushers and cruisers (Nguyen & Duncan 2002). Finally, *Steinernema riobrave* was discovered in Texas and it is also considered intermediate with respect to foraging strategy (Cabanillas, Poinar & Raulston 1994).

In addition to investigating the above EPN species, we also included a plant parasitic species as a trophic-level outgroup.

The citrus nematode, *Tylenchulus semipenetrans*, is one of the most significant parasites of plants worldwide affecting 8–12% of all citrus species. In Florida, it is estimated to affect 53–89% of described citrus species (Duncan *et al.* 1989). The life cycle of *T. semipenetrans* consists of an egg and four larval stages followed by a sexually reproducing adult stage. Second-stage larvae are the infective juveniles (IJs) 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 moult three times, increasing in size with each moult to form large, posteriorly swollen females capable of depositing *c.* 75 500 eggs per female (Munn & Munn 2002).

Above-ground plant stress elicits defensive responses in both above- and below-ground tissues (Erb *et al.* 2008; Kaplan *et al.* 2008a,b; van Dam 2009; van Dam & Heil 2011). Additionally, many studies have found an increase in the levels of shoot defences following root herbivory (Bezemer *et al.* 2004; van Dam, Raaijmakers & van der Putten 2005; Soler *et al.* 2005). Analogously, levels of root defences can be affected by shoot herbivory (Soler *et al.* 2007; Tiwari *et al.* 2009; Erb *et al.* 2011). Above-ground–below-ground cascades of plant defence can be reciprocally beneficial or detrimental between plant shoots and roots (van Dam & Heil 2011). However, it was unclear in our system whether above-ground stress induced an associated below-ground response to root feeding or vice versa. Therefore, we investigated if release of nematode attracting cues is a localized root response or whether it is also mediated by shoot herbivory.

Our ongoing investigations of herbivore-induced nematode attraction using citrus as a study system have addressed the breadth of this response among various citrus species as well as the breadth of responsiveness to the plant-produced cues by a diversity of nematode species. Additionally, the current investigation explored whether releasing a plant volatile that could potentially attract beneficial parasitoids of insect herbivores was associated with ecological cost of attracting plant pathogens. Our findings suggest that a species and hybrid line more vulnerable to phytopathogenic nematodes can reduce the associated costs by emitting nematode attracting volatiles only when it is necessary, that is, when roots are attacked by herbivores. In contrast, a species that is not susceptible to root parasites produces these cues constantly, investing more into constitutive defence.

## Materials and methods

### INSECTS

*Diaprepes 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 a commercially prepared diet (Bio-Serv, Inc., Frenchtown, NJ, USA) as described in Beavers (1982) using procedures described by Lapointe & Shapiro (1999). Larvae used in experiments were from third to sixth instars. Female adults were used two weeks after emergence.

### NEMATODES

Nematode foraging strategy and trophic level status are summarized in Table 1. The EPNs, *S. diaprepesi*, *S. riobrave*, *S. carpocapsae* and *H. indica* were isolated from *D. abbreviatus* larvae buried in commercial citrus orchards in Florida. *Steinernema riobrave* and *S. carpocapsae* isolates were descendants of commercial formulations intended for field application to manage *D. abbreviatus*. All EPN species were cultured in last-instar larvae of the greater wax moth, *Galleria mellonella*, at *c.* 25 °C according to procedures described in Kaya & Stock (1997). 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.

*Tylenchulus 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% relative humidity. *Poncirus 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, *T. semipenetrans*, 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, *C. paradisi* × *P. trifoliata*, 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 common rootstocks used for commercially grown citrus (Stover & Castle 2002). However, its susceptibility to tristeza virus and *T. semipenetrans* has

**Table 1.** Trophic level, foraging strategy and ecological status of nematodes tested

Nematode spp.	Trophic level	Foraging strategy	Ecological status
<i>Steinernema diaprepesi</i>	Entomopathogen	Intermediate	Indigenous to Florida
<i>S. carpocapsae</i>	Entomopathogen	Ambush	Commercially introduced
<i>S. riobrave</i>	Entomopathogen	Intermediate	Commercially introduced
<i>Heterorhabditis indica</i>	Entomopathogen	Cruiser	Commercially applied; indigenous to Florida
<i>Tylenchulus semipenetrans</i>	Plant parasite	Sedentary root endoparasite	Agricultural pest; citrus parasite

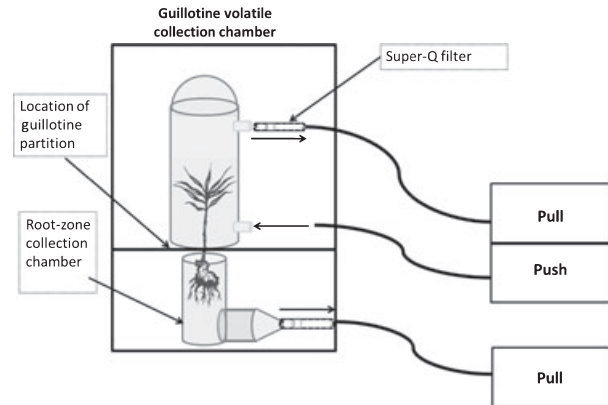
decreased its prevalence in the past decade (Stover & Castle 2002). These three rootstocks were chosen in an effort to determine the breadth of nematode attraction among diverse citrus varieties with and without hybridization.

#### NEMATODE BEHAVIOUR

The behavioural responses of nematodes to collected root samples were quantified in a two-choice sand-filled olfactometer described thoroughly by Ali, Alborn & Stelinski (2010). Briefly, the olfactometer consists 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, Alborn & Stelinski 2010). For each plant species, root volatiles were collected and extracted from the collection filters according to the methods described by Ali, Alborn & Stelinski (2010). An adsorbent trap was connected to the bottom opening of the glass root-zone chamber; treatments were non-destructively sampled with a vacuum connected to the adsorbent trap that pulled air from the chamber. Trap extracts from infested and non-infested roots 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, which were subsequently filled with 10% saturated (dry wt. sand: water volume; w/v), sterilized sand (Ali, Alborn & Stelinski 2010). The central chamber connecting the two arms of the olfactometer was also filled with sterilized and moistened sand. Nematodes (*c.* 200 IJs) were applied into the central orifice of the connecting tube and given 8 h to respond. Following the incubation period, the column was disassembled and the nematodes from the two collection jars were extracted using Baermann funnels. The experiment was replicated 10 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 BELOW-GROUND VOLATILE COLLECTIONS

By simultaneous collection of root and shoot volatiles using a head-space guillotine chamber coupled with a root-zone collection chamber (Fig. 1) we examined whether adult feeding on Swingle shoots induce a nematode-attracting plant root response analogous to that observed in response to root damage by larvae. Similarly, we investigated if typical induced root volatiles were released above ground in response to root damage by larvae. Plants were initially placed in glass root-zone chambers (ARS, Gainesville, FL, USA) filled with sand that had been autoclaved for 1 h at 250 °C and then adjusted to 10% moisture as described in Ali, Alborn & Stelinski (2010). The chambers and plants were placed below a platform on which a Teflon guillotine was attached (Fig. 1). 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 (ARS) 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<sup>-1</sup> through a trap containing 50 mg of Super Q adsorbent (Alltech Assoc., Deerfield, IL, USA). 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.



**Fig. 1.** Schematic diagram of simultaneous above- and below-ground volatile collection apparatus (ARS). The guillotine volatile collection chambers used for above-ground collections received a constant flow of charcoal-purified and humidified air, which was suctioned at a rate of 300 mL min<sup>-1</sup> through a trap containing 50 mg of Super Q adsorbent (Alltech Assoc.). Root-zone collection chambers used to collect below-ground volatiles were filled with heat-sterilized sand standardized at 10% saturation.

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 root-zone or six female adults were placed on leaves above ground. The below- and above-ground chambers of each infestation type were simultaneously sampled for three subsequent days after infestation. Beetle feeding was easily noticeable in damaged leaves above ground and was visually confirmed on roots after the feeding interval (Ali, Alborn & Stelinski 2010). Each infestation treatment was replicated five times.

#### 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 (*C. aurantium*) that were damaged by *D. abbreviatus* feeding or left undamaged. Plants were potted in sand-filled glass root-zone chambers as previously described. Seedlings were given 3 days to adjust to their sand-filled chambers. Infested plants were subjected to an additional 3 days of feeding by 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) for 24 h with a suction flow of 80 mL min<sup>-1</sup> (Ali, Alborn & Stelinski 2010). Compounds emitted from chambers were collected on adsorbent traps filled with 50 mg Super-Q, (800–1000 mesh, Alltech Assoc.) held in glass fittings between the chamber and vacuum pump (Ali, Alborn & Stelinski 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, Alborn & Stelinski 2010).

#### GC-MS ANALYSIS

All samples were injected as 1-µL aliquots of dichloromethane extracts onto a gas chromatograph (HP 6890) equipped with 30 m length × 0.25-mm internal diameter, 0.25-µm film thickness DB-1 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. Samples were introduced either by 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 40 °C for 1 min after injection and then programmed for a temperature increase of 10 °C min<sup>-1</sup> to 260 °C. The carrier gas used was helium at an average flow velocity of 30 cm s<sup>-1</sup>. Isobutane was used as the reagent gas for chemical ionization, and the ion source temperature was set at 250 °C in chemical ionization (CI) and 220 °C in electric ionization (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.

#### STATISTICAL ANALYSIS

Nematode response investigated in the two-choice bioassay chambers was analysed with a two-factor 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.

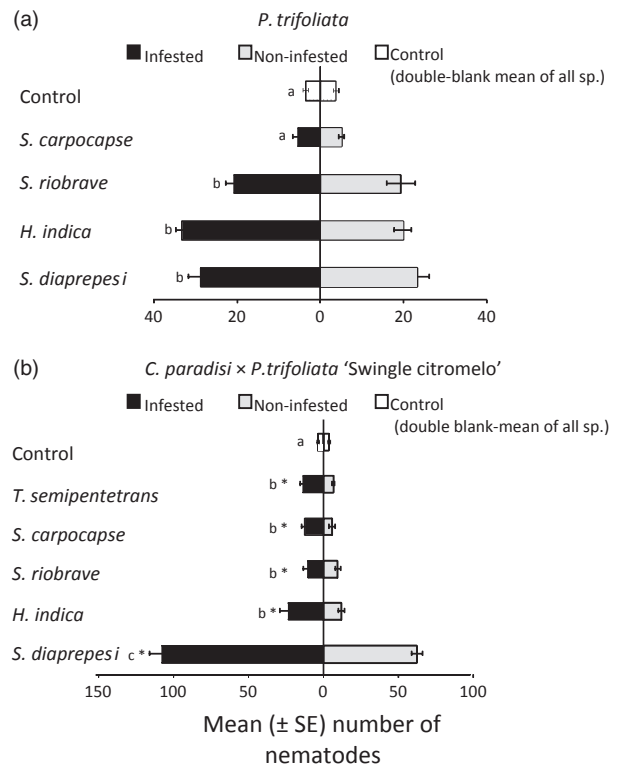
## Results

#### NEMATODE BEHAVIOUR

Entomopathogenic nematodes of all species responded similarly to *D. abbreviatus*-infested versus uninfested (negative control) *P. trifoliata* roots ( $F = 3.0$ , d.f. = 2, 72,  $P = 0.087$ ) (Fig. 2a). However, when *D. abbreviatus*-infested and uninfested *P. trifoliata* roots were tested, most nematode species preferred either root treatment over the blank control ( $F = 35.66$ , d.f. = 2, 129,  $P < 0.001$ ). The only exception was the ambush forager type *S. carpocapsae* ( $P = 0.134$ ) (Fig. 2a). All tested nematode species preferred Swingle plants infested with *D. abbreviatus* larvae over the paired uninfested controls ( $P < 0.001$ ) (Fig. 2b). In addition, movement of *S. diaprepesi* in response to *D. abbreviatus*-infested Swingle rootstocks was significantly greater than that observed for the other nematode species tested ( $P < 0.001$ ) (Fig. 2b).

#### EFFECT OF BELOW- VERSUS ABOVE-GROUND HERBIVORY ON RELEASE OF NEMATODE ATTRACTANTS

Feeding by *D. abbreviatus* larvae on citrus roots induced production of pregeijerene in the subterranean root zone; however, no pregeijerene or related compounds were found in the volatile collections of above-ground shoots in response to larval feeding (Fig. 3a). Conversion of pregeijerene to geijerene was found to be an artefact of heat exposure in a splitless GC injector and thus the total production of pregeijerene in



**Fig. 2.** Responses of *Tylenchulus semipenetrans*, *Steinernema carpocapsae*, *S. riobrave*, *S. diaprepesi* and *Heterorhabditis indica* when presented with: a) volatiles from roots of *Poncirus trifoliata* infested with *Diaprepes abbreviatus* larvae versus volatiles from undamaged *P. trifoliata* roots or b) volatiles from roots of *Citrus paradisi* × *P. trifoliata* (Swingle hybrid) infested with *D. abbreviatus* larvae vs volatiles from undamaged *C. paradisi* × *P. trifoliata* roots in two-choice olfactometer.

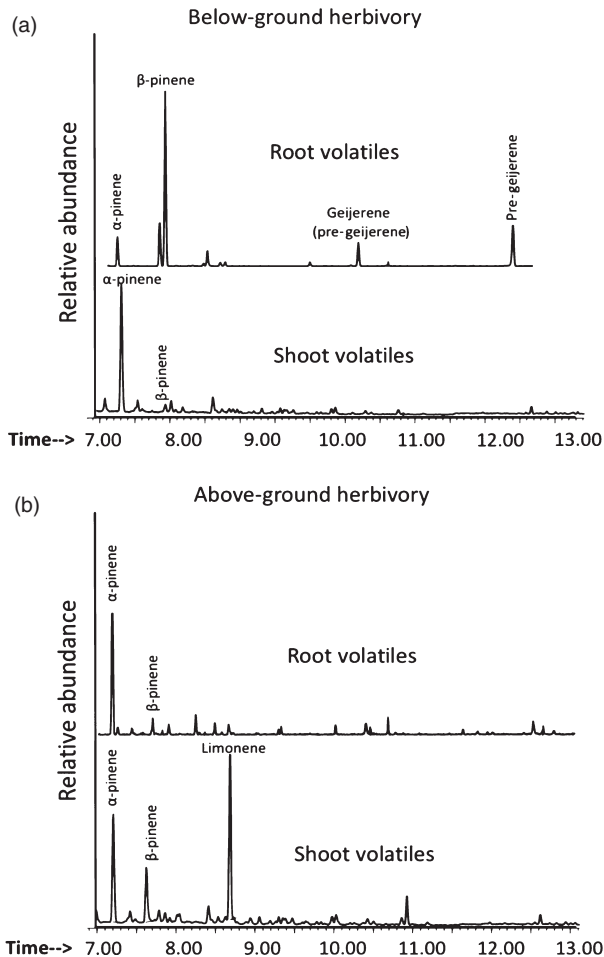
response to herbivory turned out to be the combination of the observed pregeijerene and geijerene peaks (Fig. 3a). These C<sub>12</sub> terpenes are thought to elicit nematode attraction (Ali, Alborn & Stelinski 2010). Adult beetle feeding on above-ground shoots did not induce production of pregeijerene or other volatiles typically released in response to root damage (Fig. 3b); however, release of limonene from above-ground shoots was increased (Fig. 3b).

#### SUBTERRANEAN RELEASE OF VOLATILES BY VARIOUS PLANT SPECIES

Pregeijerene was released constitutively by *P. trifoliata* roots and the release was not affected by larval *D. abbreviatus* feeding (Fig. 4a). In contrast, pregeijerene was released by Swingle roots (Table 2 and Ali, Alborn & Stelinski 2010) and sour orange rootstocks (Fig. 4b) only in response to *D. abbreviatus* larval feeding (Fig. 4b, Table 2).

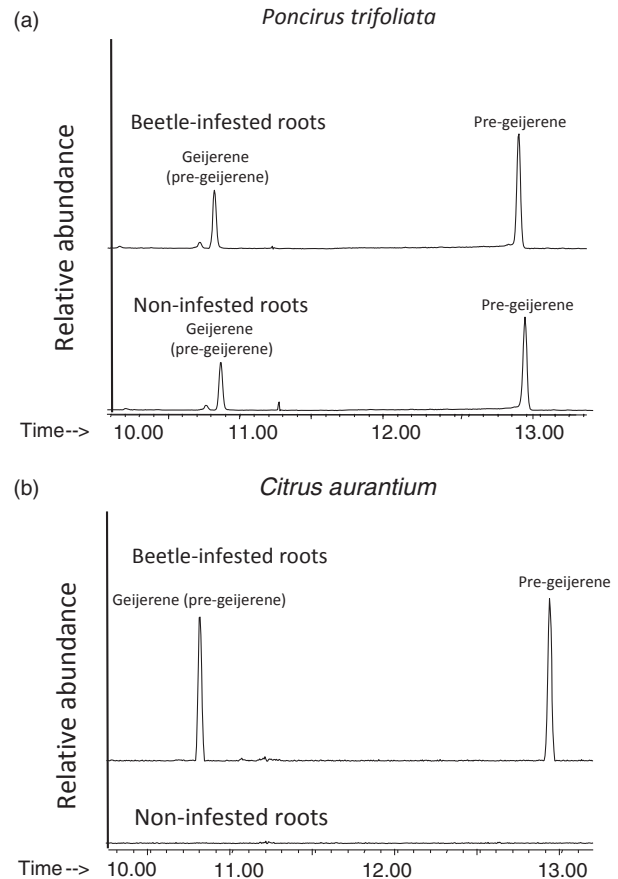
## Discussion

The rhizosphere within which nematodes forage to find resources has been the subject of investigation for several dec-



**Fig. 3.** Example chromatograms depicting volatile profiles from simultaneous collections of root and shoot volatiles of Swingle (*Citrus paradisi* × *Poncirus trifoliata*) in response to (a) below-ground and (b) above-ground herbivory by *Diaprepes abbreviatus* larvae and adults, respectively. All samples were collected for 24 h.

ades. Nematode host-searching behaviour is typically mediated by cues from host(s) or their immediate environment (Lewis *et al.* 2006) that can be either volatile and diffuse through soil or dissolved in and moving through the water film surrounding soil particles. Cues emanating from plant roots, a potential habitat for insect hosts, can also influence the behaviour of EPN nematodes (Bird & Bird 1986; Choo *et al.* 1989; Lei, Rutherford & Webster 1992; van Tol *et al.* 2001; Boff, van Tol & Smits 2002; Neveu *et al.* 2002). In addition to organic compounds, environmental factors such as temperature, substrate vibrations, electric potential, carbon dioxide and various inorganic compounds can mediate the behaviour of nematodes as they search for hosts (Jansson & Nordbringhertz 1979; Torr, Heritage & Wilson 2004). Until recently, little was known about EPN chemotaxis in response to herbivore-induced cues (Rasmann *et al.* 2005; Ali, Alborn & Stelinski 2010; Hiltbold *et al.* 2010). However, herbivore feeding triggers production of EPN-attracting volatiles in annual grasses (Rasmann *et al.* 2005) and recently, we showed that the hybrid rootstock 'Swingle citrumelo' attracts EPNs (*S. diaprepesi*) in response to herbivory by larval *D. abbreviatus*



**Fig. 4.** Example 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 24 h.

*tus* root weevils and that the attraction was due to an induced release of subterranean volatiles (Ali, Alborn & Stelinski 2010). In both cases, the nematode attractants appear to be terpenoids.

We determined that in response to herbivory, the Swingle hybrid, as well as another common non-hybridized species, sour orange (*C. aurantium*), produced pregeijerene, the proposed nematode attractant. Surprisingly, we found that one of the parents of the Swingle hybrid, *P. trifoliata*, attracted nematodes independent of herbivory and that this could be explained by constant release of pregeijerene. Thus, our observations show pregeijerene can be produced constitutively as well as in response to damage among diverse citrus varieties. It is possible that plant breeding to develop the cultivable Swingle hybrid may have created an herbivore-induced response similar to that observed with the non-hybridized sour orange (*C. aurantium*) species by loss of the trait responsible for constant signalling observed in one of its parents. A similar genetic consequence was observed in maize, where a below-ground cue found in wild relatives and European lines was lost during the breeding of North American maize lines (Köllner *et al.* 2008). Currently, we intend to utilize microarray analysis to resolve gene regulation in response to herbivory among these different citrus varieties.

**Table 2.** GC–MS identification of volatiles from various citrus rootstocks

RT	Names	CAS#	Swingle ( <i>Citrus paradisi</i> × <i>Poncirus trifoliata</i> )		Poncirus ( <i>Poncirus trifoliata</i> )		Sour orange ( <i>Citrus aurantium</i> )	
			Infested	Non-infested	Infested	Non-infested	Infested	Non-infested
7.25	A-pinene*†	000080-56-8	+	+	+	+	–	–
7.90	β-pinene*†	000127-91-3	+	+	+	+	–	–
8.69	Limonene*†	000138-86-3	–	–	–	–	–	–
12.94	Geijerene†	006902-73-4	+	–	+	+	+	–
10.81	Pregeijerene†	020082-17-1	+	–	+	+	+	–

\*Synthetic standard comparison.

†Identification was based on comparisons of retention times (RT) with standard and spectral data from Adams, EPA and Nist05 libraries.

Our results indicate that all EPN species tested exhibited attraction to herbivore-induced volatiles irrespective of their foraging strategy (Fig. 3). Specifically, the ‘ambusher’ *S. carpocapsae* (Lewis 2002), the ‘cruiser’ *H. indica* (Lewis 2002), as well as the two species thought to exhibit an intermediate behavioural foraging strategy (Lewis, Gaugler & Harrison 1992; Lewis 2002) were all attracted to *D. abbreviatus*-damaged roots of the Swingle rootstock. Analogously, the Swingle parent line, *P. trifoliata*, also attracted nematodes of all species (except for *S. carpocapsae*, ambusher) independent of damage (Fig. 2a). Thus, these results support the hypothesis that pregeijerene likely explains this attraction. Of the EPN species investigated, *S. diaprepsi* exhibited the greatest behavioural response even though this species is thought to be intermediate on the spectrum between pure ‘ambusher’ versus ‘cruiser’. However, *S. diaprepsi* is a host specialist attacking *D. abbreviatus* weevils (Nguyen & Duncan 2002) and thus it appears that specialization rather than foraging strategy may better explain this EPN’s use of HIPVs for host location.

*Steinernema carpocapsae* (ambusher) is a less effective entomopathogen of *D. abbreviatus* (Schroeder 1994; Bullock, Pelosi & Killer 1999) than *S. riobrave* (intermediate between ambusher and cruiser) (Lewis 2002). It is thought that active movement in search of sedentary hosts as opposed to the ‘sit-and-wait’ strategy may explain this difference (Grewal *et al.* 1994; Lewis, Grewal & Gaugler 1995). Nematode attraction to damaged citrus root chemicals in the current investigation appeared to differ based on foraging strategy. Our results are congruent with the proposed foraging strategy behaviours of the nematode species tested, similarly to that observed for other EPN species (Rasmann & Turlings 2008). The lone ‘pure’ ambushing species investigated (*S. carpocapsae*) did not move in the olfactometer when pregeijerene was ubiquitous and coming from each possible direction of movement (Fig. 2); however, it did respond when the cue was present in only one of the two arms (Fig. 3). In contrast, the cruising and intermediate foraging strategy species always responded to these volatiles, whether they were in one or both arms of the two-choice test chamber (Figs. 2 and 3).

To date, investigations of nematode response to below-ground HIPVs have focused on entomopathogens (Lewis, Gaugler & Harrison 1993; Lewis, Grewal & Gaugler 1995; Rasmann *et al.* 2005; Ali, Alborn & Stelinski 2010; Hiltbold

*et al.* 2010). EPN host finding is mediated by both long-range cues that facilitate finding of the root zone as well as shorter-range cues that facilitate host location within the root zone (Choo & Kaya 1991; Kanagy & Kaya 1996; Hui & Webster 2000; van Tol *et al.* 2001; Rasmann *et al.* 2005). The attraction of plant parasitic nematodes to below-ground HIPVs was hitherto unknown. It is generally accepted that plant roots release various attractants that mediate response by the infective stages of plant-parasitic nematodes (Prot 1980). A variety of physio-chemical gradients exists around physiologically active roots including amino acids, ions, pH, carbon dioxide and sugars (Perry & Aumann 1998). However, little is understood regarding the specific cues that mediate attraction of plant parasitic nematodes to preferred feeding sites. Our 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 feeding sites.

It is puzzling that the parental *P. trifoliata* line of the commercial Swingle rootstock constantly produced and released attractants for beneficial nematodes that also were utilized by plant parasitic nematodes. Selection for an herbivore-induced signalling response should be strongest in the direction toward channelling resources for production of ‘cries for help’ only when necessary because a constant release likely carries a high physiological cost (Zangerl & Rutledge 1996; Agrawal & Karban 1999; Karban *et al.* 1999; Heil 2002; Strauss *et al.* 2002; van Dam 2009). However, constant release of volatiles that attracted EPN species appeared to carry the ecological cost of also attracting a plant pathogenic species. Therefore, it is less surprising that the faster-growing Swingle commercial hybrid only released this cue upon herbivory. However, the apparent correlation between defence and growth rate needs to be carefully tested. The current laboratory-based investigation did not resolve the many potential competitive interactions between beneficial and parasitic nematodes and with their natural enemies that might occur in the field (Jansson & Nordbringhertz 1979). Costs for *P. trifoliata* resistance to *T. semipenetrans* infection require further evaluation. Exploitation of plant volatiles by their parasites may also determine whether the plant’s ‘defence’ is constitutive or induced. *Citrus aurantium* is highly susceptible to *T. semipenetrans* infection.

Therefore an induced response may have been selected for in this species given the associated ecological costs of attracting potential parasites. Costs of defences are well known above ground (Puustinen, Koskela & Mutikainen 2004; Adler & Irwin 2005). Our results are consistent with the notion that defences against diverse enemies may evolve independently but not without associated direct ecological costs in terms of reduced vigour and/or increased susceptibility to different threats and situations (Heil 2002; van Dam & Heil 2011).

Our results suggest that these terpenoid volatiles cannot be easily categorized as synomones (mutually beneficial) as was previously thought (Ali, Alborn & Stelinski 2010). It appears that in citrus, they might function as both kairomones (disadvantageous to its emitter, beneficial to its receiver) and synomones, depending on the trophic context. Resolution of their total impact on plant defence is yet to be determined. 'Nematode attractants' may serve a number of additional functions. Potential antibiotic effects and plant-microbe signalling were not investigated here. Depending on the nematode fauna in a particular location, the beneficial effect of attracting entomopathogens may be negated by concurrent attraction of plant parasites. This complex interaction occurring within the citrus system will need to be investigated in a field setting and also deserves further investigation in other below-ground systems which attempt to categorize plant volatiles. As observed previously, compounds that are characterized for defensive roles can also render plants more attractive to specialist herbivores (Dicke & van Loon 2000; Heil 2008).

Although distinct, the shoots and roots of plants act synergistically using primary resources from both above- and below-ground 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 above-ground mechanisms of plant defence (Zangerl & Bazzaz 1992; Howe & Jander 2008). However, the synergy between below- and above-ground organs associated with plant growth is likely paralleled by interactions that contribute to plant defence (Masters & Brown 1992; Bezemer *et al.* 2004; Bezemer & van Dam 2005; Erb *et al.* 2009). Roots synthesize a number of secondary metabolites that are known leaf defences, 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, Alborn & Stelinski 2010). In the current investigation, we simultaneously sampled volatiles from the above- and below-ground appendages of plants 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 below-ground herbivory by *D. abbreviatus* larvae (Fig. 3a). Neither roots nor foliage released this putative nematode attractant upon above-ground herbivory by adult beetles (Fig. 3b). Although our results indicate that the major constituent of nematode attraction is unique to

the below-ground portions of the plant, it remains possible that correlations exist between above-ground and below-ground herbivory in this system. In the current investigation, we did not address attraction of above-ground natural enemies of *D. abbreviatus* adults in response to below-ground or above-ground herbivory. However, our results suggest an above-ground HIPV release in response to adult beetle feeding (i.e. increased production of limonene from leaves; Fig. 3b), which deserves further investigation.

With respect to the influence of above-ground herbivory on below-ground plant defence, we hypothesized that adult beetle feeding may induce production of an EPN attraction cue 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 attract 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 such a priming hypothesis based on induction of nematode attracting cues as the attractants were only induced by below-ground herbivory. Yet, it is established that other responses in roots could be primed during above-ground herbivory which could facilitate defence (Rasmann & Turlings 2007; Erb *et al.* 2008, 2011; van Dam 2009). It may be possible that defence is augmented via above-ground feeding, either directly by a build-up of defensive compounds in the roots or indirectly by an increased release rate of defensive cues, both of which require further investigation.

We provide evidence that nematode-attracting cues are released by a diversity of citrus species. These cues can be released constantly or only in response to herbivore damage. A diversity of nematode species were attracted to these cues including entomopathogens and plant parasites. It seems that these nematode attractants have less effect on 'ambusher' strategists than 'cruisers', but nematode-host specialization appeared to play a more important role than foraging strategy in terms of efficiency of chemotaxis in response to these cues. The surprisingly similar response of a plant parasitic species to that of several entomopathogens suggests that these cues cannot be easily categorized as either kairomones or synomones. It seems the citrus spp. more vulnerable to phytopathogenic nematodes reduce related costs by emitting nematode attracting volatiles only when it is crucial, that is, when herbivores are feeding. In contrast, non-susceptible species invest more in constitutive defence given the lack of cost associated with attracting pathogens. This hypothesis warrants further investigation, in a context that measures the associated cost of producing this attracting cue.

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