SUBTERRANEAN CHEMICAL ECOLOGY OF TRITROPHIC INTERACTIONS: CITRUS ROOTS, ROOTS WEEVILS AND ENTOMOPATHOGENIC NEMATODES

By

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To the Monkey, the Tiger, the Rabbit, the Cock, and the Cub

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Oh yes, the Beast, the Uncooked Chicken and the Damaged Good… My brothers.

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In response to herbivore feeding, plants release odors that benefit them by attracting natural enemies of herbivorous insects. Such interactions have been thoroughly examined aboveground. It has become increasingly evident that similar interactions occur belowground. The root-weevil (*Diaprepes abbreviatus*) is a serious citrus pest. Entomopathogenic nematodes (EPNs) have varying, and unpredictable, efficacy in controlling the weevil. Interactions between the plant, insect and nematode are poorly understood. In root-zone bioassays, root-weevil infested rootstock (Swingle) recruited significantly more EPNs than non-infested or mechanically damaged roots, or larvae alone. GC-MS analysis detected unique volatiles released from roots in response to weevil feeding. We compared attraction to volatiles of infested and non-infested roots from the hybrid, Swingle rootstock, and a parent line of the hybrid, *P. trifoliata* (Pt). Volatiles from Swingle infested by weevils were more attractive to both EPNs and plant parasitic nematodes than non-infested roots irrespective of foraging strategy. Pt, attracted EPN species irrespective of insect herbivory. Analysis of root volatiles revealed that Pt released the attractive cue constitutively, regardless of weevil feeding. A different non-hybrid species (*C. aurantium*) released the attractive cue only in

response to larval feeding. Pregeijerene (1, 5-dimethylcyclodeca-1, 5, 7-triene) was identified as the major constituent of EPN attraction released from weevil-damaged roots. The release of pregeijerene by citrus roots peaked 9-12 hr after initiation of larval root feeding. Volatile collections from above/belowground portions of citrus plants revealed that aboveground adult feeding does not induce production of pregeijerene analogous to that induced by root damage nor does damage by larvae belowground induce a similar cue aboveground. Through the development of novel *in-situ* volatile sampling methods, pregeijerene release was detected from roots of mature trees in the field. In field experiments, lab-collected citrus volatiles from infested roots and isolated pregeijerene increased mortality of beetle larvae compared with controls. Using species-specific probes designed to identify EPN species, we determined by quantitative real-time PCR that field application of pregeijerene increased pest mortality by attracting four species of EPNs native to Florida. This and similar chemicals may have broad application for controlling agriculturally significant root pests.

CHAPTER 1
INTRODUCTION

Plants and Insect Herbivores

Autotrophic green plants provide virtually all of the total energy available to terrestrial organisms. Plants have been in engaged in an ‘arms race’ with herbivores over millions of years of evolution and have developed defenses that protect them from herbivory. This coevolutionary process has led to the development of tremendous biodiversity, which is highly evident in insects. Concurrent selection pressures have simultaneously pushed the evolution of resistance traits in plants and traits in insect herbivores to overcome plant defenses.

Plants have a variety of defensive strategies against insects. Chemical, physical, and biotic defenses can reduce herbivory and increase plant fitness. Physical features on the tissues of plants can drastically influence herbivore acceptance of host plants. The presence of trichomes and wax crystal structures on the plant surface, leaf thickness and toughness, sclerotization and high silica content may cause avoidance behavior. Plants may also store toxic or repellent compounds in their leaf tissues. These are all forms of constitutive defense (Karban & Baldwin 1997). Plants also produce toxic or repellent compounds only in response to insect damage, and this process is termed induced defense (Karban & Baldwin 1997). Most plants display multiple defenses, which vary in intensity and effectiveness, and can operate over different temporal and spatial scales against different attackers. These defenses can be classified as direct, when exerting a negative impact on herbivores, or indirect, when manipulating of organisms in higher trophic levels to negatively impact the herbivore.

Direct defenses may prevent herbivores from feeding due to physical (spines, thorns trichomes, and waxes), or chemical defenses (secondary plant metabolites, phenylpropanoids, terpinoids, alkaloids, and proteinase inhibitors) (Karban & Baldwin 1997). Indirect defenses are adaptations that result in the recruitment and/or preservation of organisms that protect plants against herbivores (Karban & Baldwin 1997). These can range from constitutive formation of domatia, which serve as domatia for beneficial organisms such as ants, mites, and even bacteria to the production of foliar nectaries and nutritive structures that can also be used by natural enemies of herbivores (Boethel & Eikenbary 1986; Whitman 1988). Plant indirect defenses can also be induced. During the last two decades, it has been revealed that plants respond to herbivore feeding by producing and releasing odors (herbivore induced plant volatiles or HIPVs) that are exploited by natural enemies that use these cues to locate their prey and hosts (Turlings & Wäckers 2004; Dicke & Vet 1999; Dicke *et al.* 2003).

Herbivore Induced Plant Volatiles

HIPVs are known to play various important roles in plant- arthropod interactions, in addition to natural enemy recruitment. For example they are known to deter ovipositon by Lepidoptera (Landolt 1993). There is also mounting evidence that HIPVs are involved in plant-plant communication (Engelberth *et al.* 2004; Arimura *et al*. 2000; Kessler & Baldwin 2001; Baldwin *et al.* 2002).

The composition of HIPVs is known for many plant-herbivore systems (Pare & Tumlinson 1999). Some HIPVs are taxon specific, such as glucosinolate breakdown products in Brassica species (Mattiacci *et al*. 1995), whereas others appear to be common to many different plant families (Boom *et al*. 2004). These compounds include six-carbon (C6)-volatiles or “green leaf volatiles”, generally released by plant leaves

immediately after wounding. These include isomers of hexanol, hexanal, and hexenyl actetate (Hatanaka 1993). In general, green leaf volatiles are present directly after wounding with (Z)-3-hexenyl acetate as an exception (Matsui *et al*. 2000), and they may be involved in triggering terpenoid production (Farag & Pare 2002), causing the accumulation of jasmonic acid (JA) as well as the expression of defense genes (Bate & Rothestein 1998; Engelberth *et al*. 2004). It has also been suggested that C6- volatiles play a direct role in plant defense, in addition to a possible antimicrobial function. For example C6-aldehydes and –alcohols reduce tobacco aphid fecundity (Hildebrand *et al*. 1993). In addition some C6-compounds may function as indirect defenses (Kessler & Baldwin 2001; D'Alessandro & Turlings 2005) or play a role in signaling within or between plants that results in up-regulation of genes associated with defense (Arimura *et al.* 2001). In contrast to C6- aldehydes and –alcohols, the emission of (Z)-3-hexenyl acetate can be observed a few hours after feeding or mechanical damage suggesting a similar signaling pathway as other herbivore induced terpinoids (Turlings *et al.* 1995; Arimura *et al*. 2001).

Herbivore induced leaf volatiles also include terpenoids, encompassing monoterpenes (C10), sesquiterpenes (C15) and homoterpenes (C11 or C16). All terpenoids are synthesized through the condensation of isopentyl diphosphate and its allylic isomer dimethylallyl diphosphate in either the cytosol or the plastids (Pare & Tumlinson 1999; Arimura *et al.* 2005). Indole is a common and dominating nitrogenous compound found in HIPVs, derived from the Shikemate acid pathway (Frey *et al.* 2000).

Continuous mechanical damage of plant tissues can result in the emission of volatile blends resembling those occurring after herbivore damage (Mithofer *et al.*

2005), but commonly the emission of these volatiles can be enhanced and prolonged by eliciting factors from a feeding insect. These factors also elicit odor emission when they are taken up via the stem of the plant or even via the petiole of a leaf; the response to their elicitors has been shown to be systemic (Dicke *et al.*1990, Turlings *et al.*1993). Plant defense responses have been ascribed to a wide variety of chemical elicitors that activate specific downstream signal transduction pathways (Pare *et al.* 2005). Two major classes of insect derived elicitors are the Beta-glucosidase, discovered in regurgitant of *Pieris brassicae* larvae, which facilitates the emission of glucosinolate breakdown products (Mattiacci *et al*. 1995); and the fatty acid derivative volicitin and related compounds that induce the release of the full blend of volatiles normally induced by caterpillar feeding (Alborn *et al.*1997).

The wide variety of elicitors is often the result of slight changes to chemical precursors, which can have strong effects on the volatile blend emitted from the plant (e.g. De Moraes *et al.* 2001, Kessler & Baldwin 2001, van Poecke & Dicke 2004). Moreover, biosynthesis and release of HIPVs can be affected by biotic factors such as plant hormones (Farmer 2001; Thaler *et al.* 2002), microorganisms (Piel *et al*. 1997; Cardoza *et al.* 2002), and abiotic factors such as temperature, light (Takabayashi *et al*. 1994, Gouingguene & Turlings 2002), or O_3 and CO_2 (Vuorinen *et al.* 2004).

Although the series of specific defense responses that are activated depends on the precise plant-herbivore interaction, several common global responses have emerged. Herbivore feeding usually triggers defense responses mediated by ethylene and jasmonic acid that act synergistically (Kahl *et al*. 2000; Schmelz *et al.* 2003), whereas pathogen attack typically elevates salicylic acid levels in a plant (Vranova *et al.*

2002). On the other hand, it seems that plant response signals can be highly variable depending on plant genotype (Takabayashi *et al*. 1991; Loughrin *et al.* 1995; Gouinguene *et al.* 2001), plant parts (Turlings *et al.* 1993), or growth stages of a plant (Gouinguene *et al*. 2001). Plants are additionally capable of responding differentially to specific herbivores (De Moraes *et al.*1998; Turlings *et al*. 1998), and to different life stages of the same herbivore (Takabayashi *et al.*1995).

With respect to research on HIPVs and their interactions with herbivores, substantial focus has been given to the aboveground parts of plants and only recently have interactions investigated belowground (von Tol *et al.* 2001; Rasmann *et al.* 2005; this dissertation). Van Tol *et al*. (2001) showed that plants recruit entomopahtogenic nematodes to their herbivore-damaged roots. Furthermore, maize roots infested with larvae of the Western corn rootworm (*Diabrotica virgifera*) production (*E*)-β-caryophyllene, which attracts entomopathogenic nematodes (Rasmann *et al*. 2005). Spiking soil with synthetic (*E*)-β-caryophyllene decreases the emergence of adult corn root worms from maize by half compared with untreated control plots due to enhanced nematode attraction (Rasmann *et al*. 2005).

More recently research has acknowledged that plants mediate interactions between two communities, e.i. those found above or below-ground (van Dam & Heil 2011, Erb *et al.* 2011). These interactions are highly diverse, and becoming an important aspect of investigating plant defense.

The Model System

Diaprepes abbreviatus (Linnaeus) (Coleoptera: Curculionidae), (Figure 1-1) was first introduced into Florida in 1964 (Beavers & Selhime 1975). Over the past 40 plus years, it has significantly contributed to the spread of disease and damage to citrus,

ornamental plants, and other crops. *D. abbreviatus* is a native economic pest of the Caribbean where at least 19 additional species are known within the genus (Wolcott 1936). *Diaprepes abbreviatus* has spread over a large area of central and southern Florida where it causes approximately $70 million in damage annually (Weissling *et al*. 2002; Lapointe 2000). The initial area of infestation was an estimated 6,500 acres in Apopka, FL and has now increased to an estimated 164,000 acres over 20 counties in central and southern Florida (Weissling *et al*. 2002).

Diaprepes abbreviatus has a wide host range, attacking approximately 293 different plant species including citrus, sugarcane, vegetables, potatoes, strawberries, woody fieldgrown ornamentals, sweet potatoes, papaya, guava, mahogany, containerized ornamentals, and non-cultivated wild plants (Simpson *et al.* 1996, 2000). *Diaprepes abbreviatus* damage to the vegetative portion of plants is most often seen as notching on the margins of young leaves (Fennah 1940) (Figure 1-2). This is a key trait characterizing *D. abbreviatus* infestation. Adults continue to feed on foliage and lay eggs between older leaves (Schroeder 1992; Fennah 1940). However, the greatest damage is caused by larvae feeding below ground. Upon hatching, the larvae fall to the soil and make their way to the roots of plants where later instars feed and develop (Schroeder 1992). This feeding can girdle the taproot causing damage that disables the plant from taking up water and nutrients resulting in plant death (Schroeder 1992). This type of damage also facilitates secondary infections by *Phythophora* oomycete species (Graham *et al*. 1996). Young hosts can be killed by a single larva while several larvae can result in serious decline of older, established hosts (Weissling *et al*. 2002). Since

larvae develop below ground, it is difficult to detect them before decline of above ground vegetation of the host is observed.

Current chemical control of *D. abbreviatus* includes foliar insecticides (Bullock *et al.* 1988), ovicides and oil sprays (Schroeder 1996) to reduce adult feeding, oviposition, and viable egg production. Soil applied insecticides like Brigade WSB and Capture 2EC are used as a soil barrier to decrease larval entry (Knapp 1999). Foliar chemical spray applications such as Danitol 2.4EC, Imidan 70WP, Kryocide 96 WP, and Micromite 80WGS are most effective during peak seasonal *D. abbreviatus* abundance.

Chemical controls are less effective than earlier available treatments comprised of the now banned organochlorine soil pesticides (Duncan *et al.* 1999; McCoy 1999). The most effective method for controlling the more damaging mid to late instars found on roots appears to be entomopathogenic nematodes, which are roundworms from the genera *Heterorhabditis* or *Steinernema*. They are obligate parasites that kill their host with the aid of a symbiotic bacterium (Poinar 1990). Native and introduced entomopathogenic nematodes are infectious to all larval stages and possibly adults (Adair 1994; Schroeder 1990). Releases of mass-produced entomopathogenic nematodes (EPNs) have been used by citrus growers for over 20 years (Duncan *et al.*1999). It has also been shown that use of EPNs can reduce larval populations of *D. abbreviatus* (Shroeder 1990; Downing *et al.* 1991; Schroeder 1992; Duncan *et al.* 1999; Bullock *et al.*1999) and thus resulting adult populations (Bullock *et al.* 1999, Duncan *et al.* 2007). However, improvement of the efficacy of EPN treatment is still desired. Presently *D. abbreviates* control using EPNs has been inconsistent and dependent on nematode species and soil composition (Adair 1994; Duncan *et al.* 1999). One

approach to enhance the effectiveness of EPNs and control of *D. abbreviatus*, may be to exploit plants' naturally produced chemical defenses. Exploiting herbivore induced plant volatile emissions may represent a new approaches in integrated pest management (IPM). Plants benefit by releasing HIPVs when they recruit natural enemies of subterranean herbivores (van Tol *et al.* 2001; Neveu *et al.* 2002; Aratchige *et al.* 2004). For example, entomopathogenic nematodes are attracted to exudates of Thuja plants (*Thuja occidentalis*) infested with larvae of the vine weevil (van Tol *et al.* 2001). Furthermore, maize roots infested with larvae of the Western corn root worm (*Diabrotica virgifera*) release (*E*)-β-caryophyllene, which attracts entomopathogenic nematodes (Rasmann *et al*. 2005). Spiking soil with synthetic (*E*)-β-caryophyllene decreases the emergence of adult corn root worms from maize by half compared with untreated control plots (Rasmann *et al*. 2005). Identification of the signals that mediate the interactions between *D. abbreviatus,* infested plants and entomopathogenic nematodes could advance understand of this relationship. Determining whether citrus releases specific chemicals that recruit entomopathogenic nematodes upon weevil damage may improve the efficacy of these biological control agents. Following identification, application of such chemicals to the soil may attract naturally-occurring nematodes as well as improve the host-finding capability of exogenously-applied nematodes leading to substantial improvement in the efficacy of this biological control tactic.

Biology and natural history of *Diaprepes abbreviatus*

The root weevil, *Diaprepes abbreviatus*, ranges from 3/8'' to 3/4'' in size (Wolcott, 1936). It has various color morphs that differ in hues of yellow, gray, orange and black (Lapointe USDA 2000). The larvae are white, legless and grow to about 1 inch in length

(Figure 1-3). It is native to the Caribbean region. *Diaprepes abbreviatus* became a significant pest in the early 1900s despite 500 years of cultivation of the beetle's host crops such as sugar cane (Lapointe USDA 2000). Increased incidence of *D. abbreviatus* damage may be correlated with the introduction of the mongoose as a biological control agent for rats. The mongoose failed to control the rats but successfully killed off populations of many bird and lizard species that preyed on *D. abbreviatus* (Watson 1903). *D. abbreviatus* was considered a significant pest of sugar cane in Barbados by 1921 (Bourne 1921). In 1964, it was introduced into Florida in an ornamental shipment from Puerto Rico (Woodruff 1968). It has since spread throughout Florida and may still threaten other states. *D. abbreviatus* became established in citrus groves in the Rio Grande Valley of Texas as of 2000 (Skaria & French 2001). Since 1974, *D. abbreviatus* infestation had threatened California, which is a major producer of citrus and other host plants of this polyphagous pest and has since been found in agricultural areas of California (Grafton-Cardwell *et al.* 2004).

Although adults may emerge year round, there are two peak emergence periods. The first occurs during the spring from May to June. The second peak emergence is in the fall from August to September (Duncan *et al.* 2001). Mating and egg laying occur throughout both of these periods. A single female can lay up to 5,000 eggs during her 3-4 month life span (Wolcott 1936). The eggs are laid between leaves and typically hatch within 7-10 days. The larvae will fall onto the ground and make their way into the soil to the fibrous roots of host plants where they feed until pupation begins. The period of larval to adult emergence varies from several months to more than a year (Wolcott 1936).

There are two main features of *D. abbreviatus'* life cycle that have made it difficult to control as a pest of cultivated crops. First, its life stages are active in the field throughout the majority of the annual season. Second, adults and larvae occupy separate habitats (above and below ground); therefore, each life stage must be targeted separately (Georgis *et al.* 2005). Given that adults continuously emerge from soil to produce offspring, which in turn return to the soil, control methods that target only adults or larvae will only sporadically reduce the pest population density. Because persistent insecticides (e.g., dieldrin and chlordane) are no longer available, a combination of non-persistent tactics timed to kill both life phases of the population is a strategy often used by growers (Georgis *et al.* 2005). Growers have widely adopted the use of commercially formulated entomopathogenic nematodes since they became available in 1990 to manage the soil stages of the weevil (Bullock *et al.* 1999; Schroeder 1992).

Entomopathogenic nematodes of *D. abbreviatus*

Two families of nematodes are commonly used as biological control agents: Steinermenatidae and Heterorhabditidae. These families vector a symbiotic bacterium into the body cavities of insects. The life cycle of entomopathogenic nematodes consists of these major steps: 1) penetration into the body cavity of the potential host, 2) release of bacteria, 3) development of mature adults, 4) mating and reproduction of injective juveniles, and 5) emergence of infective juveniles in search of a new host (Figure 1-4). The infective juvenile is a third-stage juvenile and is morphologically and physiologically adapted to remain for extended periods without ingesting food (Poinar 1990). Infection with entomopathogenic nematodes can result in death of their insect host within 48 hr.

Entomopathogenic nematodes have been investigated and implemented for management of *D. abbreviatus* larvae in Florida citrus for almost two decades. Early

invesitgations focused on *Steinernema glaseri*, *S. carpocapsae*, and *H. bacteriophora* for control of the weevil (Bullock & Miller 1994; Downing *et al.*1991; Schroeder 1992). Current formulations containing *S. riobrave* have become adopted commercially for *D. abbreviatus* management. Of the several species evaluated in laboratory bioassays and greenhouse trails, *S. riobrave* and a Florida isolate of *H. indica* were the most effective against the Diaprepes root weevil, and reproduction by *H. indica* in the weevil exceeded that of other species (Shapiro-Ilan & McCoy 2000a; Shapiro-Ilan & McCoy 2000b). *S. riobrave* is currently the only nematode species commercially marketed for the Florida citrus industry. *H. indica* (no longer available) was formulated as a paste and *S. riobrave* can be obtained in water dispersible granular formulations. In 1999, approximately 20% of the hectares infested with this weevil were treated with nematodes (Shapiro-Ilan *et al.* 2002). Given that reported efficacy of entomopathogenic nematodes ranges from 0% to >90% suppression (Adair 1994; Bullock *et al.* 1999; Duncan *et al.* 1999; McCoy *et al.* 2000) improved efficacy of this tactic is desired.

One potential means by which to improve the efficacy of EPNs is by gaining a better understanding of their foraging strategies in order to more effectively exploit nematode behavior. Often, nematode species can be categorized according to their foraging behavior. Ambush (sit and wait) and cruise (wide search) strategies, are generally considered as the dipoles of a continuum of saltatory search strategies (Lewis *et al*. 1992,1993; Campbell & Gaugler 1997; Grewal *et al*. 1996). Cruisers allocate more of their time to scanning for resource-associated cues as they move through the environment, and exhibit only brief pauses, and are therefore more effective at finding sedentary and cryptic hosts. Ambush foragers scan during long pauses and allocate

less time to moving through their environment. They wait for resources to come to them, making ambushers effective at finding resources with high mobility. It is important to consider these alternative foraging strategies, using a comparative approach when investigating the use of HIPVs to enhance biological control.

Objectives

Assess behavioral responses of entomopathogenic nematodes to Diaprepes-infested plants: Quantify EPN response to weevil-damaged, mechanically damaged versus undamaged plants, or weevils alone.

Identify plant-released chemicals that recruit entomopathogenic nematodes to Diaprepes-infested plants: Determine attractiveness of HIPVs to various EPN species.

Evaluate the relative efficacy of recruitment chemical(s) for improving biological control of *D. abbreviatus* in the field: Test whether HIPVs to recruit EPN to caged *D. abbreviatus* in a citrus grove.

Research Questions

The present dissertation addresses the following questions:

Do citrus roots that are attacked by larvae of the citrus root weevil produce induced volatiles that attract entomopathogenic nematodes? It has been demonstrated that aboveground, plant-produced organic volatile compounds induced by the feeding of folivores can cause the attraction of their natural enemies such as parasitoids (Turlings & Wäckers 2004). Recently the focus has gone belowground (van Tol *et al.* 2001; Rasmann *et al.* 2005). The aim of the study presented in Chapter 2 was to assess if *Diaprepes abbreviatus* infested roots produced compounds that could attract the entomopathogenic nematode, *Steinernema diaprepesi*. The chapter also

introduces a novel method for *in situ* volatile collection from roots and the bioassay of EPNs to these cues.

Does release of HIPV nematode attractant from citrus roots vary depending on citrus variety? Does response of nematodes vary depending on species, foraging strategy and trophic level? Although recent work has shown that EPNs can respond to cues emitted from roots of plants while fed-upon by their roots herbivores (Rasmann *et al.* 2005; Ali *et al.* 2010), little is known about the variation in release amongst citrus roots and variation in between response of various nematode species to these cues. Chapter 3 presents a study that evaluated various rootstock cues and responses of various nematode species, both entomopathogenic and plant parasitic, to these cues. The chapter demonstrates broad attraction of HIPVs to both plant parasitic and entomopathogenic nematodes, as well as demonstrating variation in responses to these cues based on nematode foraging strategy.

Can this cue be used to manipulate entomopathogenic nematodes in the field to increase larval mortality? Although many plants have been shown to release volatiles that attract natural enemies of their herbivores (Turling & Wäckers 2004), few studies have been able to translate these basic findings into practical field application (De Moraes *et al.* 1998;Thaler 1999; Johnson 2004). Only one study has evaluated this interaction belowground (Rasmann *et al*. 2005). In Chapter 4 isolated and purified pregeijerene was evaluated in a field trials to determine if this HIPV could increase larval mortality by attracting various species of EPNs. Moreover this study presents a novel approach to the quantification of naturally occurring EPN species that were attracted by deployment of HIPVs.

Figure 1-1. *Diaprepes abbreviatus* resting on citrus leaf. Photograph by Peggy Greb USDA-ARS 2010.

Figure 1-2. *Diaprepes abbreviatus* adults damage to citrus leaves (notching) Photograph by Jared Gregory Ali 2008.

Figure 1-3. Young (right) and older larvae (left) of the Diaprepes root weevil on cakes of an artificial diet developed by ARS. Photograph by Peggy Greb USDA-ARS.

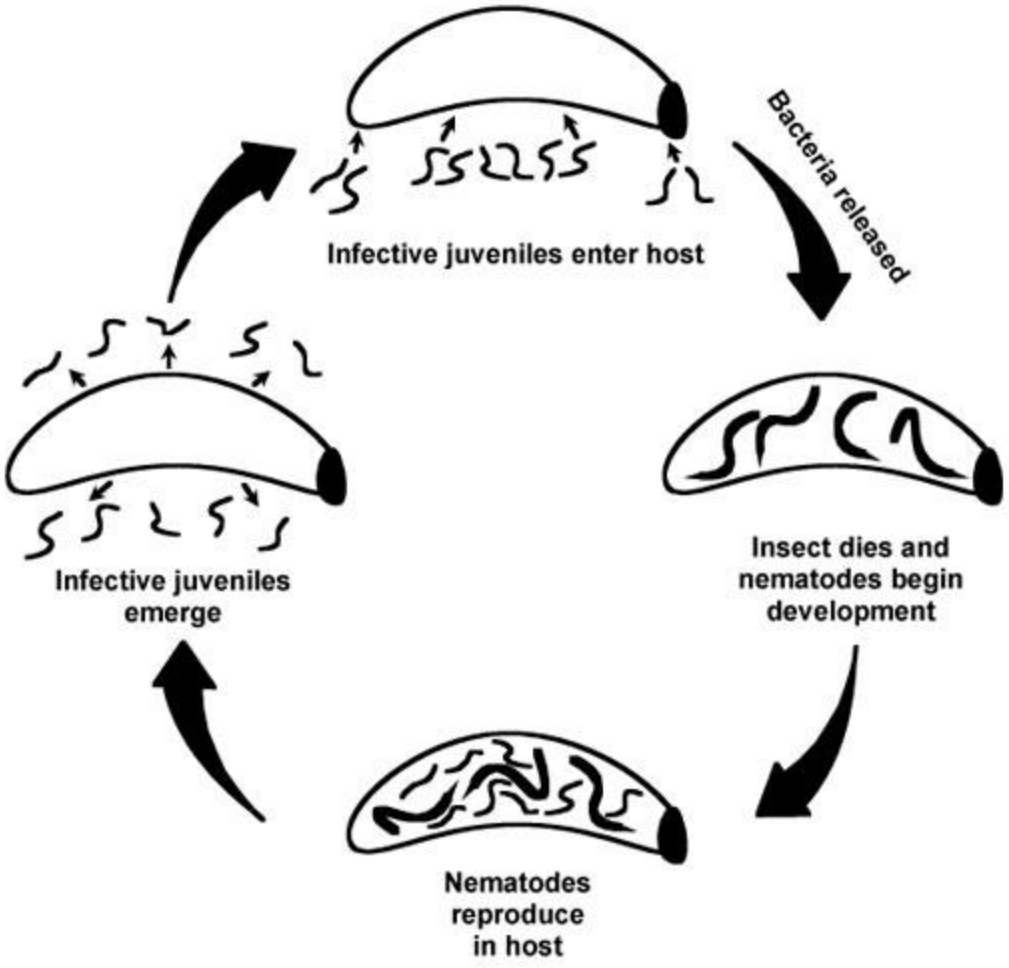


Figure 1-4. A generalized depiction entomopathogenic nematode life cycle. Diagram by David I. Shaprio-IIan, USDA-ARS, SEFTNRL, Byron,GA and Randy Gaugler, Department of Entomology, Rutgers University, New Brunswick, NJ. (From: Nematodes (Rhabditida: Steinernematidae &Heterorhabditidae. http://www.biocontrol.entomology.cornell.edu/pathogens/nematodes.html)

CHAPTER 2
SUBTERRANEAN HERBIVORE-INDUCED VOLATILES RELEASED BY CITRUS ROOTS UPON FEEDING BY DIAPREPES ABBREVIATUS RECRUIT ENTOMOPATHOGENIC NEMATODES

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 & Gershenzon 2002). Less acknowledged is the ability of a plant to manipulate the behavior of organisms to serve defensive roles (Turlings & Wäckers 2004). However, examples of such tritrophic interactions, between plants, herbivores, and natural enemies are quite common (Agrawal & Rutter 1998; Agrawal & Karban 1999; Baldwin & 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 & Dicke 1996). Oviposition can also stimulate plant exudates that are attractive to egg parasitoids; the legume, *Vicia faba* emits volatiles which 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, the plant volatile methyl salicylate attracts herbivore predators (e.g. De Boer & Dicke 2004). Volicitin, found in oral secretions of caterpillars (*Spodoptera exigua*), has been well characterized and shown to induce 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 & 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 antiherbivore substances (Bais *et al*. 2006; Tumlinson *et al*. 1992, 1999; Neveu *et al*. 2002). Plants can also benefit by releasing herbivore-induced volatile emissions that recruit natural enemies of subterranean herbivores, as recently shown by van Tol *et al*. (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). *D. abbreviatus* was first introduced into Florida in 1964 (Beavers & 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). 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 *et al*. 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 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 & 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 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 are 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 hr 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 the first experiment, we tested nematode response to weevil-infested plants versus non-infested controls. Infested plants were subjected to three days of feeding by 3^{rd}-6^{th} 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. IJ 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 a 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) 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 hr. All other experimental and sampling procedures were identical to those described for Experiment 1.

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 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 that 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 three days as previously described. Thereafter, volatiles were collected from chambers for 24 hr 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 days. 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.

To quantify EPN response to the root extracts collected, a two choice sand-filled olfactometer was used (Figure 2-1). The olfactometer consists of three detachable sections: two opposing glass jars (Figure 2-1A) (16 mL BTL, sample type 111, CLR, SNAPC, Wheaton, Millville, NJ), which contained treatments and a central connecting tube 3cm in length (Blue Max^{tm} 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 (Figure 2-1B). 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 (Figure 2-1C) 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 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) (Figure 2-2A). Infested roots attracted significantly more EPNs per arm than those containing larvae alone (F=13.78, df =1, 58, P<0.001) (Figure 2-2B). 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) (Figure 2-2C). There was no significant attraction to mechanically damaged roots as compared with the sand control (P=0.34) (Figure 2-2C).

GC-MS Analysis

Both α-pinene and β-pinene were identified in non-infested and infested plant roots by GC-MS (Table 2-1). *D. abbreviatus*-infested roots released four additional unique compounds that were not present in non-infested roots (Table 2-1). Two sesquiterpenes were the most abundant and were consistently present in infested roots.

These were geijerene and its precursor pregeijerene (Figure 2-3). 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 2-1).

EPN Response to Roots Extracts

Significantly more EPNs were found in arms containing solvent extracts of *D. abbreviatus*-infested roots than non-infested roots (P=0.03) (Figure 2-4).

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 (Duncan *et al*. 2007; Jaffee & Strong 2005). Carbon dioxide has long been known to attract nematodes to plant roots (Prot & Van Gundy 1981; Gaugler *et al*. 1980). However, functioning alone, such an ambiguous signal might not allow efficient host location by EPNs. Van Tol *et al*. (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 sesquiterpenes 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 & Devi 2007). Geijerenes have also 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 (Turlings & Ton 2006; Degenhardt *et al*. 2003, 2009). 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 to release (*E*)-β-caryophyllene to recruit EPNs and protect roots from herbivore damage (Degenhardt *et al*. 2003, 2009; Hiltpold *et al*. 2010). The currently investigated citrus rootstock system is very 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 & 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 will also 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. Furthermore, we plan 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 initially investigated 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 & 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 also 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 badly represent 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).

Table 2-1. GC-MS identification of volatiles from Swingle citrumelo rootstock *(Citrus paradise × Poncirus trifoliate*

				Infested root	Non-infested root	Larvae only
Peak #	RT	Name	CAS#	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	+	-	-

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

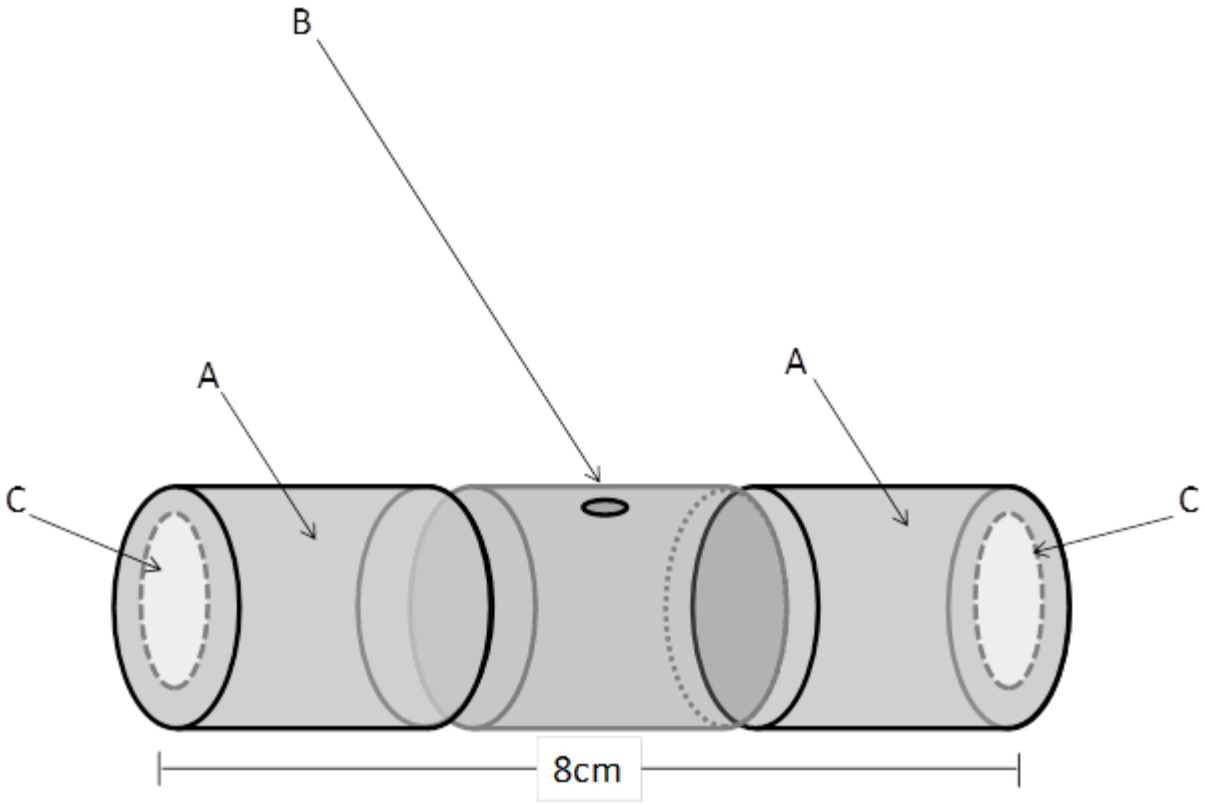


Figure 2-1. Schematic diagram of sand column assay unit. Glass jar (17 ml) with samples at base (A), connecting tube (3 cm) with hole for nematode application (B), extracts placed on filter paper (C), arena was filled with heat sterilized sand at 10% moisture for all assays.

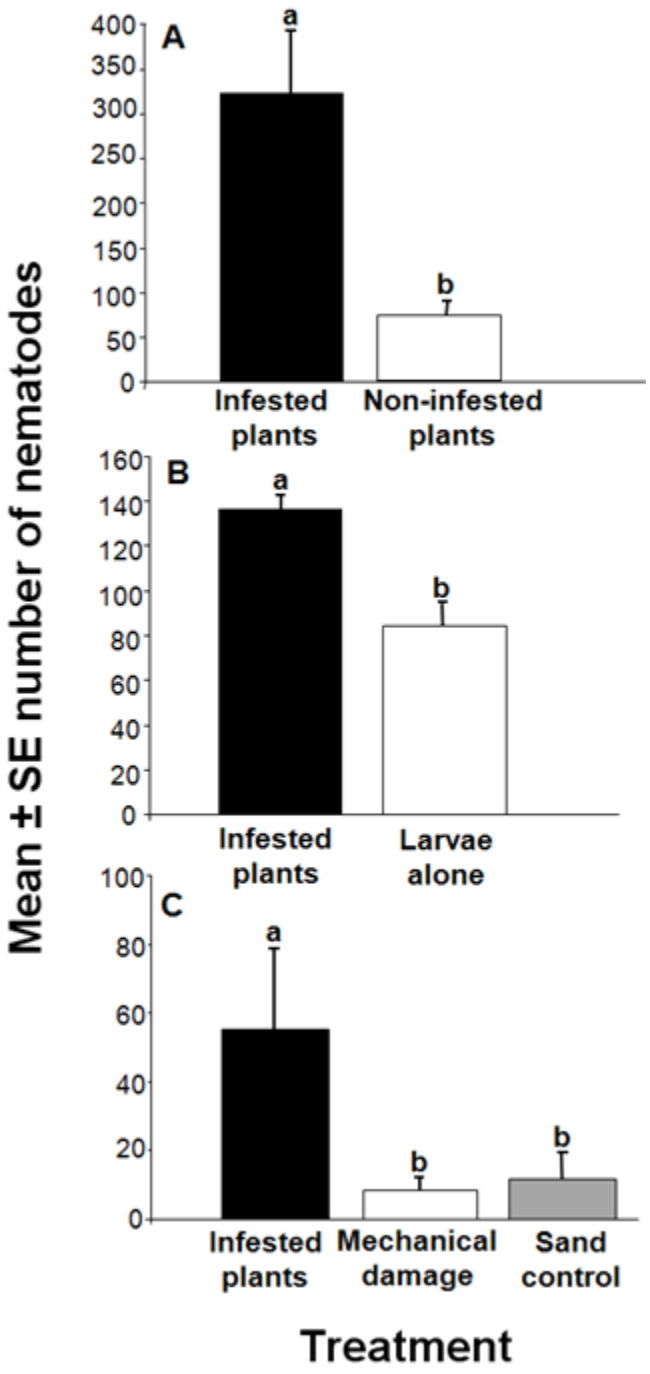


Figure 2-2. Mean number of *S. diaprepesi* attracted to chambers containing weevil-infested plants versus non-infested control plants (A), weevil-infested plants versus larvae alone (B), weevil-infested plants, mechanically damaged plants or sand control (C). Each panel represents a separate experiment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

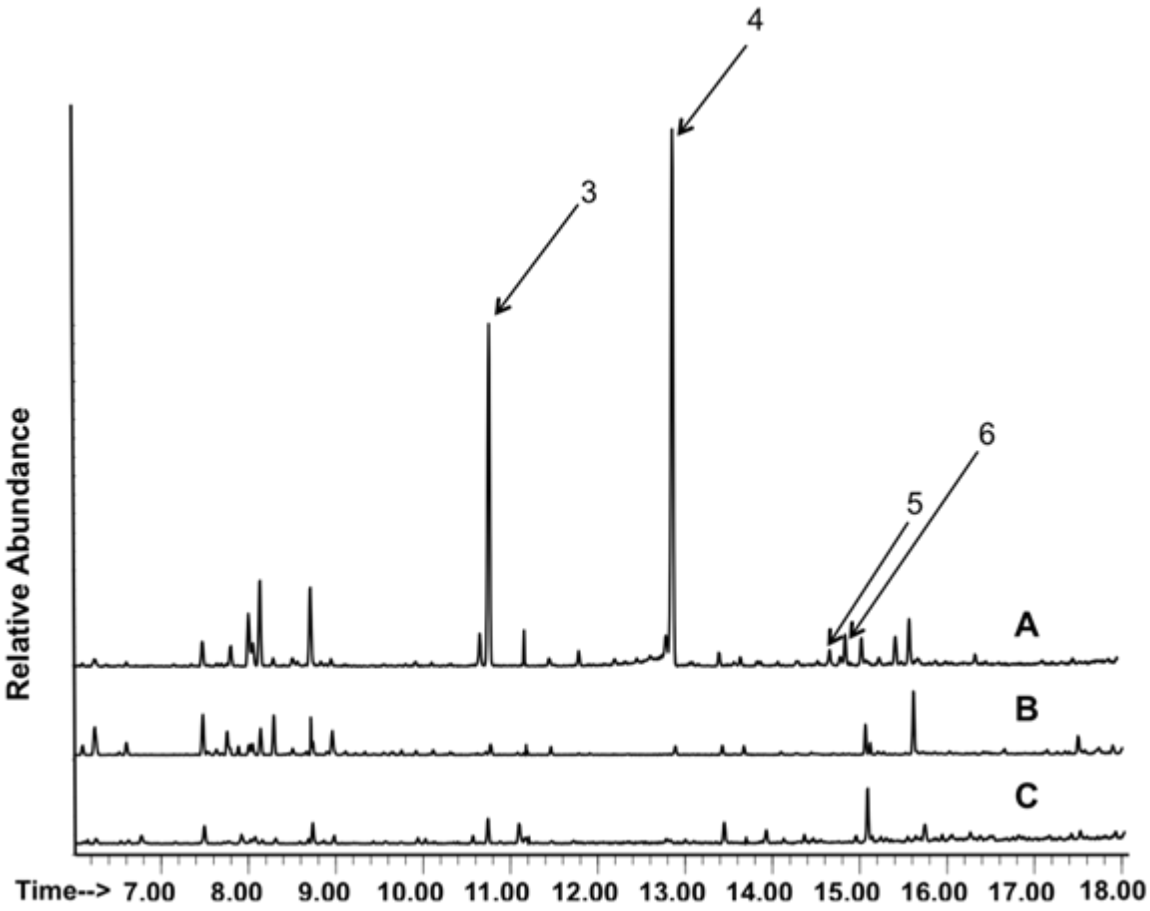


Figure 2-3. Example chromatograms showing volatile profiles of *D. abbreviatus*-infested plants, non-infested plants and larvae alone. Volatile profile of infested *Citrus paradise* × *Poncirus trifoliate* rootstock (A) Volatile profile of non-infested *Citrus paradise* × *Poncirus trifoliate* rootstock (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 2-1).

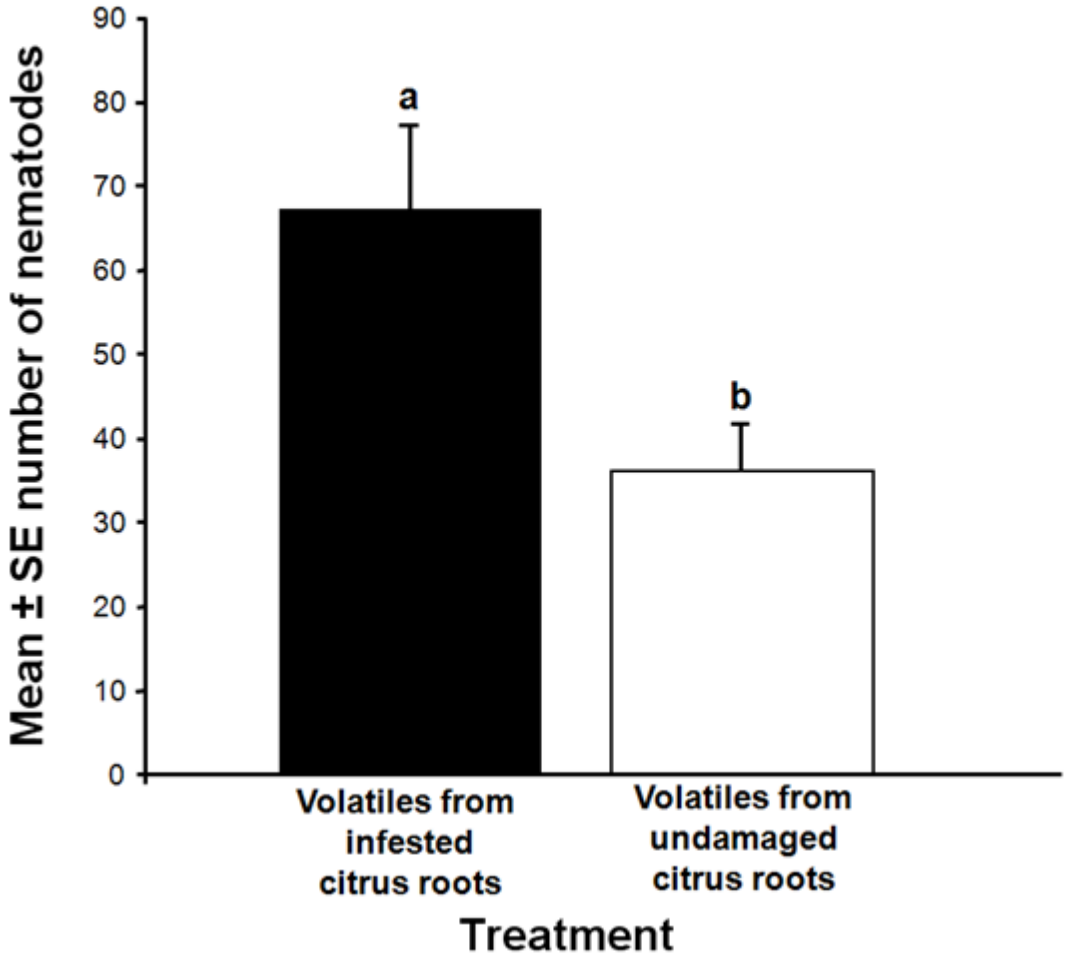


Figure 2-4. Mean number of nematodes attracted to volatiles from *D. abbreviatus*-infested roots compared with volatiles from undamaged roots.

CHAPTER 3
CONSTITUTIVE AND INDUCED SUBTERRANEAN PLANT VOLATILES ATTRACT BOTH ENTOMOPATHOGENIC AND PLANT PARASITIC NEMATODES

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 (Erlich & 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 *et al.*1990; Heil 2008; Dicke & Baldwin 2010). Above-ground plant defense 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 defense has become increasingly evident (van Tol *et al.* 2001; Aratchige *et al.* 2004; Rasmann *et al.* 2005; Ali *et al.* 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 lifecycle of the herbivore is partitioned between above- and below-ground plant zones, a unique opportunity exists for investigating plant defenses 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). Entomopathogenic nematodes (EPNs) from the genera *Heterorhabditis* or *Steinernema* (Downing *et al.* 1991; Schroeder 1992) are known to infect this insect (McCoy *et al.* 2000). Entomopathogenic nematodes 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 entomopathogenic

nematodes (*S. diaprepesi*) (Ali *et al.* 2010). We found that weevil-infested roots release volatile compounds not found in undamaged roots and suggested this to be an indirect defense associated with attraction of beneficial nematodes. Of the four main compounds released by damaged roots, the C_{12} 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 defense. Therefore, in addition to determining the extent of nematode attraction among various citrus varieties, we also investigated the breadth of responsiveness among several entomopathogenic nematode species.

Entomopathogenic nematodes 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*. 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

et al. 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* (nictating species) 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 *et al.* 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 reducing citrus yield by 6-12% worldwide. In Florida, it is estimated to affect 25% of described citrus species (Esser *et al.* 1991). 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 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 ca. 75 500 eggs per female (Munn & Munn 2002).

Above-ground plant stress elicits defensive responses in both above- and below-ground tissues (Kaplan *et al.* 2008a, b; Erb *et al.* 2008; van Dam 2009; van Dam & Heil this issue). Additionally, many studies have found an increase in the levels of shoot defenses following root herbivory (Bezemer *et al.* 2004; van Dam, Raaijmakers & van der Putten 2005; Soler *et al.* 2005). Analogously, levels of root defenses can be affected by shoot herbivory (Soler *et al.* 2007; Tiwari *et al.* 2010; Erb *et al.* 2011). Above-ground–below-ground cascades of plant defense 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 on-going 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 defense.

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) 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 3-1. The entomopathogenic nematodes, *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 approximately 25°C according to procedures described in Kaya & 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.

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 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 Behavior

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). 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 *et al.* 2010). For each plant species, root volatiles were collected and extracted from the collection filters according to the methods described by Ali *et al.* (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 *et al.* 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 hr to respond. Following the incubation period, the column was disassembled and the nematodes from the 2 collection jars were extracted using Baermann Funnels. 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 Below-ground Volatile Collections

By simultaneous collection of root and shoot volatiles using a headspace guillotine chamber coupled with a root-zone collection chamber (Figure 3-1) 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 hr at 250°C and then adjusted to 10% moisture as described in Ali *et al.* (2010). The chambers and

plants were placed below a platform on which a Teflon guillotine was attached (Figure 3-1). 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, Gainesville, FL, USA) 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^{-1} through a trap containing 50 mg of Super Q adsorbent (Alltech Assoc., Deerfield, Illinois, USA). Volatiles were collected for 24 hr 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 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 *et al.* 2010). Each infestation treatment was replicated 5 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, Gainesville, FL, USA) for 24 hr with a suction flow of 80 mL min^{-1} (Ali *et al.* 2010). 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 (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 *et al.* 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^{-1} to 260°C. The carrier gas used was helium at an average flow velocity of 30 cm s^{-1}. 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). Electric ionization 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 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

Entomopathogenic nematodes of all species responded similarly either arms of the blank negative control ($F = 3.0$, $df = 2, 72$, $P = 0.087$) (Figure 3-2A). However, when *D. abbreviatus*-infested and uninfested *P. trifoliata* roots were tested, most nematode species preferred either root treatment over the blank control ($F=35.66$, $df = 2,129$, $P<0.001$). The only exception was the ambush forager type *S. carpocapsae* ($P = 0.134$) (Figure 3-2A). All tested nematode species preferred Swingle plants infested with *D. abbreviatus* larvae over the paired uninfested controls ($P < 0.001$) (Figure 3-2B). 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 ($P < 0.001$) (Figure 3-2B).

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 (Figure 3-3A). 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 response to herbivory turned out to be the combination of the observed pregeijerene and geijerene peaks (Figure 3-3A). These C_{12} terpenes are thought to elicit nematode attraction (Ali *et al.* 2010). Adult beetle feeding on above-ground shoots did not induce production of pregeijerene or other volatiles typically released in response to root damage (Figure 3-3B); however, release of limonene from above-ground shoots was increased (Figure 3-3B).

Subteranean 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 (Figure 3-4A). In contrast, pregeijerene was released by Swingle roots (Table 3-2 and Ali *et al.* 2010) and Sour Orange rootstocks (Figure 3-4B) only in response to *D. abbreviatus* larval feeding (Figure 3-4B, Table 3-2).

Discussion

The rhizosphere within which nematodes forage to find resources has been the subject of investigation for several decades. 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 necessary habitat for many insect hosts, can also influence the behaviour of EPN nematodes (Bird & Bird 1986; Choo *et al.* 1989; Lei *et al.* 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 *et al.* 2004). Until recently, little was known about EPN chemotaxis in response to herbivore-induced cues (Rasmann *et al.* 2005; Hiltpolt *et al.* 2010; Ali *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* root weevils and that the attraction was due to an induced release of subterranean volatiles (Ali *et al.* 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). We intend to utilize microarray analysis to resolve gene regulation in response to herbivory among these different citrus varieties.

Our results indicate that all EPN species tested exhibited attraction to herbivore-induced volatiles irrespective of their foraging strategy (Figure 3-3). 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 (Lewis *et al.* 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. carpocapse,* ambusher) independent of damage (Figure 3-2A). Thus, these results support the hypothesis that pregeijerene likely explains this attraction. Of the EPN 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'. However, *S. diaprepesi* is an endemic species and may have considerable advantages in 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; Duncan *et al.*1996; Bullock *et al.* 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 *et al.*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 (Figure 3-2A); however, it did respond when the cue was present in only one of the two arms (Figure 3-2B). 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. 3-2, 3-3).

To date, investigations of nematode response to below-ground volatiles have focused on entomopathogens (Lewis *et al.* 1993; Lewis, Grewal & Gaugler 1995; Rasmann *et al.* 2005; Hiltpolt *et al.* 2010; Ali *et al.* 2010). Entomopathogenic nematode 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 exist around physiologically active roots including amino acids, ions, pH, carbon dioxide and sugars (Perry & Aummann 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 caries a high physiological cost (Zangerl & Rutledge 1996; Agrawal & Karban 1999; Karban *et al.* 1999; Strauss *et al.* 2002; Heil 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 defense 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 'defense' 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 defenses are well known above ground (Puustinen *et al.* 2004; Adler & Irwin 2005). Our results are consistent with the notion that defenses against diverse enemies may evolve independently but not without associated direct ecological costs in terms of reduced vigor 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 *et al.* 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 defense 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 entomophathogens 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 defense (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 defense (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 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, 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 (Figure 3-3A). Neither roots nor foliage released this putative nematode attractant upon above-ground herbivory by adult beetles (Figure 3-3B). 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 (Figure 3-3B), which deserves further investigation.

With respect to the influence of above-ground herbivory on below-ground plant defense, 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 defense (Rasmann & Turlings 2007; Erb *et al.* 2008; van Dam 2009; Erb *et al.* 2011). It may be possible that defense 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 phytopathogentic 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 defense 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.

Table 3-1. Trophic level, foraging strategy and ecological status of nematodes tested

Nematode spp.	Trophic Level	Foraging Strategy	Ecological Status
Steinernema diaprepesi	Entomopathogen	Intermediate	Indigenous to Florida
S. carpocapsae	Entomopathogen	Ambush	Commercially introduced
S. riobrave	Entomopathogen	Intermediate	Commercially introduced
Heterorhabditis indica	Entomopathogen	Cruiser	Commercially applied; indigenous to Florida
Tylenchulus semipenetrans	Plant parasite	Sedentary root endoparasite	Agricultural pest; citrus parasite

Table 3-2. GC-MS identification of volatiles from various citrus rootstocks

			Swingle *(Citrus paradisi × Poncirus trifoliata)*		Poncirus *(Poncirus trifoliata)*		Sour Orange *(Citrus aurantium)*	
RT	Names	CAS#	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 (RT)with standard and spectral data from Adams, EPA and Nist05 Libraries

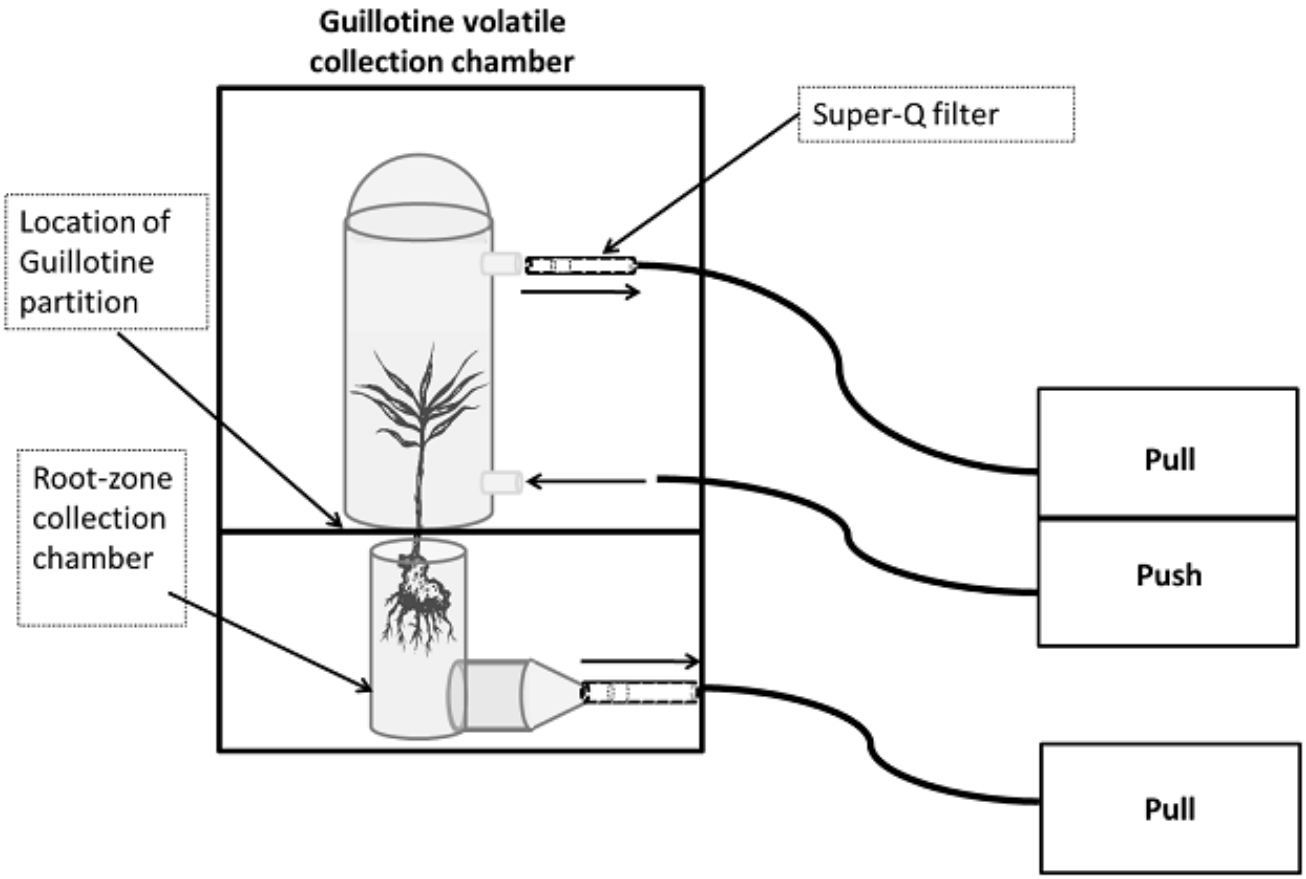


Figure 3-1. Schematic diagram of simultaneous above- and below-ground volatile collection apparatus (ARS, Gainesville, FL, USA). The guillotine volatile collection chambers used for above-ground collections received a constant flow of charcoal-purified and humidified air, which was suctioned at a rate of 300 mL min^{-1} through a trap containing 50 mg of Super Q adsorbent (Alltech Assoc., Deerfield, Illinois, USA). Root-zone collection chambers used to collect below-ground volatiles were filled with heat-sterilized sand standardized at 10 % saturation.

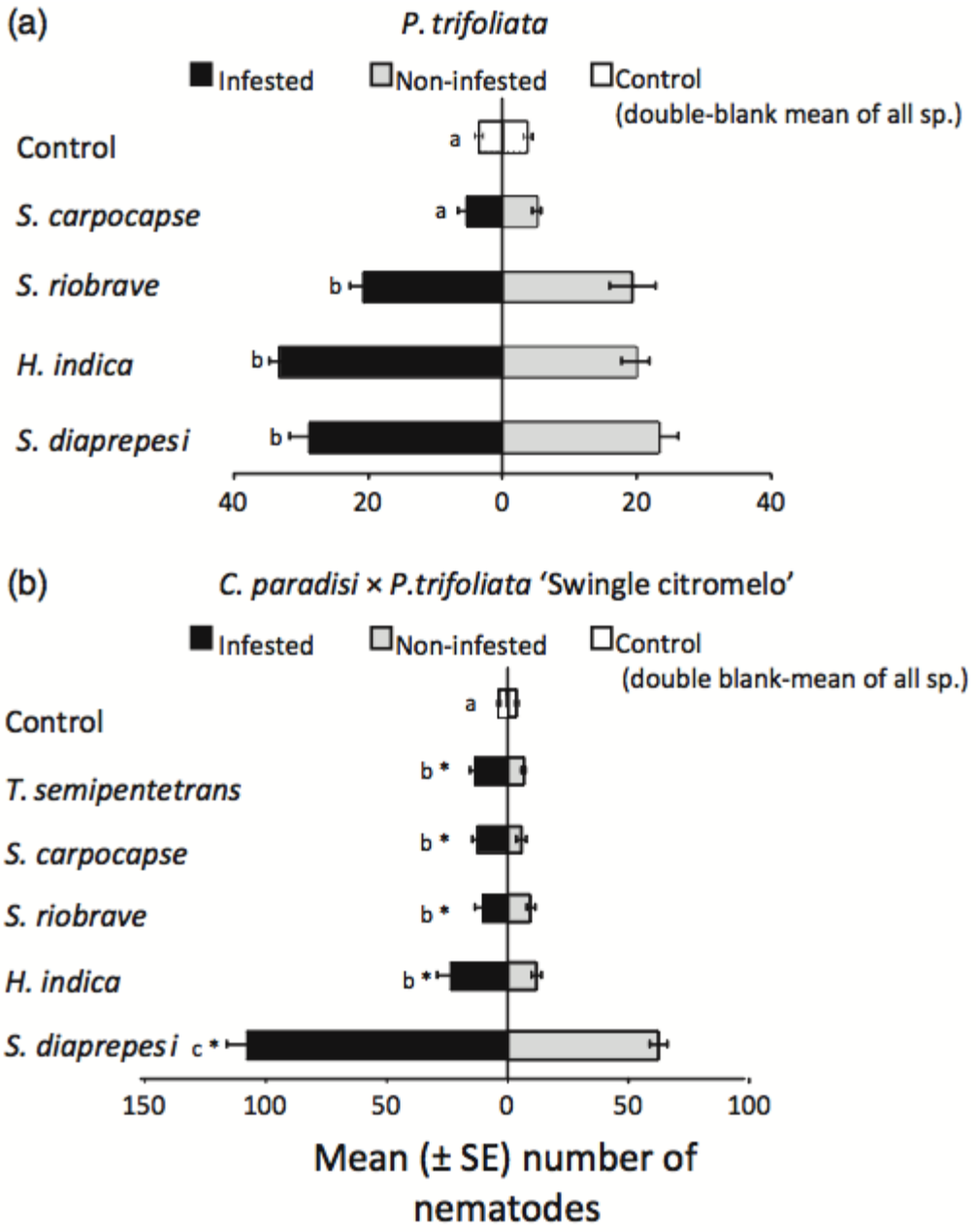


Figure 3-2. Responses of Tylenchulus semipenetrans, Steinernema carpocapse, S. riobrave, S. diaprepesi, and Heterorhabditis indica when presented with: A) Volatiles from roots of Poncirus trifoliata infested with Diaprepes abbreviatus lavare vs. volatiles from undamaged P. trifoliata roots or B) Volatiles 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.

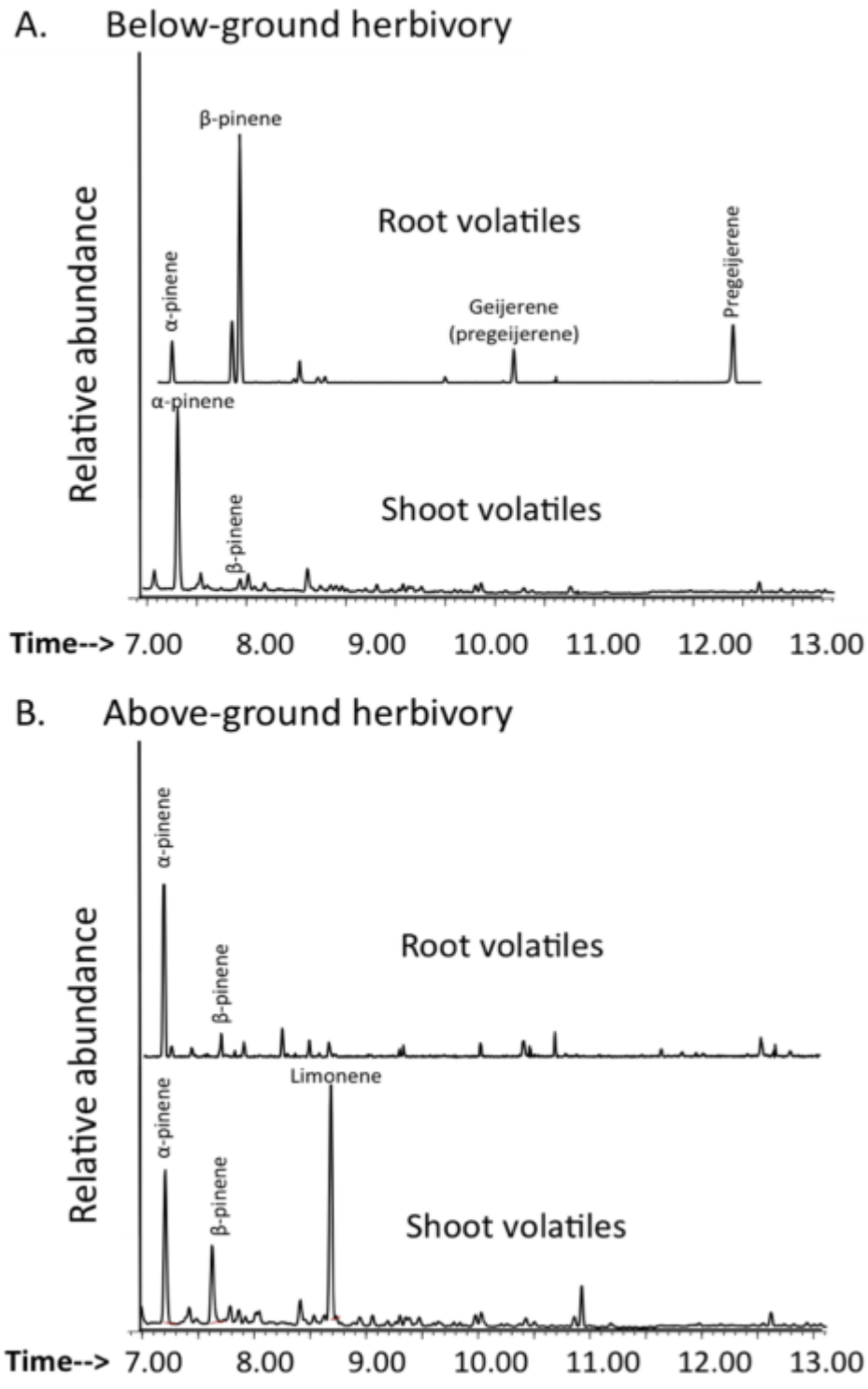
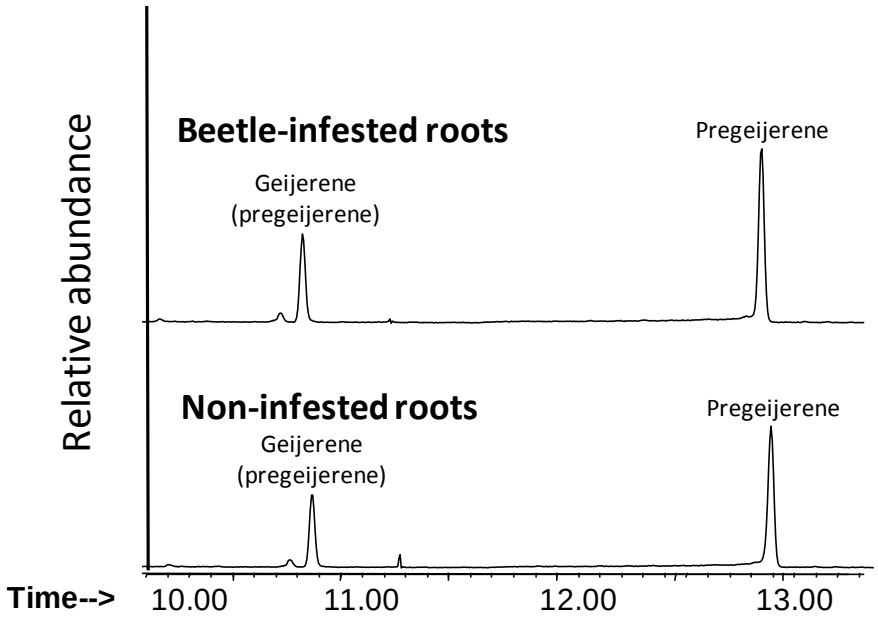


Figure 3-3. Example chromatograms depicting volatile profiles from simultaneous collections of root and shoot volatiles of Swingle (*Citrus paradisi* × *Poncirus trifoliata*) in response to A) Below-ground and B) Above-ground herbivory by *Diaprepes abbrevatus* larvae and adults, respectively. All samples were collected for 24 hr.

A. *Poncirus trifoliata*



B. *Citrus aurantium*

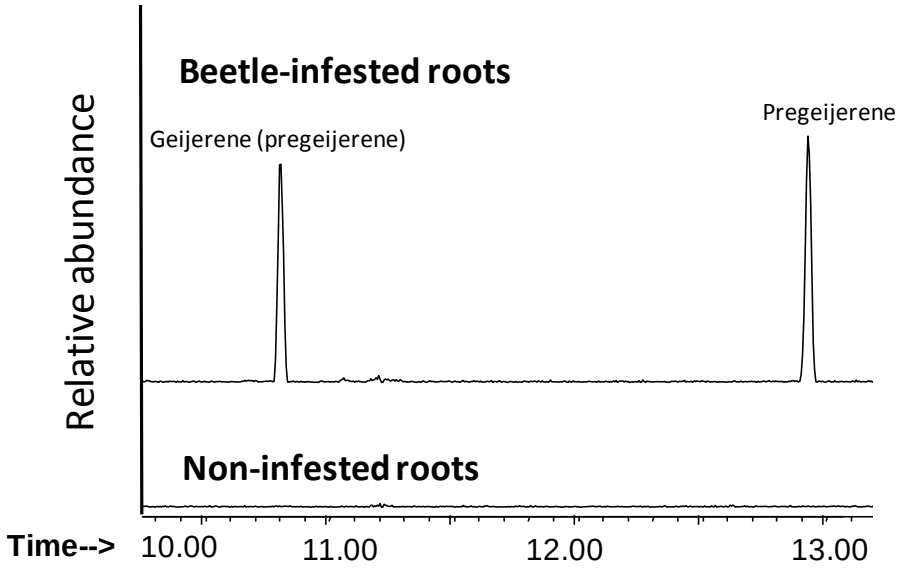


Figure 3-4. Example chromatogram showing volatile profiles from roots of A) *Poncirus trifoliata* or B) Sour orange (*Citrus aurantium*) in response to *Diaprepes abbrevatus* herbivory upon roots or undamaged controls. All samples were collected for 24 hr.

CHAPTER 4
MANIPULATING NATIVE POPULATIONS OF ENTOMOPATHOGENIC NEMATODES WITH HERBIVORE INDUCED PLANT VOLATILES TO ENHANCE PEST CONTROL

Natural enemies of herbivores use flexible foraging strategies that often incorporate environmental cues from the herbivore's host. Above ground phytodistress signals that mediate interactions between herbivore-damaged plants and species within the third trophic level are well documented (e.g. Turlings *et al.* 1990; Takabayashi & Dicke 1996; Tumlinson *et al.* 1993; Turlings & Wackers 2004). However, it has recently been shown that subterranean defenses also mediate HIPVs and reduce herbivore performance and population densities (De Moraes *et al.*1998; Thaler 1999; Kessler & Baldwin 2001, 2004) by attracting natural enemies of the herbivore. Thus below ground induced defense might be as complex and important as above ground induced plant defense (van Tol *et al.* 2001; Rasmann *et al*. 2005; Hiltpold *et al.* 2010; Ali *et al*. 2010; 2011). Furthermore, it could be utilized in agroecosystems to enhance the effectiveness of natural enemies (Pickett & Poppy 2001; Degenhardt *et al*. 2003; Aharoni *et al*. 2005; Turlings & Ton 2006) as has been demonstrated for maize (Rasmann *et al*. 2005). The disparity in the number of aboveground investigations versus analogous belowground research on indirect defense is largely due to technical limitations rather than lack of interest or relevance (Hunter 2001; Rasmann & Agrawal 2008).

Larvae of the weevil *Diaprepes abbreviatus* (L), that was first found in Florida in 1964 (Beaver & Selhime 1978), feed on the roots of more than 290 plant species including citrus, sugarcane, potatoes, strawberries, woody field-grown ornamentals, sweet potatoes, papaya, guava, mahogany, containerized ornamentals, and non-cultivated wild plants (Simpson *et al*. 1996). Over the past 40 years, it has contributed

significantly to the spread of disease and agricultural damage (Graham *et al*. 2002). Pesticide applications are expensive, environmentally hazardous and often ineffective (Bullock *et al*. 1999; Duncan *et al*. 1999). Currently, the most effective method for controlling *D. abbreviatus* is with application of commercially formulated entomopathogenic nematodes (EPNs) from the genera *Heterorhabditis* and *Steinernema* (Downing *et al*. 1991; Schroeder 1994). EPNs are obligate parasites that kill their host with the aid of a symbiotic bacterium (Poinar 1990). Over 20 years of use, the mass release of EPNs as a biopesticide for *D. abbreviatus* has been reported as varying and unpredictable with an efficacy ranging anywhere between 0% to >90% (Downing *et al*.1991; Schroeder 1994; Graham *et al*. 2002; Duncan *et al*. 1999). In addition many orchards in Florida, especially on the central ridge harbor rich communities of naturally occurring EPN species capable of suppressing weevil populations below economic thresholds (Stuart *et al*. 2008).

Promoting plant attractiveness to natural enemies is a novel agrochemical alternative to traditional broad-spectrum pesticides that indiscriminately kill predators and parasitoids leading to subsequent pest resurgence and secondary pests (Bruce 2010). The use of natural products to enhance biocontrol is typically compatible with integrated pest management; deploying HIPVs above ground by controlled release dispensers has been shown to increase plant recruitment and retention of beneficial parasites or predators (Thaler 1996; James & Grasswitz 2005). In an analogous belowground investigation, EPN infection of *Diabrotica virgifera virgifera* larvae was increased by spiking soil surrounding maize roots with the HIPV, (*E*)-β-caryophyllene (Rasmann *et al*. 2005). We have also recently shown that some citrus root stocks

(*Citrus paradisi* Macf.×*Poncirus trifoliata* L. Raf. and *Citrus aurantium*) release HIPVs in response to feeding by the weevil, *D. abbreviatus,* that attract EPN species endemic to Florida (Ali *et al*. 2010, 2011).

In this investigation, we identify the specific HIPV attractant as 1, 5-dimethylcyclodeca-1, 5, 7-triene (pregeijerene) and show its real-time release in response to herbivory. We also demonstrate that field application of this volatile increases mortality of belowground root feeding weevils by attracting their natural enemies. Furthermore, we demonstrate the presence of this compound in the root zone of fully grown trees in root weevil infested orchards. Recently developed qPCR primers and probes were used to detect and enumerate cryptic species of EPNs allowing for species-specific quantification of nematode response to attractants belowground. The use of plant produced signals, such as damage induced release of pregeijerene along with conservation biological control strategies could extend the usefulness of EPNs in citrus and other crops damaged by belowground herbivores. Given the broad effect of pregeijerene on a plurality of EPN species, it is possible that this chemical could be widely used for enhancing EPN-based biological control of subterranean insect pests of agricultural and urban plants.

Materials and Methods

Insect Larvae

Diaprepes abbreviatus 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) as

described in Beavers (1982) using procedures described by Lapointe and Shapiro (1999). Larvae used in experiments were from third to sixth instars.

Plants

'Swingle citrumelo' (*Citrus paradisi* Macf.×*Poncirus trifoliata* L. Raf.) rootstock is very prominent in commercial citrus production (Castle & Stover 2001). 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 ± 2°C, and 60-80% RH.

Ruta graveolens L. was purchased as fully grown plants 18- 24'' in height. The plants were immediately bare rooted and rinsed removing as much soil material as possible, and placed in vials containing Dichloromethane for further extractions and purification. The remaining plant material was discarded.

Nematodes used for Laboratory Bioassays and qPCR

The entomopathogenic nematodes, *Steinernema diaprepesi*, *S. riobrave*, *S. glaseri(x)*, and *Heterorhabditis indica* were isolated from *D. abbreviatus* larvae buried in commercial citrus orchards in Florida. *S. riobrave* 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 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.

In situ Volatile Collection from Infested Roots

Citrus plants (Swingle citrumelo, *Citrus paradisi* × *Poncirus trifoliata*) were grown and maintained at the CREC in Lake Alfred, FL, U.S.A. in a greenhouse at 26°C, and 60–80% RH. Six plants were initially placed in glass root-zone chambers (ARS, Gainesville, FL, U.S.A.) filled with sand that had been autoclaved for 1 hr at 250°C and then adjusted to 10% moisture as described in Ali *et al.* (2010). All seedlings were given three days to adjust to their sand filled chambers. Three of the plants were subjected to three days of feeding by weevil larvae. During this period each of the six root-zone chamber were connected to a vacuum pump (ARS, Gainesville, FL, U.S.A.) 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 mesh, Alltech Deerfield, IL, U.S.A.) held in glass fittings between the chamber and vacuum pump (2010). Super-Q traps were changed every 3h for a 72h 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, Palo Alto, CA, U.S.A., part number: 392611549 equipped with 500 µL glass inserts) (Ali *et al*. 2010). The undamaged plants served as a control.

In situ Volatile Collection from Infested Roots in the Field

Volatiles were collected from the soil beds surrounding citrus trees in a non-managed, privately owned field site. A soil probe (Figure 4-1) was used to sample at a depth of 20 cm and at distances of 1 m or 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 30min. Compounds were collected on adsorbent traps filled with 50 mg Super-Q, (800–1000 mesh, Alltech Deerfield, IL, U.S.A.) attached to the top of the soil probe (Figure 4-1).

The Super-Q traps were subsequently eluted with 150 µL of dichloromethane into individual 2.0 mL clear glass vials (Varian, Palo Alto, CA, U.S.A., part number: 392611549 equipped with 500 µL glass inserts).

GC-MS Analysis

All samples were injected as 1 µL aliquots of dichloromethane extracts 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, Palo Alto, CA, U.S.A.), in both electron impact and chemical ionization modes. Samples were introduced using either splitless injection at 220°C or by cold on column injection. In the second case, a 1m 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 at 10°C/min to 260°C. The carrier gas used was helium at 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, Adams 1995) and the NIST05 library. When available, mass spectra and retention times were compared to those of authentic standards in addition to internal standard (nonyl-acetate (4µg/µL)).

Isolation and Purification of Pregeijerene

Although pregeijerene (1, 5-dimethylcyclodeca-1, 5, 7-triene) was collected from citrus roots damaged by *D. abbreviatus*, it was necessary to find an alternative and abundant source of the pure compound for laboratory bioassay and field testing.

Hydrodistilled common rue (*Ruta graveolens*) essential oil contains geijerene as a major constiuent (67% of the total volatile compounds) (Kuzovkina *et al.*2008). However, at temperatures exceeding 120ºC (Kubeczka 1974, Figure 4-2) the macrocyclic pregeijerene will rearrange to geijerene thus by on-column analyses of common rue root extracts we, as anticipated, found large quantities of pregeijerene rather than geijerene. For isolation of pregeijerene, rue roots were crushed in dichloromethane. GC/MS analyses showed pregeijerene to constitute about 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-suspending it in 4mL of pentane. After centrifugation, the supernatant was again gently concentrated and re-suspended in 4ml 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 (Figure 4-3). The two remaining impurities were removed by first repeatedly partitioning the hexane extract with methanol followed by a slow filtering through a quartenary amin ion exchange column (Figure 4-3). The final hexane solution was analyzed by GC-MS for purity and by GC/FID with nonyl acetate as an internal standard for quantification. Serial dilutions were made from this extract providing five concentrations (30µL aliquots) of pregeijerene (8.0µg/µL; 0.80µg/µL; 0.08µg/µL; 0.008µg/µL; 0.0008µg/µL).

Two-choice Bioassay to Determine Optimal Dosage to Attract EPNs

The behavioral responses of nematodes to collected pregeijerene were quantified in a two choice sand-filled olfactometer described thoroughly by Ali *et al.* (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. Dilutions from the purified *R. gravleolens* root extract were placed on filter paper, which was allowed to dry 30 s for solvent evaporation. Thereafter, filter papers were placed on the bottoms of each glass jar, which were subsequently filled with 10% saturated (dry wt. sand: water volume; W/V), 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 IJs) were applied into the central orifice of the connecting tube and given 8 hr to respond. Following the incubation period, the column was disassembled and the nematodes from the 2 collection jars were extracted using Baermann Funnels. The experiment was replicated five times for each dilution for two species of EPN, *S. riobrave* and *H. indica*.

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 we detected a significant proportion of EPNs attracted to the treatment arm was selected for our field trial.

Application of HIPVs in the Field

The experiment was conducted in a sandy soil citrus grove at the Citrus Research and Education Center, in Lake Alfred (28 07 26.84 N, 81 42 55.31 W; 97:2:1, sand:silt:clay; pH 7.1; 0.1% OM). 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 larva of *D. abbreviatus* (reared on artificial diet for 3 to 5 weeks) were buried 20 cm deep beneath the tree canopies. Cages were made of 225-mesh stainless steel cylinders (7 × 3-cm diam.) secured at each end with polypropylene snap-on caps (McCoy *et al*. 2000). Six cages were placed equidistant from one another in a circle pattern (48cm diam.) for each treatment (n=10) (Figure 4-4). The cages contained one of two treatments (1) Soil with a single *D. abbreviatus* larva and infested roots volatiles, or (2) Soil with a single *D. abbreviatus* larva and blank solvent control. Treatments were applied as 30 µL aliquots to 3-cm diameter filter paper discs (Whatman, Maidstone, U.K.). 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 hr. Eight soil core samples (2.5 cm dia. X 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 hr after removal from soil.

We also investigated the effect of isolated pregeijerene on weevil mortality. The methods for this experiment were similar to that 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 arena were also homogenized and stored for nematode DNA extraction (n=10) (Figure 4-4). DNA extraction took place after sucrose centrifugation extraction of all nematodes (see further materials and methods). DNA extracted from

both soil samples were analyzed using species-specific primers for five EPN species known to be present in Florida's central ridge, and compared to an established standard curve (Campos-Herrera *et al*. 2011). In the second experiment, cages were left buried for 96hr prior to sampling. For both experiments a student's *t*-tests were used to determine the effect of treatment on mean weevil mortalities (*df* = 18).

Detection, Identification and Quantification of Entomopathogenic Nematodes using Real Time qPCR

Real time qPCR was used to identify and quantify attraction of naturally occurring EPN species to volatiles applied in the field and to identify nematodes to species. This technique targeted six EPN species (*Steinernema diaprepesi*, *S. riobrave*, *S. scapterisci, Heterorhabditis indica*, *H. zealandica*, and an undescribed species in the *S. glaseri*–group(x)) (Campos-Herrera *et al*. 2011a, b). Briefly, species–specific primers and TaqMan® probes were designed from the ITS rDNA region using sequences of the target steinernematid and heterorhabditid 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 (Larkin *et al*. 2007) to select areas of variability in the ITS region. The designed primers and probes (Primer–Blast, Rozen & Skaletsky 2000) provided 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 (Ultra Clean Soil™ DNA kit, MO BIO) and mixed to avoid the differences between DNA elution in the final step of extraction (Torr *et al*. 2007, Campos-Herrera *et al*. 2011). Dilutions corresponding with 100, 30, 10, 3 and 1 were prepared using serial dilution of the appropriate DNA.

Nematodes from soil samples were extracted by sucrose centrifugation (Jenkins 1964) from aliquots of 500 cm^3 from the mixed composite sample. Each nematode community was concentrated in an 1.5 mL Eppendorf tube. DNA was processed using the UltraClean™ soil DNA extraction Kit and quantification was performed for each DNA extraction using the nanodrop system with the control program ND-1000 v3.3.0. All DNA samples were adjusted to a 0.2 ng/µL that is required for nematode quantification (Campos-Herrera *et al*. 2011).

Real–time PCR was performed in optical 96–well reaction plates (U.S.A. Scientific, Orlando, FL, U.S.A.) on an ABI Prism 7000 (Applied Biosystem). All reactions were performed in a final volume of 20 µL, with 10 µLof TaqMan® Universal PCR Master Mix (AB, manufactured by Roche, Branchburg, NJ), using the appropiate primer and probe concentration for each species previously described (Campos-Herrera *et al*. 2011). In all tests, a negative control was included by adding sterile de–ionized water instead of template DNA, and the positive control was the corresponding standard curve. Thermal cycling was performed as described in Campos-Herrera *et al*. (2011), using 59ºC as annealing temperature and 35 cycles. All the samples/controls were run in duplicate. Data from the standard curves were log (x) transformed and a linear regression was performed of log (number of nematodes) and threshold cycle value (Ct) was performed to estimate the efficieny and accuracy of the system (SPSS® 18.0 software for Windows XP®, SPSS Inc., Chicago, IL, U.S.A.). Then, a correction factor was applied to transform the qPCR data to the real value according with each dilution. The resulting real values were analyzed with an 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 discriminate among means in the software R (R Development Core Team 2004).

NMR Analysis of Pregeijerene

Pregeijerene was purifed for NMR using prepGC as a mixture of pregeijere and geirene 70:30 ratio. The pregeijerene and geijerene mixture (~60 ug) in ~150 µL of C_6D_6 (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 ^{13}C). Residual C_6D_6 was used to reference chemical shifts to $\delta(C_6H_6) = 7.16$ ppm for ^1H and $\delta(C_6H_6) = 128.2$ ppm for ^{13}C (Fulmer *et al.* 2010). NMR spectra were processed using Bruker Topspin 2.1 and MestreLabs MestReNova software packages. Numbering is based on Jones and Southerland (1968). The H and ^{13}C NMR data in C_6D_6 are presented for pregeijerene and geijerene in Tables 4-2 and 4-3 because the original NMR data was obtained in carbon tetrachloride solution.

Results

***In situ* Volatile Collection from Infested Roots in the Field**

Volatiles collected from 1m and 10m distances detected both pregeijerene and geijeriene (Figure 4-5). Thus, demonstrating the presence of this cue under natural field conditions.

Release and Purification of 1, 5-Dimethylcyclodeca-1, 5, 7-Triene

Volatiles were non-destructively sampled every 3 hours from seedlings in sandy soil using root-zone chambers (ARS, Gainesville, FL, U.S.A.) as previously described (Ali *et al.* 2010, 2011). Three hours after the introduction of *D. abbreviatus* larvae to citrus roots, 1, 5-dimethylcyclodeca-1, 5, 7-triene (pregeijerene) was identified as a dominating root volatile, reaching a maximum release between 9 and 12 hr after initiation of larval feeding (Figure 4-6). There was no appreciable increase of any additional volatiles. After the initial spike, the release of pregeijerene decreased progressively over time (Figure 4-6). Initially it was a challenge to find sufficient amounts of pregeijerene for bioassays and field testing. However, it was previously established (Kuzovkina *et al*. 2008) that a hydrodestillate of common rue (*Ruta graveolens*) roots contained the related terpene geijerene as a major constituent (67% of the total volatile compounds). It is known that pregeijerene easily converts to geijerene, for example at temperatures exceeding 120ºC (Kubeczka 1974) (Figure 4-2) , thus on-column GC/MS analyses confirmed pregeijerene as the naturally occurring main terpene in roots of common rue that easily could be extracted and purified from crushed roots using a series of solid phase extractions (Figure 4-3).

Identification of Pregeijerene

Pregeijerene isolated from common rue and in the citrus root volatiles was found to be identical by EI and 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 analyses (see NMR results).

Optimum Pregeijerene Concentration

Serial 10-fold dilutions were made in dichloromethane from purified pregeijerene providing five concentrations. The behavioral responses of EPNs to pregeijerene were quantified in two choice sand-filled olfactometers (Ali *et al*. 2010, 2011) identifying 8ng/µL (in 30µL aliquots) as an optimally attractive dosage to EPNs (*S. riobrave* and *H. indica*) (Figure 4-7).

Field Verification of Increased Beetle Mortality by Belowground HIPVs

Field tests were conducted to determine whether application of infested root volatiles affects EPN inflicted mortality of sentinel *D. abbreviatus* larvae deployed in a citrus orchard by increasing response of naturally occurring and introduced EPN species. Cylindrical mesh cages containing autoclaved sandy soil (McCoy *et al*. 2000) contained one of two treatments (1) Soil with a single *D. abbreviatus* larva and a standardized collection of infested roots volatiles, or (2) Soil with a single *D. abbreviatus* larva and blank solvent control. Cages were buried 20 cm below ground between citrus tree canopies (Figure 4-4). Mortality of larvae placed in cages with the infested root volatiles was significantly higher than that for larvae placed in cages with solvent alone (Figure 4-8).

A second experiment was conducted to test whether also pregeijerene alone would increase mortality of larvae by attracting naturally occurring EPN species. Two treatments were deployed within the root-zone of mature citrus as described above. EPNs were quantified from soil samples taken within cages and from the surrounding soil of each treatment arena using real-time qPCR. Nematodes were extracted from collected soil and analyzed using species specific primers for EPN species known to be present in Florida's central ridge, and compared to an established standard curve

(Campos-Herrera *et al* .2011a, b). Average mortality of larvae buried with purified pregiejerene was significantly higher than that of larvae buried with the solvent control (Figure 4-9A). The number of EPNs recovered from cages containing the purified compound was significantly higher than that from cages with the solvent control (Figure 4-9B). There were also significantly more EPNs found in the soil samples surrounding cages containing pregeijerene than in solvent control cages (Figure 4-9C).

Real-time qPCR Determination of EPN Diversity, and Attraction to HIPVs

Real-time qPCR was employed to quantify the attraction of naturally occurring entomopathogenic nematodes (EPN) in the field and identify them to species. In this study, we employed a technique for identification of six EPN species known to either naturally occur in Florida (*Steinernema diaprepesi, Heterorhabditis indica*, *H. zealandica*, as well as an undescribed species in the *S. glaseri*–group (x)) or which were commercially applied in citrus groves in Florida (*S. riobrave*, *S. scapterisci*) (Campos-Herrera *et al*. 2011) (Table 4-1). Species–specific primers and TaqMan® probes were designed from the ITS rDNA region using sequences of the target steinernematid and heterorhabditid species as well as closely related species recovered from the NCBI database (http://www.ncbi.nlm.nih.gov/Genbank/) or generated by the authors in that study.

Comparisons of EPN species were based on standard curve points obtained from DNA dilutions (Holeva *et al*. 2006, Leal *et al*. 2007, Torr *et al*. 2007, Campos-Herrera *et al*. 2011). *Steinernema glaseri(x), S. diaprepesi*, *H. indica*, and *H. zealandica* were detected in soil samples in which mortality of *D. abbreviatus* was increased by the presence of HIPVs (Table 1). Tukey HSD test indicated *H. indica* and *H. zealandica* were significantly more abundant than the *S. glaseri(x) and S. diaprepesi* EPN species

($P < 0.0001$ in all comparisons). Species that could have been exogenously applied biopesticides, *S. riobrave* and *S. scapterisci*, were not detected in any of the samples (Table 4-1).

NMR Analysis of Pregeijerene

The ^{1}H NMR data (Table 4-2) for pregeijerene with reported proton chemical shifts and J-couplings for pregeijerene A (Jones & Southerland 1968) are consistent, but not with pregeijerene B (Cool & Adams 2003). Jones and Southerland (1968) 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 ^{1}H and ^{13}C NMR data agreed with germacrene C (Colby *et al.* 1998) except for carbons adjacent to C8 as expected. The two-dimentional 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 (Faraldos *et al.* 2007). Therefore, NOE difference experiments were done 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/.94 of C3. The NOE results agree with pregeijerene and flexible cyclodecadiene ring structures (Jones & Southerland 1968; Colby *et al.* 1998; Faraldos *et al.* 2007). In Addition, we found that chemical shifts of protons at C2, C7 and C8 are sensitive to temperature changes.

Discussion

The present research identifies pregeijerene as an HIPV associated with the indirect defense of citrus plant roots. Field application of this compound increased mortality of root weevils by its corresponding attraction of EPN. In addition to citrus roots, other rutaceus plant species are known to produce pregeijerene (Santos *et al.* 1998, Kuzovkina *et al.* 2008). (*E*)-β-caryophyllene is the only other identified volatile terpenoid known to attract EPNs (Rasmann *et al*. 2005). While the sesquiterpene (*E*)-β-caryophyllene is known to play an ecological role for numerous arthropod and nematode species (Turlings *et al*. 1998, Kigathi *et al.* 2009), this is the first description of an ecological role for the C_{12} terpene pregeijerene.

In this investigation we combined both recent and novel techniques of *in situ* detection of belowground cues and enumeration of cryptic EPN species using real-time qPCR to describe this subterranean interaction in detail. Two major obstacles account for difficulties in evaluating applied volatiles for the attraction of belowground natural enemies in the field. First, quantifying mortality of the target pest is usually measured by emerging adults (Degenhardt *et al*. 2009, Rasmann *et al*. 2011). In field experiments there is a high potential for low recovery of applied herbivores. This technique also gives no confirmation for the specific cause of mortality. Second, it can be difficult to quantify populations of naturally occurring EPNs. The number of EPNs in soil is usually estimated indirectly by baiting soil with sentinel insects (Koppenhofer *et al.*1998; Mracek *et al*. 2005). Such estimates are imprecise because infection rates are species specific and are dependent on environmental conditions such as soil moisture, temperature and porosity (Stuart *et al*. 2006). Low recovery of EPNs after application can make accurate quantitative comparisons of EPN attraction to treatments difficult, in addition to the time

consuming and intensive task of identifying the recovered nematodes to species, which very few have the expertise to do accurately. Quantitative real-time PCR is an efficient method for quantifying cryptic organisms such as bacteria, fungi, and nematodes from soil samples (Klob *et al*. 2003, Atkins *et al*. 2005, Zhang *et al*. 2006) and has been recently employed to investigate EPN diversity in natural habitats (MacMillan *et al*. 2006, Torr *et al*. 2007, Campos-Herrera *et al*. 2011a, b). This technique allowed quantification of increased mortality of weevil larvae in response to field application of the HIPV, pregeijerene. Furthermore, we proved that weevil mortality was caused by EPN species naturally occurring in Florida rather than those which could have been previously applied in the form of biopesticides. These results are not due to a lack of behavioral response by the commercially formulated species entirely, as shown in the laboratory experiment (Figure 4-6).

Non- native EPM species have been introduced into Florida citrus in the form of biopesticides. In our field tests, only native species of nematodes responded to field applications of the HIPV. Therefore manipulation of naturally occurring EPNs with pregeijerene without the need for exogenous application of non-native EPNs appears to be a viable tactic. Furthermore, in orchards with established EPN populations, large scale introduction of non-native species may displace native populations due to trophic cascades and limited resources or may cause an increase in populations of nematophagous fungi that eliminate EPN populations (Duncan *et al.* 1996, Koppenhofer *et al*. 1998, McCoy *et al*. 2000, Stuart *et al*. 2008). Although it is known that the artificially reared and commercially formulated EPN can persist, it is possible that natives have advantages associated with habitat acclimation and response to HIPVs

(Hiltpolt *et al*. 2010) thus further investigation of enhancing conservation biological control of belowground pests in concert with behavioral modification via HIPVs is warranted.

The obstacles of investigating above-belowground chemically mediated interactions between plants and animals are being overcome and refocused (van Dam 2009, Johnson 2008, van Dam & Heil 2011). One of the most important areas of focus in both aboveground and belowground systems remains understanding induced plant responses to herbivory that can indirectly reduce preference or performance of herbivores. Although it was originally postulated as a potential novel approach to pest management in agricultural systems (Green & Ryan 1972) and insect herbivore population regulation (Haukioja & Hakala 1975), few studies (Khan *et al*. 1997,De moraes *et al*. 1998, Birkett *et al*. 2000, Kessler & Baldwin 2001, Ockroy *et al*. 2001) of induced responses (particularly volatile) have addressed this practical application beyond fundamental concepts in ecology and evolutionary biology (Hunter 2002, van Dam & Heil 2011). There are even fewer attempts to investigate these dynamics belowground. At least half of all plant biomass is attacked by underground herbivores and pathogens, living amongst a complex ecological foodweb (De Deyn & van der Putten 2005). HIPVs are likely important mediators of tritrophic interactions that afford indirect plant defense within the root zone. Direct field sampling of root volatiles is a promising method for evaluating these belowground interactions in real time.

We also for the first time detected not only direct increases in larval insect mortality associated with use of a belowground HIPV attractant, but demonstrated a corresponding quantitative increase in subterranean natural enemies. Our previous

research suggests that volatile production in response to herbivore feeding differs between citrus species (Ali *et al*. 2010). Our findings could have broad impact on rootstock selection in commercial agriculture, by screening and recommending or developing rootstocks that release attractants which promote accumulation of EPN communities. Further investigation is needed to evaluate genetic expression of this response, which could optimize development of transgenic rootstocks to attract beneficial nematodes in response to pest damage. Pregeijerene may have extensive application for enhancing native biological control of root feeding insects given its broad attractiveness to a plurality of nematode species, including those which attack a wide range of belowground herbivores (Choo 2002).

Table 4-1. Species of entomopathogenic nematodes identified and quantified in response to HIPV deployment in the field.

EPN Species	Ecological Status	Detected	Representation (%)
Steinernema diaprepesi	Native	+	1
S. glaseri(x)	Native	+	1
S. scapterisci	Commercial product	-	0
S. riobrave	Commercial product	-	0
Heterorhabditis indica	Native	+	54
H. zealandica	Native	+	44

Table 4-2. ^{1}H (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 $\delta(^{1}H) = 7.16$ ppm for ^{1}H and $\delta(C_6D_6H) = 128.2$ ppm for ^{13}C.

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

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

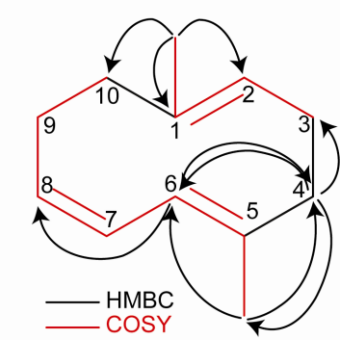
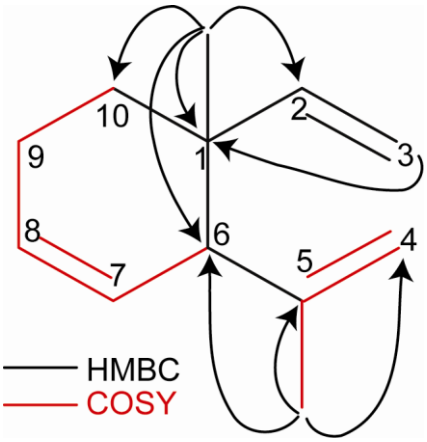


Table 4-3. ^{1}H (600 MHz), ^{13}C (151 MHz), HMBC and NOESY NMR spectroscopic data for geijerene in C_6D_6. ^{13}C was also detected directly (126 MHz) using a 5 mm Cryoprobe. Chemical shifts referenced to residual proton signal in C_6D_6 benzene $\delta(^{1}H)$ = 7.16 ppm for ^{1}H and $\delta(C_6D_6H)$ = 128.2 ppm for ^{13}C. For convenience, the pregeijerene numbering is retained after cope rearrangement to geijerene.

Position	δ ^{13}C [ppm]	δ ^{1}H [ppm]	J coupling constants [Hz]	HMBC correlations (C.No)	Unique NOESY peaks
1	38.0				
2	149.0	1H 5.86	dd J = 17.5, 10.8		
		2H 4.99,	4.99, 1H, dd J=17.5, 1.3	4.99 - C1	
3	110.6	4.94	4.94, 1H, dd J = 10.8, 1.3	4.95 - C1	4.99 - 0.96
		2H 4.82,	4.82, 1H, br s		
4	114.2	4.97	4.97, 1H, m		
5	146.7				
6	51.5	1H 2.7	quintet J = 2.7		
7	126.2	1H 5.66	dddd J = 10.1, 2.2, 3.5, 3.5		
8	129.9	1H 5.59	dddd J = 10.1, 3.2, 2.1, 2.1		
9	22.6	2H 1.91	m		0.96
10	33.4	2H 1.43	m		
CH3-C1	20.9	3H 0.96	s	C1, C2, C6, C10	1.72
CH3-C5	24.3	3H 1.72	br s	C5, C4, C6	0.96



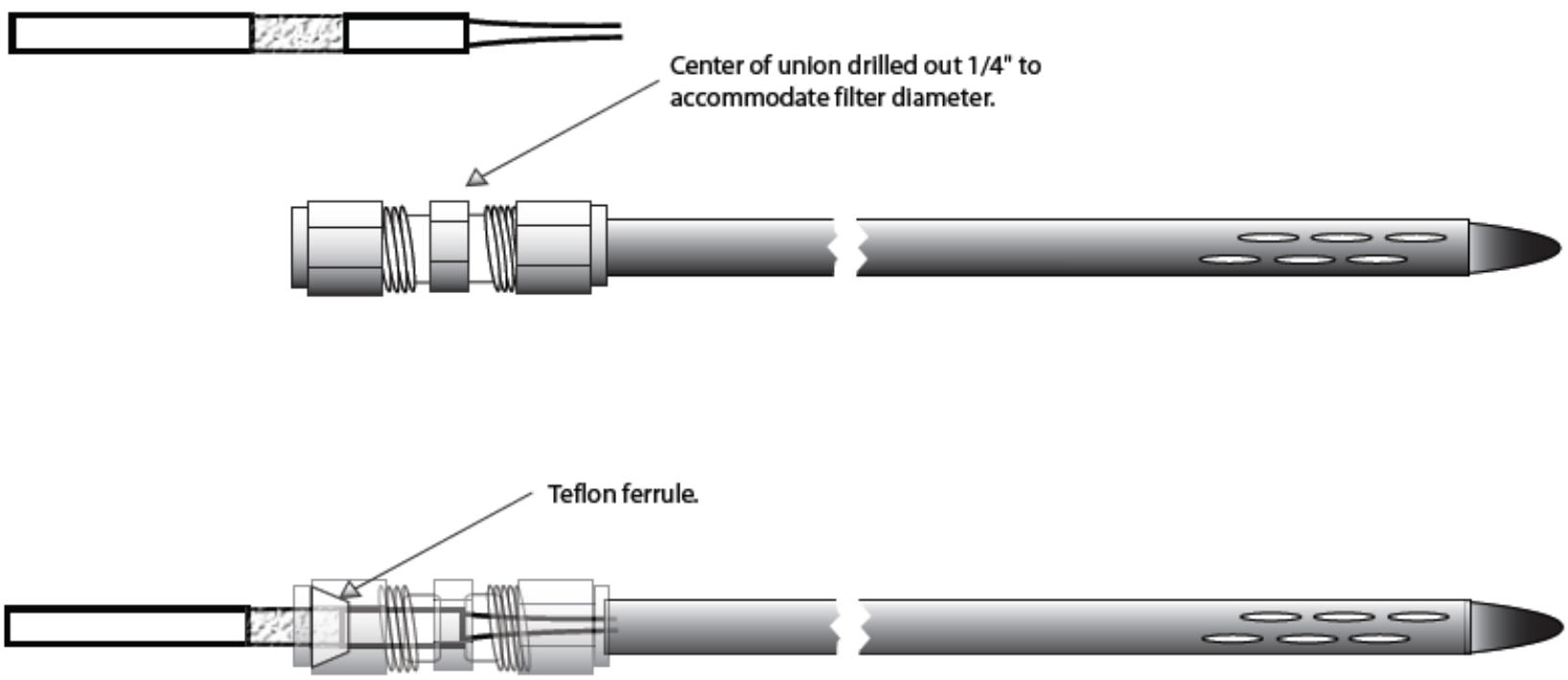


Figure 4-1. Representation of soil probe design used to sample volatiles belowground. Probe is inserted into soil and connected to a vacuum pump.

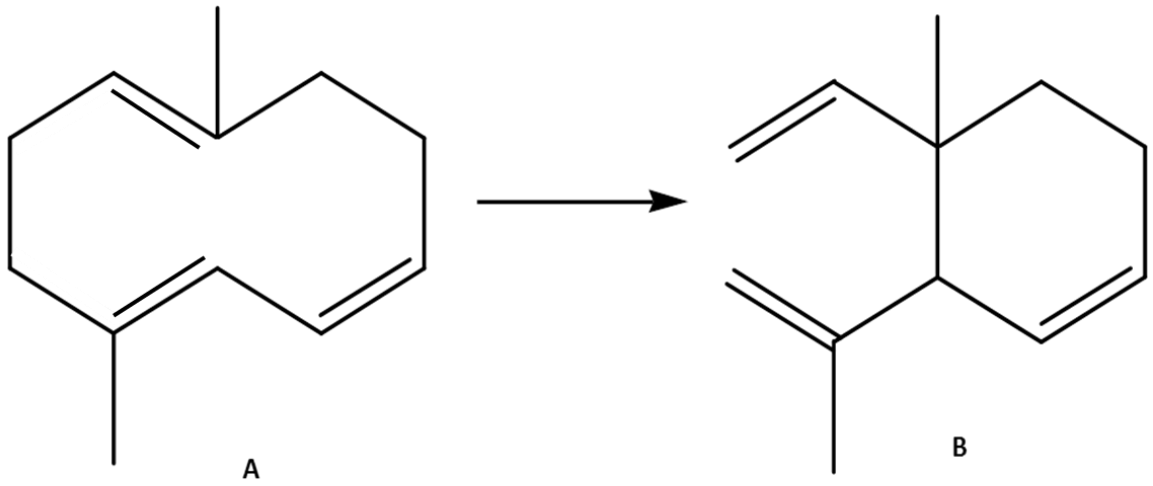


Figure 4-2. Conversion of Pregeijerene(A) to Geijerene(B).

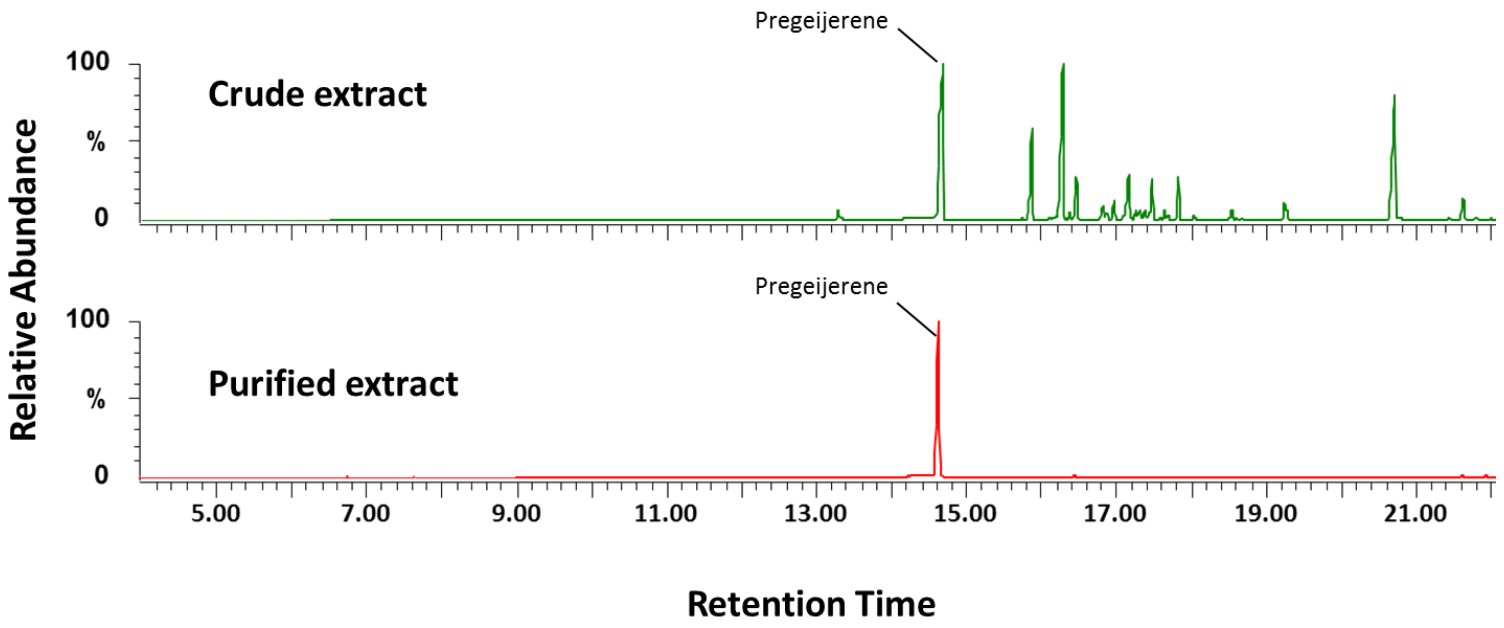


Figure 4-3. Chromatograms showing the initial crude extract prior to purification and final purified Pregeijerene.

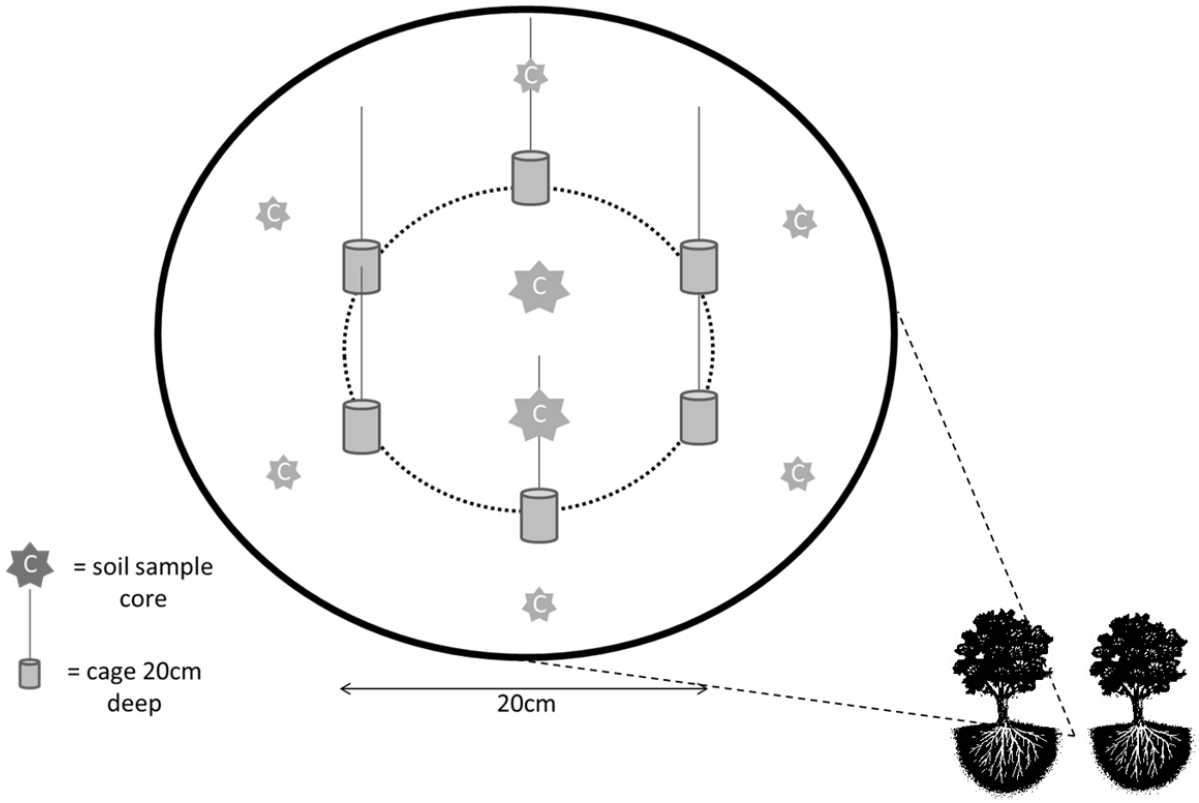


Figure 4-4. Schematic diagram of the deployment and sampling procedure for field experiments in which sentinel traps with root weevils were deployed with or without HIPVs. One treatment replicate is depicted.

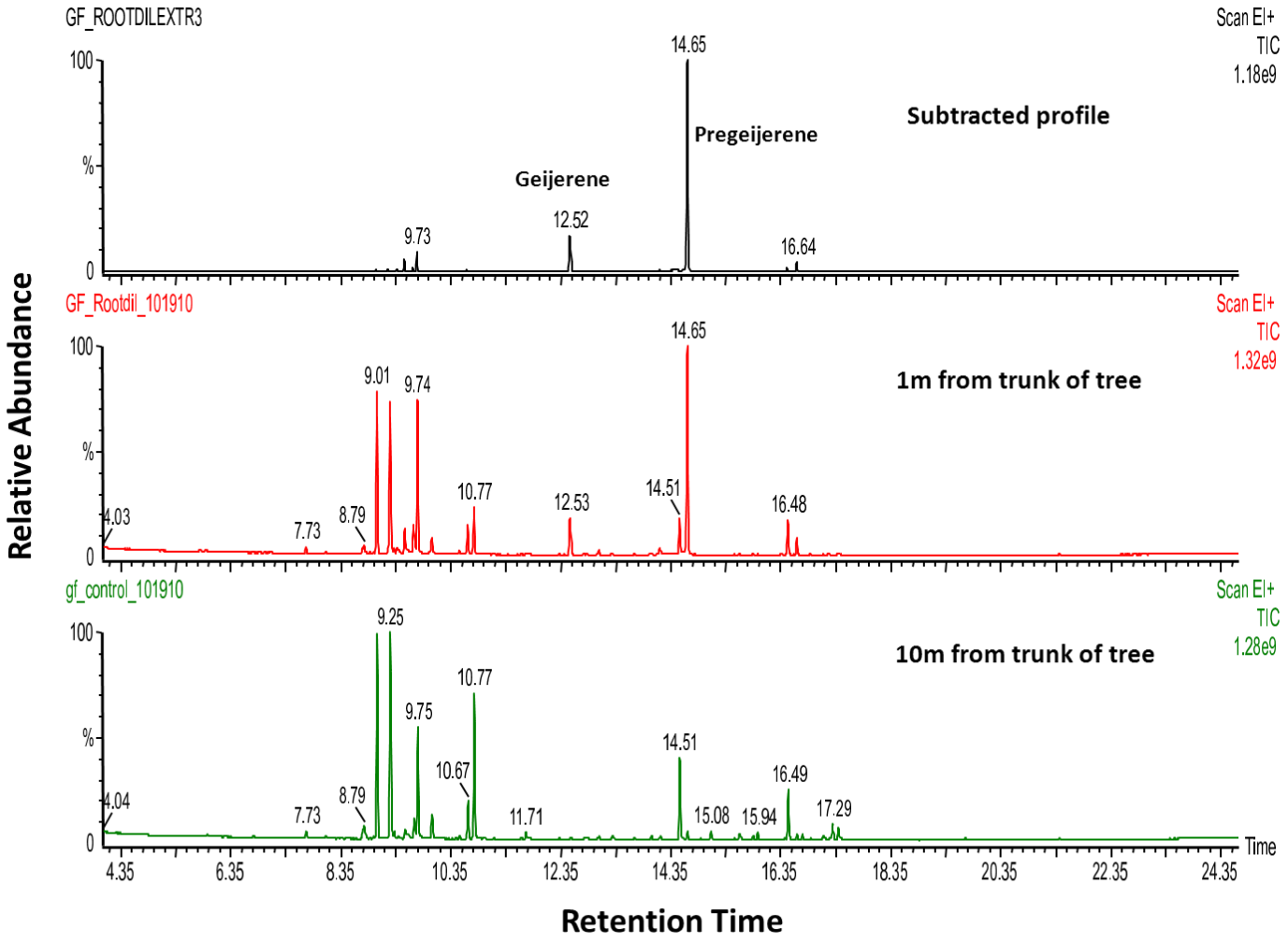


Figure 4-5. Chromatograms of volatiles taken from intact citrus roots in the field at 1m and 10m distances from the trunk of the tree.

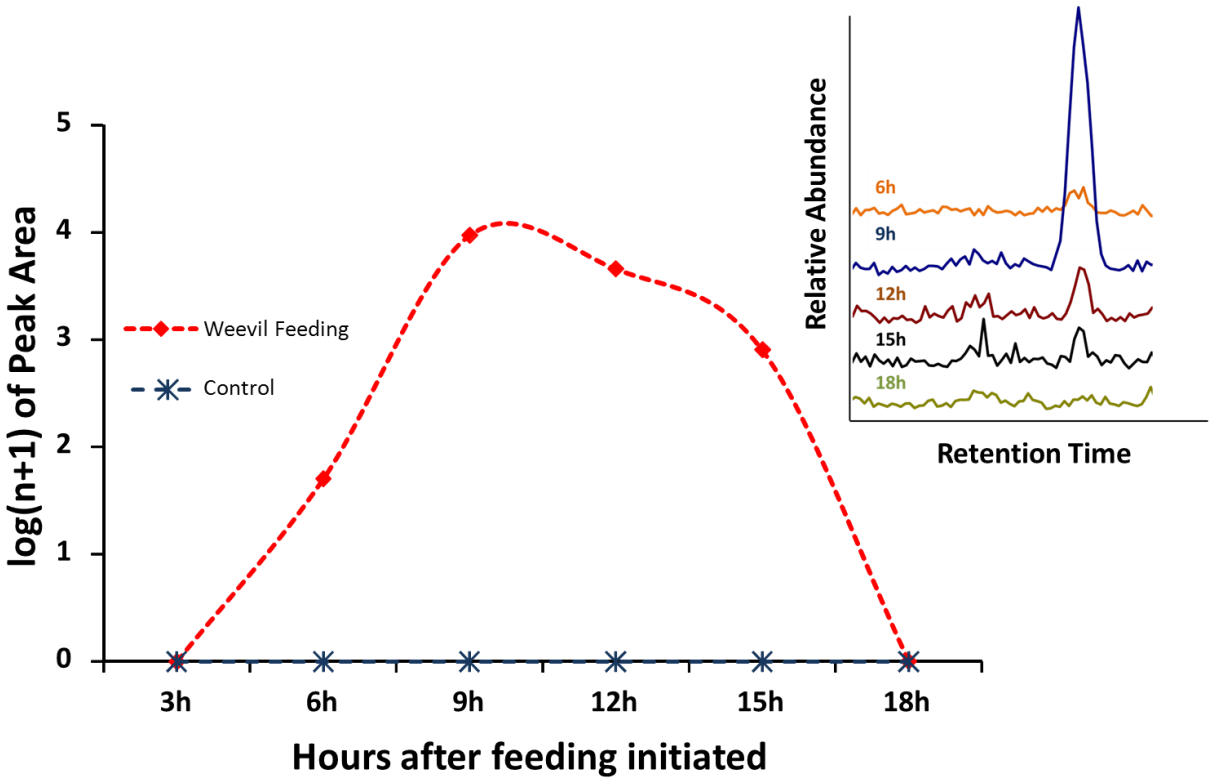


Figure 4-6. Time course of pregeijerene (1, 5-dimethylcyclodeca-1, 5, 7-triene) release following initiation of root weevil (D. abbreviatus) feeding on citrus roots. Closed diamonds represents beetle damaged roots and X's represent non-fed controls.

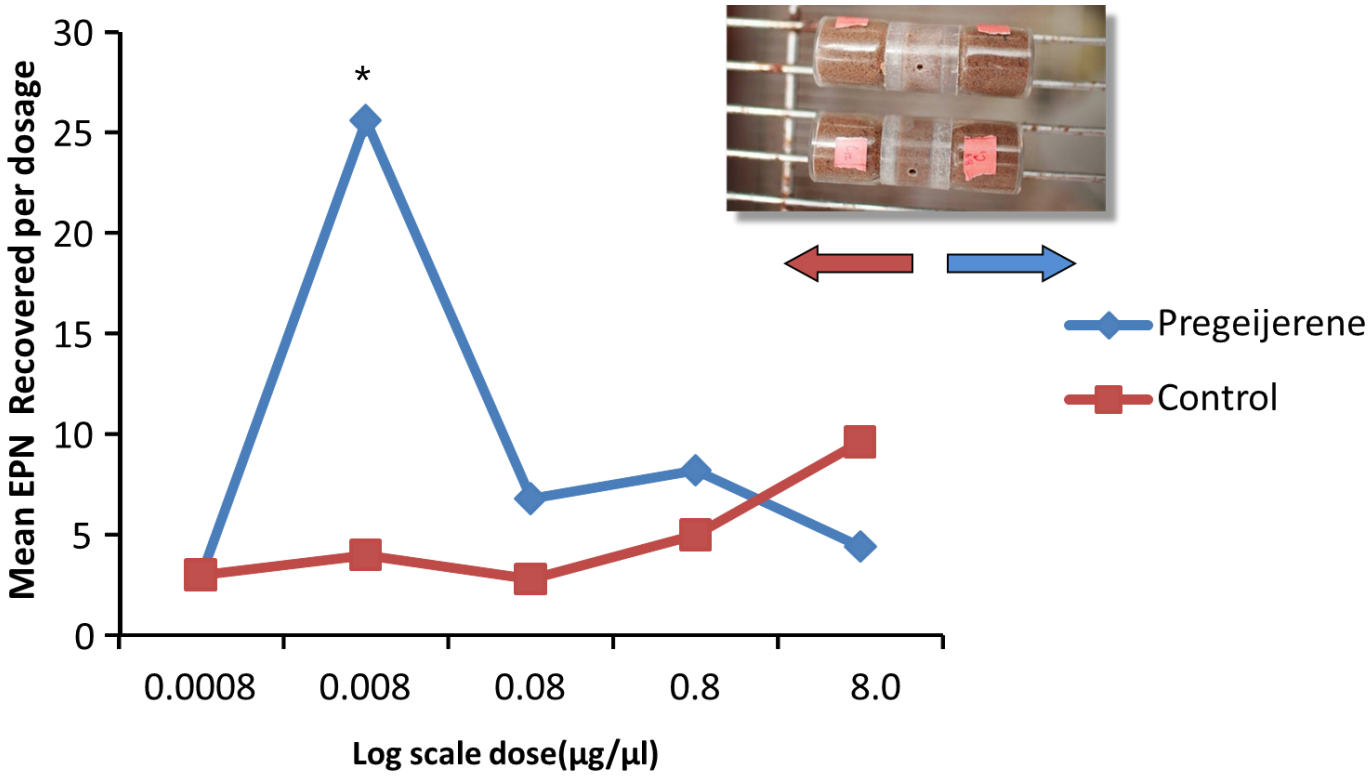


Figure 4-7. Optimal dosage of pregeijerene (1, 5-dimethylcyclodeca-1, 5, 7-triene) for attracting entomopathogenic nematodes (*S. riobrave* and *H. indica*) based on the log scale dilution of purified compound. Picture in upper right displays sand filled two-choice olfactometers used for nematode bioassays. * = P-value < 0.05.

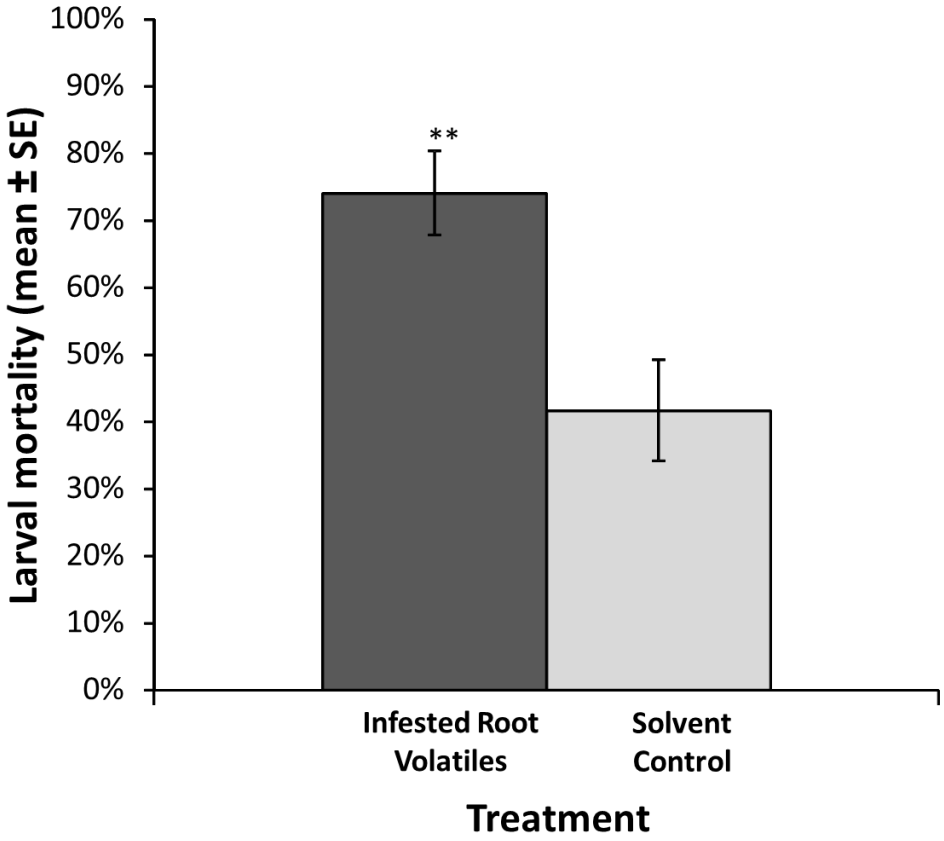


Figure 4-8. Mean percentage of larval mortality for treatments with or without *D. abbreviatus* fed-upon root volatiles. ** = P-value < 0.01. (N = 10, t = 3.25, P = 0.005)

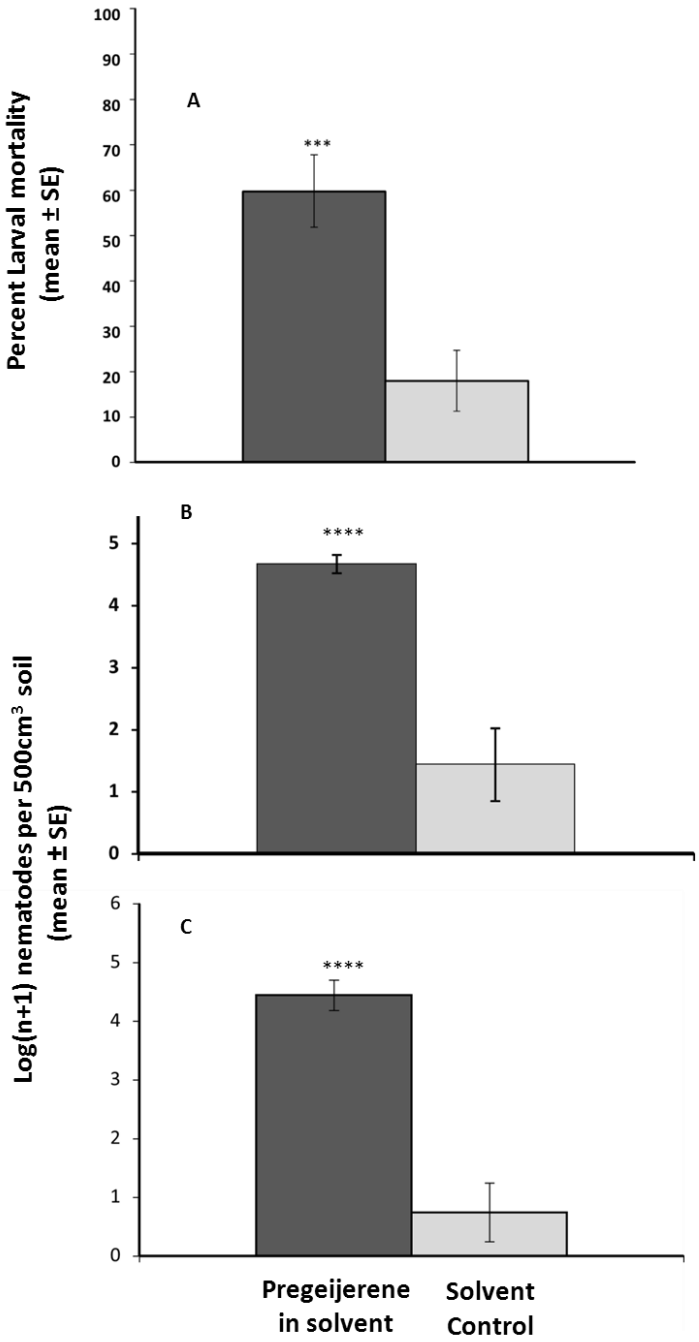


Figure 4-9. Effect of pregeijerene on weevil mortality and associated attraction of EPN species. A) Average mortality of larvae buried with purified pregeijerene compared with the solvent control (N = 10, *t* = 4.01, *P* = *0.0008*) B) Mean number of EPNs recovered from cages containing the purified pregeijerene compared with cages containing the solvent control (N = 10, *t* = 5.33, P = *0.00005*). C) Mean number of EPNs recovered from soil samples surrounding cages containing pregeijerene compared with the solvent control (N = 10, *t* = 5.67, *P* = *0.00003*).

CHAPTER 5
CONCLUSIONS

Plants are under constant pressure from higher trophic levels which attempt to exploit their autotrophic resources. Herbivorous insects (primary consumers) are the most successful herbivores harvesting primary resources, as evident by their overwhelming numbers and diversity. However, the success and abundance of primary consumers causes selection pressure for evolution of plant defense. This has resulted in the evolution of complex interactions between plants, insect herbivores, and their natural enemies.

As stated by Price *et al.* (1980) all terrestrial communities based on living plants are composed of at least three interacting trophic levels: plants, herbivores, and natural enemies. This seminal paper introduced an important relationship, which has led to over 30 years of investigations by ecologists and entomologists focusing on predators and parasitoids of herbivores. There is increasing evidence of plants using natural enemies as bodyguards. Mechanisms of this relationship are found in plants' production of food and shelter for these natural enemies or by producing herbivores induced volatiles that expose the presence of herbivores.

The chapters outlined in this dissertation provide evidence for the use of herbivore induced volatiles by entomopathogenic nematodes to locate their hosts. This is only the second study to identify a belowground cue in a tritrophic interaction. However, to fully define this relationship as a plant defense additional terms must be met. Understanding the evolution of this potential defensive interaction will require a connection to the plant trait which affects the entomopathogen, and the direct/indirect effects the entomopathogen has on the fitness of the plant. Observations made in the

studies of this work can be evaluated on their implications for both agricultural biological control strategies and the evolutionary consequences of these relationships.

Chapter 2 demonstrated, in a root zone bioassay, that root weevil infested rootstock (Swingle citrumelo) recruited significantly more EPN (*Steinernema diaprepesi*) than non-infested or mechanically damaged roots, or larvae alone. By dynamic *in situ* collection and GC-MS analysis of volatiles from soil, in combination with a two choice sand-column bioassay it was found that Swingle citrus roots release induced volatiles in response to herbivore feeding and that some of these induced volatiles function as attractants for EPNs. This study was the first step in drawing a connection to a trait which affects the entomopathogen. Although we introduce an *in situ* method for detecting belowground signals and correlate these specific infested root volatiles to the attraction of EPNs, further work was necessary to reveal the breadth of this interaction.

Chapter 3 examined the extent to which belowground recruitment signals modify behavior of nematode species representing various foraging strategies, and trophic levels. We compared attraction to extracts of infested roots and non-infested roots from hybrid, Swingle citrus rootstock, and a parent line of the hybrid, *P. trifoliata* (Pt). Swingle roots infested by weevils attracted more nematodes than non-infested roots irrespective of nematode foraging strategy and trophic status. The parental line of the swingle rootstock, Pt, attracted all nematode species irrespective of insect herbivory. Dynamic *in situ* collection and GC-MS analysis of soil volatiles revealed that Pt roots released recruitment signals constitutively, regardless of weevil feeding. A different non-hybrid citrus species (Sour orange, *C. aurantium*) released nematode recruitment signals only in response to larval feeding. Volatile collections from above/belowground portions of

citrus plants revealed that aboveground feeding does not induce production of nematode recruitment signals analogous to that induced by root damage nor does damage by larvae belowground induce a similar signal aboveground. This study demonstrated that roots can have induced or constitutive release of nematode cues. It also becomes evident that these cues aren't entirely associated with the attraction of beneficial nematodes. The plants susceptible to the plant parasites seemed to have a strategy which circumvents the ecological cost of attracting its parasites by only releasing these cues when fed upon by the root weevils. The roots which constitutively release these cues were not susceptible to the phytopathogens. The cue thus seems to be heavily context dependent and should be evaluated for use only in systems which are not threatened by citrus specific plant parasites. It became evident that evaluations of the attractants' ability to increase mortality of root weevils would be necessary to further define this cue as an indirect defense.

In the fourth chapter, the main constituent released by damaged citrus roots was identified as pregeijerene (1, 5-dimethylcyclodeca-1, 5, 7-triene) and field assays of lab-collected citrus root HIPVs proved attraction of native EPN and associated increased mortality of beetle larvae compared with controls was possible. We determined by quantitative real-time PCR that field application of pregeijerene increased pest mortality by attracting four species of EPNs native to Florida, U.S.A. This was a first step in evaluating the attractant's potential to reduce herbivore populations. Although it holds promising implications for biological control, we must still evaluate this interaction from the prespective of the plant's fitness/yield. This study identified a specific belowground cry for help and then showed how this cue could reduce herbivore numbers, but we are

yet to show protection of the plant via reduced damage and thus increased yield. Future studies must show this protection relationship for the ecological requirements of defining this response as a defense and for the implementation of these responses in biological control strategies.

These studies have made a number of contributions to the field of chemical ecology and biological control. By developing methods for the underground detection of responses to herbivory, in real-time, we have made the first direct quantifications of belowground HIPV release. Combining both recent and novel techniques of *in situ* detection of belowground cues and enumeration of cryptic EPN species using real-time qPCR it became possible to describe this subterranean interaction in detail. (*E*)-β-caryophyllene is the only other identified volatile terpenoid known to attract EPNs (Rasmann *et al*. 2005). While the sesquiterpene (*E*)-β-caryophyllene is known to play an ecological role for numerous arthropod and nematode species (Turlings *et al*. 1998, Kigathi *et al.* 2009), this is the first description of an ecological role for the C_{12} terpene pregeijerene. The next steps of this investigation must test the potential for this compound to protect citrus roots from damage.

This research has two very different aspects and implications. First, the observations in this dissertation have implications in the fields of natural defense ecology and evolutionary biology. Second, we are examining the phenomena within an agroecological system with domesticated crops. These two points bring about a paradox in their respective methodological applications. Can we impose observations of domesticated plants in an agricultural context on evolutionary theory of natural plant

defenses? We must first take into consideration the properties associated with agricultural systems.

Traditional farming practices thrived on large genetic crop diversity (Marshall 1977). Although, outbreaks of insect pests often devastated yields, the high level of resistance maintained allowed for lower, but consistent and sustainable levels of production. Decades of plant breeding to meet the demands of growers, processors, packers and consumers has produced dramatic increases in yield and qualities of crop plants (Marshall 1977). This consequentially led to an increasing level of uniformity in viable crop products. Locally adapted genotypes have been substituted with widely adapted cultivars that are characterized by insufficient defense against attack by specialized insects. Feeny (1976) developed an extension of coevolution defense theory, based on apparency. Apparent plants could be referred to as, “bound to be found”, they are predicted to be well-defended and not readily susceptible to counter adaptation. Unapparent plants, on the other hand, are not predictably distributed and defenses are susceptible to counter adaptation. The plight of modern agriculture has been to domesticate unapparent plants and make them apparent.

Managed ecosystems are characterized by low plant diversity and low genetic variability compared with natural ecosystems. It is well known that reduced heterogeneity can enhance the colonization of plants by insect pests (Andow 1990), and it can also influence the performance of natural enemies (Price *et al.* 1980, Price 1986). However, biological control studies are often viewed as "ecological experiments on a grand scale, and illustrate both the 'escape' of pest species relieved of natural enemies and their demise when enemies are restored to the system" (Strong *et al.* 1984), and

many early biological control practitioners (e.g., DeBach and Rosen 1991, Waage 1992) considered there to be no fundamental ecological difference between successful classical biological control and the action of native natural enemies ('naturalcontrol' *sensu* DeBach [1964]).

A distinguishing feature instantly appears as we consider the indirect defense interactions in agroecosystems. The objective of biological control in agriculture is to maximize the effectiveness of a natural enemy complex in suppressing pests and ultimately in enhancing crop yield (Debach & Rosen, 1991; Norris et al. 2003; Denno *et al.* 2008). In ecological contexts, trophic cascades are predator-prey interactions that indirectly alter the abundance, biomass or productivity of a community across more than one trophic link in a food web (Hawkins *et al.* 1999; Pace *et al.* 1999). In this way the extent to which herbivore populations are constrained by natural enemies in agroecosystems is significantly different and their broad ecological implications may be limited.

Although the findings of this, largely agricultural, study may have limited ecological relevance, there are too few investigations of belowground community interactions to neglect this research's potential "grand scale" implications on plant defense strategy. The majority of terrestrial studies, investigating enemy propagated trophic cascades have focused on arthropods or vertebrates as predators in aboveground food webs, whether in natural or managed ecosystems (Rosenheim *et al.*1995; Hawkins *et al.* 1999; Snyder *et al.* 2005; Rasmann & Agrawal 2008). Soil-dwelling organisms comprising belowground food webs have been virtually ignored (Hunter 2001; Rasmann & Agrawal 2008). Nematodes, despite their prevalence in

natural and agricultural habitats, are highly under-represented in studies of population and food-web dynamics and in particular in those investigating trophic cascades (Stuart *et al.* 2006). Thus based on the knowledge obtained from the study of aboveground arthropod food webs, analogous information is critical for understanding the broader ecological and evolutionary mechanisms involved in EPN-herbivore-plant indirect defense.

Thus the broader evolutionary implications of research presented in this work are open to debate. However there is a common focal point for the future directions of this study which would be relevant to both evolutionary relationships and agricultural applications. Next it will be most important to study how such interactions enhance or reduce plant biomass and yield. As stated earlier, it is clear how a link to this 'defensive' trait must be made between the natural enemy and the fitness of the plant to develop evolutionary predications on the adaptive role of this relationship. Additionally, investigating this EPN-herbivore-plant interaction (and the manipulation of cues within it) to potentially influence yield of citrus and other crops is of paramount importance to agriculture and biological control.

LIST OF REFERENCES

Adair, R.C. 1994. A four-year field trial of entomopathogenic nematodes for control of *Diaprepes abbreviatus* in Flatwoods citrus grove. *Proceedings Florida State Horticultural Society,* **107**, 63-68.

Adams, R.P. 1995. Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry. Allured, Carol Stream, IL.

Adler, L.S., & Irwin, R.E. 2005 Ecological costs and benefits of defenses in nectar. *Ecology,* **86**, 2968-2978.

Agrawal, A. A., & Karban, R. 1999. Why induced defenses may be favored over constitutive strategies in plants. In *The Ecology and Evolution of Inducible Defenses*, eds. R. Tollrian and C. D. Harvell, pp. 45–61. Princeton: Princeton University Press.

AgrawaL, A.A., & Rutter, M.T. 1998. Dynamic anti-herbivore defense in ant–plants: the role of induced responses. *Oikos,* **83**, 227–236.

Aharoni, A., Jongsma, M.A., & Bouwmeester, H.J. 2005. Volatile science? Metabolic engineering of terpenoids in plants. *Trends in Plant Science*, **10**, 594-602.

Alborn, H.T., Jones, T.H., Stenhagen, G.S., & Tumlinson, J.H. 2000. Identification and synthesis of volicitin and related components from beet armyworm oral secretions. *Journal Chemical Ecology,* **26**, 203-220.

Alborn, H.T., **Turlings**, **T.C.J., Jones, T.H., Stenhagen, G., Loughrin, J. H., & Tumlinson, J. H.** 1997. An elicitor of plant volatiles from beet armyworm oral secretion*. Science,* **276**,945-949.

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Ali, J.G., Alborn, H.T. & Stelinski, L.L. 2010. Subterranean herbivore-induce volatiles released by citrus roots attract entomopathogenic nemtaodes. *Journal of Chemical Ecology*, **36**, 361-368.

Ali J.G., Alborn, H.T., Stelinski, L.L. 2011. Constitutive and Induced Subterranean Plant Volatiles Attract Both Entomopathogenic and Plant Parasitic Nematodes. *Journal of Ecology*, **99**, 26-35.

Andow, D.A. 1990 Vegetational Diversity and arthropod response. Annual Review of Entomology, **36**, 561-586.

Aratchige, N. S., Lesna, I., & Sabelis, M. W. 2004. Below-ground plant parts emit herbivore-induced volatiles: olfactory responses of a predatory mite to tulip bulbs infested by rust mites. *Experimental and Applied Acaro*logy, **33**, 21-30.

Arimura, G.-i., Kost, C., & Boland, W. 2005. Herbivore-induced, indirect plant defenses. *Biochimica and Biophysica Acta,* **1734**, 91-111.

Arimura, G., Ozawa, R., Horiuchi, J., Nishioka, T., Takabayashi, J. 2001. Plant-plant interactions mediated by volatiles emitted from plants infested by spider mites. *Biochemical Systematics and Ecology*, **29**, 1049-1061.

Arimura, G., Ozawa, R., Shimoda, T., Nishioka, T., Boland, W., & Takabayashi, J. 2000. Herbivory-induced volatiles elicit defense genes in limabean leaves. *Nature*, **406**, 512-515.

Atkins S.D., Clark I.M., Pande S., Hirsch P.R., Kerry B.R. 2005. The use of real–time 466 PCR and species– specific primers for the identification and monitoring of *Paecilomyces lilacinus*. *FEMS Microbiology Ecology*, **51**, 257–264.

Bais, H.P., Weir, T.L., Perry, L.G., Gilroy, S., & Vivanco, J.M. 2006. The role of root exudates in rhizosphere interactions with plants and other organisms. *Annual Review of Plant Biology,* **57**, 233–236.

Baldwin, I.T., Kessler,A., & Halitschke, R. 2002. Volatile Signaling in plant-plant-herbivore interactions: what is real? *Current Opinion in Plant Biology*, **5**, 351-354.

Baldwin, I.T. & Preston, C.A. 1999. The eco-physiological complexity of plant responses to insect herbivores. *Planta,* **208**, 137-145.

Bate, N. J., & Rothstein, S. J. 1998. C-6-volatiles derived from the lipoxygenase pathway induce a subset of defense-related genes. *Plant Journal,* **16**, 561-569.

Beavers, J. B. 1982. biology of Diaprepes abbreviatus (Coleoptera: Curculionidae) reared on an artificial diet. *Florida Entomologist.* **65**, 263-269.

Beavers, J. B., & Selhime, A. G. 1975. Development of Diaprepes Abbreviatus on Potted Citrus Seedlings. *Florida Entomologist,* **58**, 271-273.

Bezemer, T.M. & van Dam, N.M. 2005. Linking aboveground and belowground interactions via induced plant defenses. *Trends in Ecology & Evolution,* **20**, 617-624.

Bezemer, T.M., Wagenaar, R., van Dam, N.M., van der Putten, W.H. & Wäckers, F.L. 2004. Above- and below-ground terpenoid aldehyde induction in cotton, *Gossypium herbaceum*, following root and leaf injury. *Journal of Chemical Ecology*, **30**, 53-67.

Bird, A.F. & Bird, J. 1986. Observations on the use of insect parasitic nematodes as a means of biological control of root-knot nematodes. *International Journal for Parasitology*, **16**, 511-516.

Birkett, M.A., Campbell, C.A.M., Chamberlain, K., Guerrieri, E., Alastair, J.H., Martin, J.L., Matthes, M., Napier, J.A., Petterson, J., Pickett, J.A., Poppy, G.M., Pow, E.M., Pye, B.J., Smart, L.E., Wadhams, G.H., & Woodcock, C.M. 2000. New roles for cis-jasmone as an insect semiochemical and inplant defense. *Proceedings of the National Academy of Sciences, U.S.A*., **97**, 9329-9334.

Blossey, B. & Hunt-Joshi, T. R. 2003. Belowground herbivory by insects: influence on plants and aboveground herbivores. *Annual Review of Entomology*, **48**, 521-547.

Boethel, D.J. & Eikenbary, R.D. 1986. Interactions of plant resistance and parasitoids and predators of insects. Chichester, Ellis Horwood limited.

Boff, M. I. C., van Tol, R. H. W. M., & Smits, P.H. 2002. Behavioural response of *Heterorhabditis megidis* towards plant roots and insect larvae. *Biocontrol,* **47**, 67-83.

Bourne, B.A. 1921. Insect attacks reported or observed. *Annual Report of the Department of Agriculture*: Barbados, 1919-1920, pp. 12-13.

Bullock, R.C., McCoy, C.W., & Fojtik. J. 1988. Foliar sprays to control adults of thecitrus root weevil complex in Florida. *Proceedings of Florida State Horticultural Society,* **101**, 1-5.

Bullock, R.C. & Miller, R.W.1994. Suppression of *Pachnaeus litus* and *Diaprepes abbreviatus* (Coleoptera: Curculionidae) adult emergence with *Steinernema carpocapsae* (Rhabditida: Steinernematidae) soil drenches in field evaluations, *Proceedings of the Florida State Horticultural Society,* **107**, pp. 90–92.

Bullock, R.C., Pelosi, R.R., & Killer. E.E. 1999. Management of citrus root weevils (Coleoptera: Curculionidae) on Florida citrus with soil-applied entomopathogenic nematodes (Nematoda: Rhabditida). *Florida Entomologist,* **82**,1-7.

Bruce, T.J.A. 2010. in *Plant Communication from Ecological Perspective,* eds Ninkovic & V, Baluška F (Springer-Verlag, Heidelberg), pp 215-227.

Cabanillas, H.E., Poinar, G.O. & Raulston, J.R. 1994. *Steinernema riobravis* n. sp. (Rhabditida: Steinernematidae) from Texas. *Fundamental & Applied Nematology*, **17**, 123-131.

Campbell, J.F. & Gaugler R. 1997. Nictation behavior and its ecological implications in the host search strategies of entomopathogenic nematodes (Heterorhabditidae and Steinernematidae). *Behaviour*, **126**, 3-4.

Campos-Herrera R, Johnson, E.G., El-Borai, F.E., Stuart, R.J., Graham, J.H., Duncan, L.W. 2011. Long-term stability of entomopathogenic nematode spatial

patterns in soil as measured by sentinel insects and real-time PCR *Annals of Applied Biology,* **158**, 55-68.

Cardoza, Y. J., Alborn, H.T., & Tumlinson, J.H. 2002. In vivo volatile emissions form peanut plants induced by simultaneous fungal infection and insect damage. *Journal of Chemical Ecology,* **28**,161-174.

Choo, H.Y. & Kaya, H.K. 1991. Influence of soil texture and presence of roots on hostfinding by *Heterorhabditis bacteriophora*. *Journal of Invertebrate Pathology,* **58**, 279-280.

Choo, H.Y., Kaya, H.K., Burlando, T.M. & Gaugler, R. 1989. Entomopathogenic nematodes host-finding ability in the presence of plant-roots. *Environmental Entomology,* **18**, 1136-1140.

Colazza, S., McElfresh, J.S., & Millar, J.G. 2004. Identification of volatile synomones, induced by *Nezara viridula* feeding and oviposition on bean spp., that attract the egg parasitoid *Trissolcus basalis*. *Journal Chemical Ecol*ogy, **30**, 945-964.

Colby, S.M., Crock, J., Dowdle-Rizzo, B., Lemaux, P.G., & Croteau, R. 1998. Germacrene C synthase from Lycopersicon esculentum cv. VFNT cherry tomato: cDNA isolation, characterization, and bacterial expression of the multiple product sesquiterpene cyclase. *Proceedings of the National Academy Sciences*, U S A. **95**, 2216-2221.

Cool, L.G., & Adams, R.P. 2003. A pregeijerene isomer from Juniperus erectopatens foliage. *Phytochemistry,* **63**,105-108.

D'Alessandro, M., & Turlings, T. C. J. 2005. *In situ* modification of herbivore-induced plant odors: A novel approach to study the attractiveness of volatile organic compounds to parasitic wasps. *Chemical Senses*, **30**, 739-753.

De Bach, P. 1964. Biological control of insects pests and weeds. Reinhold, New York.

De Bach, P. & Rosen, D. 1991. Biological control by natural enemies, 2nd Ed. Cambrige University Press, Cambrige, MA.

De Boer, J.G., & Dicke, M. 2004. The role of methyl salicylate in prey searching behavior of the predatory mite *Phytoseiulus persimilis*. *Journal of Chemical Ecology,* **30**, 255-271.

De Deyn, G.B. & van der Putten, W.H. 2005. Linking above and belowground biodiversity. *Trends in Ecology and Evolution,* **20**, 625-633.

Degenhardt, J., Gershenzon, J., Baldwin, I.T. & Kessler, A. 2003. Attracting friends to feast on foes: engineering terpene emission to make crop plants more attractive to herbivore enemies. *Current Opinions in Biotechology,* **14**, 169-176.

Degenhardt, J., Hiltpold, I., Köllner, T.G., Frey, M. Gierl, A., Gershenzon, J., Hibbard, B.E., Ellersieck, M. R., & Turlings, T.C.J. 2009. Restoring a maize root signal that attracts insect-killing nematodes to control a major pest, *Proceedings of the National Academy Sciences*, U S A. **106,** 13213–13218.

De Moraes, C., M.C. Mescher, & Tumlinson, J.H. 2001. Caterpillar-induced nocturnal plant volatiles repel conspecific females. Nature, **410**: 577-580.

De Moraes, C.M., Lewis, W.J., Pare, P.W., Alborn, H.T., Tumlinson J.H. 1998. Herbivore-infested plants selectively attract parasitoids. *Nature,* **393**,570-573.

Denno, R.F., Gruner, D.S., and Kaplan, I. 2008. Potential for entomopathogenic nematodes in biological control: A meta-analytical synthesis and insights from trophic cascade theory. Journal of Nematology **40**, 61-72.

Dicke, M. & Baldwin, I.T. 2010. The evolutionary context for herbivore-induced plant volatiles: beyond the 'cry for help'. *Trends in Plant Science,* **15**,167-175.

Dicke, M., de Boer, J.G., Hofte, M., & Rocha-Granados, M.C. 2003. Mixed blends of herbivore-induced plant volatiles and foraging success of carnivorous arthropods. *Oikos* **101**, 38-48.

Dicke, M. & Sabelis, M.W. 1988. How plants obtain predatory mites as bodyguards. *Netherlands Journal of Zoology*, **38**, 148-165.

Dicke, M., Sabelis, M.W., Takabayashi, J., Bruin, J., & Posthumus, M.A. 1990. Plant strategies of manipulating predator-prey interactions through allelochemicals: prospects for application in pest control. *Journal of Chemical Ecology*, **16**, 3091-3119.

Dicke, M. & van Loon, J. 2000. Multitrophic effects of herbivore-induced plant volatiles in an evolutionary context. *Entomologia Experimentalis et Applicata*, **97**, 237-249.

Dicke, M., van Poecke, R. M. P., & de Boer, J.G. 2003. Inducible indirect defense of plants: from mechanisms to ecological functions. *Basic and Applied Ecology* **4**, 27-42.

Dicke, M. & Vet, L.E.M. 1999. Plant-carnivore interactions: evolutionary and ecological consequences for plant, herbivore and carnivore. Herbivores: between plants and predators. H. Olff, V. K. Brown and R. H. Drent. Oxford, Blackwell Science**:** 483-520.

Downing, A.S., Erickson, S.G., & Kraus, M.J. 1991. Field evaluation of entomopathogenic nematodes against citrus root weevils (Coleoptera: Curculionidae) in Florida citrus. *Fla. Entomol.* **74**, 584–586.

Duncan, L.W., Graham, J.H., Zellers, J., Bright, D., Dunn, D.C., El-Borai, F. E., & Porazinska, D.L. 2007. Food web responses to augmenting the entomopathogenic nematodes in bare and animal manure–mulched soil. *J. Nematol*ogy, **39**,176-189.

Duncan, L.W., McCoy, C.W., & Terranova, A.C. (1996) Estimating sample size and persistence of entomogenous nematodes in sandy soils and their efficacy against the larvae of *Diaprepes abbreviatus* in Florida. *Journal of Nematology*, **28**, 56–67.

Duncan, L.W. Shapiro, D.I. McCoy, C.W., & Graham, J.H. 1999. Entomopathogenic nematodes as a component of citrus root weevil IPM, pp. 69-78. *In* S. Polavarapu [ed], Optimal Use of Insecticidal Nematodes in Pest Management. Rutgers University Press, New Brunswick, NJ.

Ehrlich, P.R. & Raven, P.H. 1964. Butterflies and plants: a study in coevolution. *Evolution,* **18**, 586-608.

Engelberth, J., Alborn, H.T., Schmelz, E.A., & Tumlinson, J.H. 2004. Airborne signals prime plants against insect herbivore attack. *Proceedings of the National Academy of Science USA*, **101**, 1781-1785.

Erb, M., Lenk, C., Degenhardt, J. & Turlings, T.C.J. 2009. The underestimated role of roots in defense against leaf attackers. *Trends in Plant Science*, **14**, 653-659.

Erb, M., Ton, J., Degenhardt, J. & Turlings, T.C.J. 2008. Interactions between arthropod-induced aboveground and belowground defenses in plants. *Plant Physiology, **146**, 867-847.*

Erb, M., Robert, C.A.M., Hibbart, B.E. & Turlings, T.C.J. (2011) Sequence of arrival determines plant-,mediated interactions between herbivores. *Journal of Ecology*, **99**,7-15.

Farag, M.A. & Paré, P.W. 2002. C6-Green leaf volatiles trigger local and systemic VOC emission in tomato. *Phytochemistry*, **61**, 545-554.

Faraldos, J.A., Wu, S., Chappell, J., & Coates, R.M. 2007. Conformational Analysis of (+)-Germacrene A by Variable Temperature NMR and NOE Spectroscopy. *Tetrahedron*, **63**, 7733-7742.

Farmer, E.E. 2001. Surface-to-air signals. Nature, **411**: 854-856.

Fennah, R.G. 1940. Observations on behaviour of citrus root-stocks in St. Lucia, Dominica and Montserrat. *Tropical Agriculture,* **17**, 72-76.

Feeny, P. 1976. Plant Apparency and Chemical Defense, pp 1-40, in J.W. Wallace and R.L. Nansel (eds). Biological Interactions Between Plants and Insects. Recent Advances in Phytochemistry, Vol 10. Plenum Press, NY.

Frey, M., Stettner, C., Pare, P.W., Schmelz, E.A., Tumlinson, J.H., & Gierl, A. 2000. An herbivore elicitor activates the gene for indole emission in maize. Proceedings of the National Academy of Science, U.S.A. **97**, 14801-14806.

Fulmer, G. R., Miller, A. J. M., Sherden, N. H., Gottlieb, H. E., Nudelman, A., Stoltz, B. M., Bercaw, J. E., & Goldberg, K. I. 2010. NMR Chemical Shifts of Trace Impurities: Common Laboratory Solvents, Organics, and Gases in Deuterated Solvents Relevant to the Organometallic Chemist . *Organometallics. **29***, 2176.

Hiltpold, I., Toepfer, S., Kuhlmann, U., & Turlings, T.C.J. 2010. How maize root volatiles affect the efficacy of entomopathogenic nematodes in controlling the western corn rootworm. *Chemoecology,* **20**, 155-162.

Gardner, F.E. & Horanic, G.E. 1967. *Poncirus trifoliata* and some of its hybrids as rootstocks for Valencia sweet orange. *Proceedings of the Florida State Horticultural Society*, **53**, 85-87.

Gaugler, R., Lebeck, I., Nakagaki, B., & Boush, G.M. 1980. Orientation of the entomopathogenic nematode *neoaplectana carpocapse* to carbon dioxide. *Environment Entomol*ogy **9**, 649-652.

Georgis, R., Koppenöfer, A.M., Lacey, L.A., Bélair, G., Duncan, L.W., Grewal, P.S., Samish, M., Tan, L., Torr, P., & van Tol R.W.H.M. 2006. Successes and failures in the use of parasitic nematodes for pest control. *Biological Control*, **38**, 103-123.

Grafton-Cardwell, E. E., Godfrey, K. E., Pena, J. E., McCoy, C. W., & Luck, R. F. 2004. Diaprepes Root Weevil. University of California Agriculture and Natural Resources Publication 8131. Oakland, CA. 8 pp.

Graham, J.H., Bright, D.B., McCoy, C.W. 2002. Phytophthora-Diaprepes weevil complex: *Phytophthora* spp. relationship with citrus rootstocks. *Plant Dis*ease, **87**, 85–90.

Graham, J.H., McCoy, C.W., & Rogers, J.S. 1996. Insect-plant pathogen interactions:Preliminary studies of *Diaprepes* root weevils injuries and *Phytophthora* infections. *Proceedings of the Florida State Horticultural Society*, **109**, 57-62.

Green, T.R., & Ryan, C.A. 1972. Wound-induced proteinase inhibitor in plant leaves: a possible defense mechanism against insects. *Science,* **175**, 776–777.

Grewal, P.S., Lewis, E.E., & Gaugler, R. 1996. Response of infective stage parasites (Rhabditida Steinernematidae) to volatile cues from infected hosts. *Journal of Chemical Ecology **23**, 503-515.*

Grewal, P.S., Lewis, E.E., Gaugler, R., & Campbell, J.F. 1994. Host finding behaviour as a predictor of foraging strategy in entomopathogenic nematodes. *Parasitology*, **108**, 207-215.

Gouinguene, S., Degen, T., & Turlings, T. C. J. 2001. Variability in herbivore-induced odour emissions among maize cultivars and their wild ancestors (teosinte). *Chemoecology*, **11**, 9-16.

Hatanaka, A. 1993. The biogeneration of green odor by green leaves. *Phytochemistry,* **34**, 1201-1218.

Haukioja, E., & Hakala, T. 1975. Herbivore cycles and periodic outbreaks. *Reports from the Kevo Subarctic Research Station.* **12**, 1–9.

Hawkins, B.A., Mills, N.J., Jervis, M.A. & Price, P.W. 1999. Is the biological control of insects a natural phenomenon? *Oikos*, **86**, 493–506.

Heil, M. 2002. Ecological costs of induced resistance. *Current Opinion in Plant Biology*, **5**, 345-350.

Heil, M. 2008. Indirect defense via tritrophic interactions. *New Phytologist*, **178**, 41-61.

Hildebrand, D. F., Brown, G. C., Jackson, D. M., & Hamilton-Kemp, T. R. 1993. Effects of some leaf-emitted volatile compounds on aphid population increase. *Journal of Chemical Ecology,* **19**, 1875-1887.

Hiltpold, I., Toepfer, S., Kuhlmann, U., & Turlings, T. C. J. 2010. How maize root volatiles affect the efficacy of entomopathogenic nematodes in controlling the western corn rootworm. *Chemoecology,* **20**, 155-162.

Holeva, R., Phillips, M. S., Neilson, R., Brown, D. J., Young, V., Boutsika, K., & Blok, V. C. 2006. Real-time PCR detection and quantification of vector trichodorid nematode and Tobacco rattle virus. *Molecular and Cell Probes,* **20**, 203-211.

Howe, G. A., & Jander, G. 2008. Plant immunity to insect herbivores. *Annual Review Plant Biology,* **59**, 41-66.

Hui, E., & Webster, J. M. 2000. Influence of insect larvae and seedling roots on the host-finding ability of *Steinernema feltiae* (Nematoda: Steinernematidae). *Journal of Invertebrate Pathology,* **75**, 152-162.

Hunter, M. D. 2001. Out of sight, out of mind: The impacts of root- feeding insects in natural, managed systems. *Agricultural and Forest Entomology,* **3**, 3-10.

Hunter, M. D. 2002. A breath of fresh air: beyond laboratory studies of plant volatile-natural enemy interactions. *Agricultural and Forest Entomology*, **4**, 81-86.

Hutchinson, D. J. 1974. Swingle citrumelo- a promising rootstock hybrid. *Proceedings of the Florida State Horticultural Society*, **87**, 89-91.

Jaffee, B. A., & Strong, D. R. 2005. Strong bottom–up and weak top–down effects in soil: nematode–parasitized insects and nematode–trapping fungi. *Soil Biology and Biochemistry,* **37**, 1011–1021.

James, D. G., & Grasswitz, T. R. 2005. Synthetic herbivore-induced plant volátiles increase field captures of parasitic wasps. *Biocontrol*, **50**, 871-880.

Jansson, H. B., & Nordbringhertz, B. 1979. Attraction of nematodes to living mycelium of nematophagous fungi. *Journal of General Microbiology,* **112**, 89-93.

Jenkins, W. R. 1964. A rapid centrifugal–flotation technique for separating nematodes from soil. *Plant Disease Reporter*, **48**, 492.

Jones, R. V. H., & Surtherland, M. D. 1968. Terpenoid chemistry. XV. 1,5-Dimethylcyclodeca-1,5,7-triene, the precursor of geijerene in Geijera parviflora. *Australian Journal of Chemistry,* **21**, 2255-2264.

Kahl, J., Siemens, D. H., Aerts, R. J., Gäbler, R., Kühnemann, F., Preston, C. A., & Baldwin, I. T. 2000. Herbivore-induced ethylene suppresses a direct defense but not a putative indirect defense against an adapted herbivore. *Planta*, **210**, 336-342.

Kanagy, J. M. N., & Kaya, H. K. 1996. The possible role of marigold roots and alpha terthienyl in mediating host-finding by steinernematid nematodes. *Nematologica*, **42**, 220-231.

Kaplan, I., Halitschke, R., Kessler, A., Sardanelli, S., & Denno, R. F. 2008a. Constitutive and induced defenses to herbivory in above- and belowground plant tissues. *Ecology*, **89**, 392-406.

Kaplan, I., Halitschke, R., Kessler, A., Rehill, B. J., Sardanelli, S. & Denno, R. F. 2008b. Physiological integration of roots and shoots in plant defense strategies links above- and belowground herbivory. *Ecology Letters*, **11**, 841-851.

Karban, R. **& Baldwin, I.T.** 1997. Induced responses to herbivory, University of Chicago Press, Chicago, IL.

Karban, R., Agrawal, A. A., Thaler, J. S., & Adler, L. S. 1999. Induced plant responses and information content about risk of herbivory. *Trends in Ecology & Evolution,* **14**, 443-447.

Kaya, H. K., & Stock, S. P. 1997. Techniques in insect nematology, In L. A. Lacey [ed.], Manual of Techniques in Insect Pathology. Academic, San Diego, CA.

Kessler, A., & Baldwin, I. T. 2001. Defensive function of herbivore-induced plant volatile emissions in nature. *Science,* **291**, 2141-2144.

Kessler, A., & Baldwin, I. T. 2004. Herbivore-induced plant vaccination. Part I. The orchestration of plant defenses in nature and their fitness consequences in the wild tobacco *Nicotiana attenuate. The Plant Journal,* **38**, 639-649.

Khan, Z. R., Ampong-Nyarko, K., Chiliswa, P., Hassanali, A., Kimani, S., Lwande, W., Overholt, W. A., Picketta, J. A., Smart, L. E., & Woodcock, C. M. 1997. Intercropping increases parasitism of pests. *Nature*, **388**, 631-632.

Kigathi, R., Unsicker, S. B., Reichelt, M., Kesselmeier, J., Gershenzon, J., & Weisser, W. W. 2009. Emission of volatile organic compounds after herbivory from trifolium pretense under laboratory and field conditions. *Journal of Chemical Ecology*, **35**, 1335-1348.

Kiran, R. S., & Devi, P. S. 2007. Evaluation of mosquitocidal activity of essential oil and sequiterpenes from leaves of *Chloroxylon swietenia* DC. *Parasitology Research*, **101**, 413-418.

Kiran, S. R., Reddy, A. S., Devi, P. S., & Reddy, K. J. 2006. Insecticidal, antifeedant, and oviposition deterrent effects of the essential oil and individual compounds from leaves of Chloroxylon swietenia DC. *Pest Management Sci*ence, **62**, 1116-1121.

Klob, S., Knief, C., Stubner, S., & Conrad, R. 2003. Quantitative detection of methanotrophs in soil by novel pmoa–targed real–time PCR assays. *Applied and Environmental Microbiology*, **69**, 2423–2429.

Knapp, J. L. 1999. Florida Citrus Pest Management Guide. Cooperative Extension Service - IFAS, SP-43, Gainesville, FL.

Köllner, T. G., Held, M., Lenk, C., Hiltpold, I., Turlings, T. C. J., Gershenzon, J. & Degenhardt, J. 2008. A Maize (E)-{beta}-Caryophyllene Synthase Implicated in

Indirect Defense Responses against Herbivores Is Not Expressed in Most American Maize Varieties. *The Plant Cell*, **20**, 482-494.

Koppenhöfer, A. M., Campbell, J. F., Kaya, H. K., & Gaugler, R. 1998. Estimation of entomopathogenic nematode population density in soil by correlation between bait insect mortality and nematode penetration. *Fundamental and Applied Nematology*, **21**, 95–102.

Kubeczka, K. H. 1974. Pregeijeren, Hauptkomponente desätherischen Wurzelöls von Ruta graveolens. *Phytochemistry*,**13**, 2017-2018.

Kuzovkina, I. N., Szarka, Sz., Héthelyi, É., Lemberkovics, E., & Szöke, É. 2008. Composition of essential oil in genetically transformed roots of *Ruta graveolens*. *Russian Journal of Plant Physiology,* **56**, 846-851.

Landolt, P. J. 1993. Effects of host plant leaf damage on cabbage-looper moth attraction and oviposition. *Entomologia Experimentalis Et Applicata*, **67**, 79-85.

Lapointe, S. L. 2000. History and importance of *Diaprepes* to agriculture in the Caribbean region. *Diaprepes* short course. 8-12. Citrus Research and Education Center, Lake Alfred, FL.

Lapointe, S. L., & Shapiro, J. P. 1999. Effect of soil moisture on development of *Diaprepes abbreviatus* (Coleoptera: Curculionidae). *Florida Entomologist*, **82**, 291-299.

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J., Higgins, D. G. 2007. ClustalW2 and ClustalX version 2. *Bioinformatics*, **23**, 2947–2948.

Leal, I., Green, M., Allen, E., Humble, L., & Rott, M. 2007. Application of a real-time PCR method for the detection of pinewood nematode, Bursaphelenchus xylophilus, in Wood samples from lodgepole pine. *Nematology*, **9**, 351-36.

Lei, Z., Rutherford, T. A., & Webster, J. M. 1992. Heterorhabditid behavior in the presence of the cabbage maggot, *Delia radicum*, and its host plants. *Journal of Nematology,* **24**, 9-15.

Lewis, E. E. 2002. Behavioural Ecology. In: Gaugler, R. [ed.] Entomopathogenic Nematology. pp. 205-223. CAB International**.**

Lewis, E. E., Campbell, J. F., Griffin, C., Kaya, H. K., & Peters, A. 2006. Behavioral ecology of entomopathogenic nematodes. *Biological Control*, **38**, 66-79.

Lewis, E. E., Gaugler, R., & Harrison, R. 1992. Entomopathogenic nematode host finding- response to contact cues by cruise and ambush foragers. *Parasitology*, **105**, 309-315.

Lewis, E. E., Gaugler, R., & Harrison, R. 1993. Response of cruiser and ambusher entomopathogenic nematodes (Steinernematidae) to host volatile cues. *Canadian Journal of Zoology/Revue Canadienne De Zoologie,* **71**, 765-769.

Lewis, E. E., Grewal, P. S., & Gaugler, R. 1995. Hierarchical order of host cues in parasite foraging strategies. *Parasitology*, **119**, 207-213.

Loughrin, J. H., Manukian, A., Heath, R. R., & Tumlinson, J. H. 1995. Volatiles emitted by different cotton varieties damaged by feeding beet armyworm larvae. *Journal of Chemical Ecology*, **21**, 1217-1227.

Marshall, **D. R.** 1977. The Advantages and Hazards of Genetic Homogeneity. Annals of the New York Academy of Sciences, **287**, 1-20.

Masters, G. J., & Brown, V. K. 1992. Plant-mediated interactions between two spatially separated insects. *Functional Ecology*, **6**, 175–179.

Matsui, K., Kurishita, S., Hisamitsu, A., & Kajiwara, T. 2000. A lipid hydrolysing activity involved in hexenal foramtion. *Biochemical Society Transactions*, **28**, 857-860.

Mattiacci, L., Dicke, M., & Posthumus M. A. 1995. Beta-glucosidase: an elicitor of herbivore-induced plant odor that attracts host-searching parasitic wasps. *Proceedings of the National Academy of Science of the United States of America,* **92**, 2036-2040.

McCoy, C. W. 1999. Arthropod pests of citrus roots. In “Citrus Health Management” (L.W. Timmer and L.W. Duncan,Eds.), pp. 149-156. Aps Press, St. Paul, MN.

McCoy, C. W., Shapiro, D. I., Duncan, L. W., & Nguyen, K. 2000. Entomopathogenic nematodes and other natural enemies as mortality factors for larvae of Diaprepes abbreviatus (Coleoptera: Curculionidae). *Biological Control,* **19,** 182-190.

Mithöfer, A., Wanner, G., & Boland, W. 2005. Continuous mechanical wounding resembling insect feeding is sufficient to elicit herbivory-related volatile emission. *Plant Physiology,* **137**, 1160-1168.

Mráček, Z., Bečvář, S., Kindlmann, P., & Jersákova, J. 2005. Habitat preference for entomopathogenic nematodes, their insect hosts and new faunistic records for the Czech Republic. *Biological Control*, **34**, 27–37.

Munn, E. A., & Munn, P. D. 2002. Feeding and digestion. In: Lee D.L. [Ed.], The Biology of Nematodes. pp. 211-232. Taylor and Francis, London, UK.

Neveu, N., Grandgirard, J., Nenon, J. P., & Cortesero, A. M. 2002. Systemic release of herbivore-induced plant volatiles by turnips infested by concealed root-feeding larvae Delia radicum L. *Journal of Chemical Ecol*ogy, **28**, 1717-1732.

Nguyen, K. B., & Duncan, L. W. 2002. *Steinernema diaprepesi* n. sp. (Rhabditida: Steinernematidae), a parasite of the citrus root weevil *Diaprepes abbreviatus* (L) (Coleoptera: Curculionidae) *Journal of Nematology*, **34**, 159-170.

Norris RF, Caswell-Chen EP, Kogan M. 2003. Concepts in integrated pest management Prentice Hall:Upper Saddle River, NJ.

Ockroy, M. L. B., Turlings, T. C. J., Edwards, P. J., Fritzsche-Hoballah, M. E., Ambrosetti, L., Bassetti, P., & Dorn, S. 2001. Response of natural populations of predators and parasitoids to artificially induced volatile emissions in maize plants (Zea mays L.). *Agricultural and Forest Entomology*, **3**, 201-209.

Pace, M. L., Cole, J. J., Carpenter, S. R., Kitchell, J. F. 1999. Trophic cascades revealed in diverse ecosystems.*Trends in Ecology and Evolution.* **14**, 483–488.

Paré, P.W., & Tumlinson, J.H. 1999. Plant Volatiles as a Defense against Insect Herbivores. *Plant Physiology*, **121**, 325-331.

Pichersky, E., & Gershenzon, J. 2002. The formation and function of plant volatiles: perfumes for pollinator attraction and defense. *Current Opinions in Plant Biology*, **5**, 237-243.

Pickett, J.A., & Poppy, G.M. 2001. Switching on plant genes by external chemical signals. *Trends in Plants Science.* **6**,137–139.

Piel, J., Atzorn, R., Gäbler, R., Kühnemann, F., & Boland, W. 1997. Cellulysin from the plant parasitic fungus *Trichoderma viride* elicits volatile biosynthesis in higher plants via the octadecanoid signaling cascade. *FEBS Letters* **416**, 143-148.

Perry, R.N., & Aumann, J. 1998. Behaviour and sensory responses. In: Perry, R.N. & Wright, D.J. [Eds]. The physiologyand biochemistry of free-living and plant-parasiticnematodes. pp. 75-102. Wallingford, UK, CAB International.

Poinar, G. O. 1990. Taxonomy and biology of Steinernematidae and Heterorhabditidae. Entomopathogenic nematodes in biological control. R. Gaugler and H. K. Kaya. Boca Raton, CRC.

Price, P. W. 1986. Ecological basis of host plant resistance and Biological control: interactions among three trophic levels- In Boethell, D.J. & Eickenbery R.D. (eds), interaction of plant resistance and parasitiods and predators of insects. Wiley, New York, NY.

Price, P.W., Bouton, C.E., Gross, P., McPheron, B.A., Thompson, J.N., & Weis, A.E. 1980. Interactions among three trophic levels: influence of plants on interactions between insect herbivores and natural enemies. *Annual Review of Ecology and Systematics*. **11**, 41-65.

Prot, J-C., & van Gundy, S.D. 1981. Effects of soil texture and the clay component on migration of Meloidogyne incognita second-stage juveniles. *Journal Nematology*. **13**, 213-217.

Puustinen, S., Koskela, T., & Mutikainen, P. 2004. Direct and ecological costs of resistance and tolerance in the stinging nettle. *Oecologia*, **139**, 76-82.

R Development Core Team. 2004. R: a language and environment for statistical computing. R Foundation for Statistical Computing,Vienna, Austria (http://www.R-project.org).

Rasmann, S., Erwin, A.C., Halitschke, R., & Agrawal, A.A. 2011. Direct and indirect root defenses of milkweed (*Asclepias syriaca*): trophic cascades, trade-offs and novel methods for studying subterranean herbivory. *Journal of Ecology*. **99**,16–2.

Rasmann, S., Köllner, T.G., Degenhardt, J., Hiltpold, I., Töpfer, S., Kuhlmann, U., Gershenzon, J., & Turlings, T.C.J. 2005. Recruitment of entomopathogenic nematodes by insect-damaged maize roots. *Nature* **43,** 732-737.

Rasmann, S. & Turlings, T.C.J. 2007. Simultaneous feeding by aboveground and belowground herbivores attenuates plant-mediated attraction of their respective natural enemies. *Ecology Letters*, **10,** 926-936.

Rasmann, S. & Turlings, T.C.J. 2008. First Insights into Specificity of Belowground Tritrophic Interactions. *Oikos*.**117**, 362-369.

Rosenheim, J. A., Kaya, H. K., Ehler, L. E., Marois, J.J., Jaffee, B.A. 1995. Intraguild predation among biological-control agents: Theory and evidence. *Biological Control.* **5**, 303–335.

Rozen S., & Skaletsky H.J. 2000. Primer3 on the WWW for general users and for biologist programmers. In *Bioinformatics Methods and Protocols: Methods in Molecular Biology*, pp 365–386 Eds. S. Krawetz and S. Misener. Totowa, NJ, USA: Humana Press.

Santos, P.M., Figueiredo, A.C., Oliveira, M.M., Barroso, J.G., Pedro, L.G., Deans, S.G., Younos, A.K.M., & Scheffer, J.J.C. 1998. Essential oils from hairy root cultures and from fruits and roots of *Pimpinella anisum*, *Phytochemistry* **48**, 455–460.

Schmelz, E.A., Alborn, H.T., Engleberth, J., & Tumlinson, J.H. 2003. Nitrogen deficiency increases volicitin-induced volatile emission, jasmonic acid accumulation, and ethylene sensitivity in maize. *Plant Physiology* **133**, 295-306.\

Schnee, C., Köllner, T.G, Held, M., Turlings, T.C.J., Gershenzon, J. & Degenhardt, J. 2006. The products of a single maize sesquiterpene synthanse from a volatile defense signal that attracts natural enemies of maize herbivores. *Proceedings National Academy Science USA.* **103**,1129-1134.

Schroeder W. J. 1990. Suppression of Diaprepes abbreviates (Coleoptera: Curculionidae) adult emergence with soil application of entomopathogenic nematodes (Nematoda: Rhabditida). Florida. Entomologist **73,** 680–683.

Schroeder, W.J. 1992. Entomopathogenic nematodes for control of root weevils of citrus. *Florida Entomologist.* **73**, 563-567.

Schroeder, W.J. 1994. Comparison of two steinernematid species for control of the root weevil *Diaprepes abbreviatus. Journal of Nematology*, **26**, 360-362.

Shapiro-Ilan, D.I., & McCoy, C.W. 2000a. Virulence of entomopathogenic nematodes to *Diaprepes abbreviatus* (Coleoptera: Curculionidae) in the laboratory, *Journal Economic Entomology.* 93**,** 1090–1095.

Shapiro-Ilan, D.I., & McCoy, C.W. 2000b. Susceptibility of *Diaprepes abbreviatus* (Coleoptera: Curculionidae) larvae to different rates ofentomopathogenic nematodes in the greenhouse, *Florida. Entomology.* **83**, 1-9.

Shapiro-Ilan, D.I., Gouge, D.H., & Koppenhöfer, A.M. 2002. Factors affecting commercial success: case studies in cotton, turf and citrus. In: Gaugler, R. (Ed.), Entomopathogenic Nematology. CABI Publishing, Wallingford UK, pp. 333–355.

Skaria, M., & French, J.V. 2001. Phytophtora disease of citrus associated with root weevils in Texas. *Phytopathology* 91:S203. Publication no. P-2001-0016-s0a.

Simpson, S.E., Nigg, H.N., Coile, N.C., & Adair, R.A. 1996. *Diaprepes abbreviatus* (Coleoptera: Curculionidae): host plant associations. *Environmental Entomology.* **25**, 333-349.

Simpson, S. E., Nigg, H. N., & Knapp, J. L. 2000. Host plants of Diaprepes root weevil and their implications to the regulatory process. *Diaprepes* short course. 19-37. Citrus Research and Education Center Lake Alfred, FL.

Snyder, W. E., Chang, G. C., Prasad, R. P. 2005. Conservation biological control: Biodiversity influences the effectiveness of predators. In: Barbosa P, Castellanos I, editors. *Ecology of predator-prey interactions.* London, UK: Oxford University Press. pp. 324–343.

Soler, R., Bezemer, T.M., Cortesero, A.M., Van Der Putten, W.H., Vet, L.E.M. & Harvey, J.A. 2007. Impact of foliar herbivory on the development of a root-feeding insect and its parasitoid. *Oecologia*, 152, 257-264.

Soler, R., Bezemer, T M., van Der Putten, W.H., Vet, L E.M., & Harvey, J A. 2005. Root herbivore effects on above-ground herbivore, parasitoid and hyperparasitoid performance via changes in plant quality. *Journal of Animal Ecology*. 74, 1121–1130.

Southey, J.F. 1986. Laboratory Methods for Work with Plant and Soil Nematodes. Her Majesty's Stationery Office. London,UK.

Southwood, T. R. E. & Comins, H. N. 1976. A Synoptic Population Model. *J. Anim. Ecol.* **45**, 949-65.

Stover, E., & Castle, W. 2002. Citrus rootstock usage in the Florida Indian River region. *Hortocultural Technology.* **12**,143-147.

Strauss, S. Y., Rudgers, J. A., Lau, J. A., & Irwin, R. A. 2002. Direct and ecological costs of resistance to herbivory. *Trends Ecology & Evolution.* **17**, 278–85.

Strong, D. R., Lawton, J.H. & Southwood, T.R.E. 1984. Insects on Plants. Oxford: Oxford University Press.

Stuart, R.J. Barbercheck, M.E., Grewal, P.S., Taylor, R.A.J., & Hoy, C.W. 2006 Population biology of entomopathogenic nematodes: Concepts, issues, and models. *Biological Control*. **38**, 80–102.

Stuart, R.J., El-Borai, F.E., & Duncan, L.W. 2008. From Augmentation to Conservation of Entomopathogenic Nematodes: Trophic Cascades, Habitat Manipulation and Enhanced Biological Control of *Diaprepes abbreviatus* Root Weevils in Florida Citrus Groves. *Journal of Nematology.* **40**,73-84.

Takabayashi, J., & Dicke, M. 1996. Plant-carnivore mutualism through herbivore-induced carnivore attractants. *Trends in Plant Science.* **1**, 109-113.

Takabayashi, J., Dicke, M., & Posthumus, M. A. 1991. Variation in composition of predator-attracting allelochemicals emitted by herbivore-infested plants: relative influence of plant and herbivore. *Chemoecology*. **2**, 1-6.

Takabayashi, J., Takahashi, S., Dicke, M., & Posthumus, M. A. 1995. Developmental stage of herbivore *Pseudaletiaseparata* affects production of herbivore-induced synomone by corn plants. *Journal of Chemical Ecology* **21**, 273-287.

Thaler, J. S. 1996. Exogenous jasmonates simulate insect wounding in tomato plants (Lycopersicon esculentum) in the laboratory and field. *Journal Chemical Ecology* **22**, 1767-1781.

Thaler, J. S.1999. Jasmonate-inducible plant defenses cause increased parasitism of herbivores. *Nature,* **399**, 686-688.

Thaler, J. S., Farag, M., Pare, P., & Dicke, M. 2002. Jasmonate deficient tomato mutant has reduced direct and indirect defense. *Ecology Letters*, **5,** 764-774.

Tiwari, S., Youngman, R. R., Lewis, E. E., & Eisenback, J. D. 2009. European corn borer (Lepidoptera: Crambidae) stalk tunneling on Root-knot nematode (tylenchida: Heteroderidae) fitness on corn. *Journal of Economic Entomology*, **102**, 602-609.

Torr, P., Heritage, S., & Wilson, M. J. 2004. Vibrations as a novel signal for host location by parasitic nematodes. *International Journal for Parasitology*, **34**, 997-999.

Torr, P., Spiridonov, S. E., Heritage, S., & Wilson, M. J. 2007. Habitat associations of two entomopathogenic nematodes:a quantitative study using real-time quantitative polymerase chain reactions. *Journal of Animal Ecology*, **76**, 238–245.

Tumlinson, J. H., Paré, P. W., Alborn, H. T., & Lewis, W. J. 1999. Chemically mediated tritrophic plant -insect interactions. In de Wit, P. J. G. M., Bisseling, T., and Stiekema, W.J. (Eds.) Biology of Plant-Microbe Interactions, Vol. 2. Proc. 9th Intl. Cong. on Molecular Plant -Microbe Interactions, pp 378-383.

Tumlinson, J. H., Turlings, T. C. J., & Lewis, W. J. 1992. The semiochemical complexes that mediate insect parasitoid foraging. *Agricultural Zoology Review,* **5**, 221–252.

Turlings, T. C. J., Alborn, H. T., Loughrin, J. H., & Tumlinson, J. H. 2000. Volicitin, an elicitor of maize volatiles in the oral secretion of Spodoptera exigua: its isolation and bio-activity. *Journal of Chemical Ecology,* **26**, 189-202.

Turlings T. C. J., Davison, A. C., & Tamo, C. 2004. A six-arm olfactometer permitting simultaneous observation of insect attraction and odour trapping. *Physiology Entomology*, **29**, 45–55.

Turlings, T. C. J., Lengwiler, U. B., Bernasconi, M. L., & Wechsler, D. 1998. Timing of induced volatile emissions in maize seedlings. *Planta*, **207**, 146-152.

Turlings, T. C. J., Loughrin, J. H., McCall, P. J., Röse, U. S., Lewis, W. J., & Tumlinson, J. H.1995. How caterpillar-damaged plants protect themselves by

attracting parasitic wasps. *Proceedings of the Natural Academy of Science United States of America,* **92**, 4169-4174.

Turlings, T. C. J., McCall, P. J., Alborn, H.T., & Tumlinson, J.H. 1993. An elicitor in caterpillar oral secretions that induces corn seedlings to emit chemical signals attractive to parasitic wasps. *Journal of Chemical Ecology,* **19**, 411-425.

Turlings, T. C. J., & Ton, J. 2006. Exploiting scents of distress: the prospect of manipulating herbivore-induced plant odours to enhance the control of agricultural pests. *Current Opinions in Plant Biology*, **9**, 421-427.

Turlings, T. C. J., Tumlinson, J. H., & Lewis, W. J. 1990. Exploitation of herbivore-induced plant odors by host-seeking parasitic wasps. *Science*, **250**, 1251-1253.

Turlings, T. C. J., & Wäckers, F. L. 2004. Recruitment of predators and parasitoids by herbivore-damaged plants. In (R. T. Cardé and J. Millar eds.) Advances in Insect Chemical Ecology. Cambridge University Press, pp. 21-75.

Turlings, T. C. J., Wäckers, F. L., Vet, L. E. M., Lewis, W. J., & Tumlinson, J. H.1993. Learning of host-finding cues by hymenopterous parasitoids. Insect Learning: Ecological and Evolutionary Perspectives. D. R. Papaj and Lewis. New York, Chapman & Hall**,** pp. 51-78.

van Dam, N. M. 2009. Belowground herbivory and plant defenses. *Annual Review Ecology & Systematics,* **40**, 373-91.

van Dam, N. M. & Heil, M. 2011. Multitrophic interactions below ground and above ground: *en route* to the next level. *Journal of ecology*, **99**, 77-88.

van Dam, N. M., Raaijmakers, C. E., & van der Putten, W. H. 2005. Root herbivory reduces growth and survival of the shoot feeding specialist *Pieris rapa*e on *Brassica nigra. Entomologia Experimentalis et Applicata*, **115**, 161-170.

van den Boom, C. E., van Beek, T. A., Posthumus, M. A., de Groot, A., & Dicke, M. (2004). Qualitative and quantitiative variation among volatile profiles induced by *Tetranychus urticae* feeding on plants from various families. *Journal of Chemical Ecology,* **30**, 69-89.

van Poecke, R. M., & Dicke, M. 2004 Indirect defense of plants against herbivores: Using Arabidopsis thaliana as a model plant. *Plant Biology,* **6**, 387-401.

van Tol, R., & Sommen, A. T. C. 2001. Plants protect their roots by alerting the enemies of grubs. *Ecology Letters,* **4**, 292-294.

Vet, L. E. M., & Dicke, M. 1992. Ecology of infochemical use by natural enemies in a tritrophic context. *Annual Review of Entomology,* **37**, 141–172.

Vet, L. E. M., Wäckers, F. L., & Dicke, M. 1991. How to hunt for hiding hosts: the reliability-detectability problem in foraging parasitoids. *Netherlands Journal of Zoology,* **41**, 202–213.

Vuorinen, T., Nerg, M. A., Ibrahim, Reddy, G.V.P. & Holopainen, J.K. 2004. Emission of *Plutella xylostella*-induced compoundsfrom cabbages grown at elevated CO2 and orientation behavior of the natural enemies. *Plant Physiology,* **135,** 1984-1992.

Vranova, E., Inze, D. & Breusegem, F. 2002. Signal transduction during oxidative stress. *Journal of Experimental Botany* **53**, 1227-1236.

Watson, N.B. 1903. The root-borer of sugar-cane. *West Indian Bulletin,* **4**, 37-47.

Weissling, T. J., Peña, J. E., Giblin-davis, R. M. J. R., & Knapp, J. L. 2002. Sugarcane rootstock borer weevil, *Diaprepes abbreviatus* (L.). *Featured creatures*, Univ. of Florida, Gainesville, FL.

White, G. 1927. A method for obtaining infective nematode larvae from culture. *Science* **66**, 302–303.

Whitman, D. W. 1988. Allelochemicals interactions among plants, herbivores, and their predators, John Wiley and Sons.

Woodruff, R.E. 1964. A Puerto Rican weevil new to the United States (Coleoptera: Cuculionidae). Florida Department of Agriculture. *Plant Industry Entomology Circular,* **77**, 1-4.

Zangerl, A.R. & Bazzaz, F.A. 1992. Theory and pattern in plant defense allocation. In: RS Fritz & EL Simms [eds] Plant Resistance to Herbivores and Pathogens. Ecology, Evolution, and Genetics, pp. 363–91. Chicago: University of Chicago Press.

Zangerl, A.R. & Rutledge, C.E. 1996. The probability of attack and patterns of constitutive and induced defense: a test of optimal defense theory. *American Naturalist*, **147**, 599–608.

Zhang L.M., Liu X.Z., Zhu S.F., Chen S.Y. 2006. Detection of the nematophagous fungus *Hirsutella rhossiliensis* in soil by real time qPCR and parasitism bioassay. *Biological Control*, **36**, 316–323.

BIOGRAPHICAL SKETCH

Jared Gregory Ali was born in Philadelphia, Pennsylvania. Jared was educated in Quaker schools for elementary, middle, and high school. Here he developed a longing to explore an alternative path of knowledge. Jared left high school his junior year and traveled through the United States, Canada and Mexico. Reading many classic works of philosophy and science he came across Thomas Lewis' 'The Lives of a Cell'. This book inspired Jared to approach his education through the study of biological interactions and evolution. After completing his GED and writing an expressive letter to the University of Delaware, he was offered admission to the College of Arts and Sciences. He majored in biological sciences as an undergraduate and continued on to complete his M.Sc. in *Entomology & Wildlife Ecology* under the advisement of Dr. Douglas W. Tallamy, studying sexual selection and chemical communication. He moved to Florida to pursue a Ph.D. under Dr. Lukasz L. Stelinski, where his research encompassed belowground multitrophic interactions and chemical ecology. Upon the completion of his Ph.D. at the University of Florida, Jared accepted an opportunity to study plant defense and multitrophic interactions in the department of *Ecology & Evolutionary Biology* at Cornell University, under the supervision of Dr. Anurag Agrawal.