Mechanisms of Olfaction in Parasitic Wasps: Analytical and Behavioral Studies of Response of a Specialist (*Microplitis croceipes*) and a Generalist (*Cotesia marginiventris*) Parasitoid to Host-Related Odor

by

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Abstract

Parasitic wasps (parasitoids) are known to utilize as host location cues various types of host-related volatile signals. These volatile signals could be plant-based, originate from the herbivore host, or be produced from an interaction between herbivores and their plant host. The success of parasitoids in suppressing pest populations depends on their ability to locate hosts in a complex olfactory and visual environment. Despite the intense interest in host-parasitoid interactions, certain aspects of olfactory communication in this group of insects are not well understood. This study was conducted to characterize mechanisms of olfaction and response to host-related odor in two parasitic wasps (Hymenoptera: Braconidae) with different degrees of host specificity, *Microplitis croceipes* (Cresson) (specialist) and *Cotesia marginiventris* (Cresson) (generalist), using an integration of analytical, behavioral and electrophysiological techniques. Specific objectives are: (1) Electroantennogram (EAG) responses of *M. croceipes* and *C. marginiventris* and their lepidopteran hosts to a wide array of odor stimuli: Correlation between EAG response and degree of host specificity?; (2) Comparative GC-EAD responses of a specialist (*M. croceipes*) and a generalist (*C. marginiventris*) parasitoid to cotton volatiles induced by two caterpillar species; (3) Effects of plant growth-promoting rhizobateria (PGPR) on the induction of cotton volatiles and consequences for response of parasitoids; and (4) Sexual and species differences in the behavioral response of a specialist and generalist parasitoid species to host-related volatiles.

In chapter II, studies were conducted in order to test whether the electroantennogram (EAG) response spectrum of an insect correlates to its degree of specificity. We recorded EAG responses of two parasitoid species with different degrees of host specificity, *M. croceipes* (specialist) and *C. marginiventris* (generalist) and their lepidopteran hosts (Lepidoptera: Noctuidae) (*Heliothis virescens* Fab. and *Spodoptera exigua* Hübner), to a wide array of odor stimuli. The compounds tested included green leaf volatiles (GLVs), herbivore-induced plant volatiles (HIPVs), ecologically irrelevant plant volatiles, and several types of host specific odor stimuli including synthetic host sex pheromones and extracts of host caterpillar and body frass. The specialist parasitoid showed greater EAG responses than the generalist to host-specific odor and one HIPV (*cis*-3-hexenyl butyrate), whereas the generalist showed relatively greater EAG responses to the GLVs and unrelated plant volatiles. There were no differences in the EAG responses of *H. virescens* and *S. exigua* to any of the tested odors. In Chapter III, a comparative study was done to determine similarities and differences in GC-EAD (coupled gas chromatography electroantennogram detection) responses of both parasitoid species to headspace volatiles of cotton plants damaged by *H. virescens* (a host species for both parasitoids) vs. *S. exigua* (a host species of *C. marginiventris*). Thirty volatile components were emitted by cotton plants in response to feeding by either of the two caterpillars, however, 18 components were significantly elevated in the headspace of *H. virescens* damaged plants. Sixteen components consistently elicited GC-EAD responses in both parasitoids. *Cotesia marginiventris* showed significantly greater GC-EAD responses than *M. croceipes* to most green leaf volatile components, whereas several herbivore-induced volatile components elicited comparatively greater responses in *M. croceipes*. Results suggest that differences in the ratios of identical

volatile compounds between similar volatile blends may be used by specialist parasitoids to discriminate between host-plant and non-host plant complexes.

In Chapter IV, studies were conducted to evaluate the potential of plant growth-promoting rhizobacteria (PGPR) on the induction of cotton volatiles and consequences for response of parasitoids. Three PGPR treatments were evaluated: i) *Bacillus pumilis* strain INR-7, ii) Blend 8, and iii) Blend 9. An untreated (water) control was also tested. There were quantitative and qualitative differences in headspace volatiles collected from PGPR treated and untreated cotton plants. A total of eleven peaks were detected from headspace of PGPR treated cotton plants but only three peaks were detectable in untreated cotton plants. Differences in root growth between PGPR treated vs. untreated plants were recorded, with Blend 9 recording the highest root growth. PGPR treated plants were also very highly attractive to parasitoids, with Blend 9 being the most attractive. In Chapter V, studies were done to determine if there were sexual and species differences in the behavioral response of a specialist and generalist parasitoid species to host-related volatiles. Y-tube olfactometer bioassays were conducted to compare responses of naïve females and males of both parasitoid species to select synthetic plant-based host-related volatiles; two GLVs (hexanal and (*Z*)-3-hexen-1-ol) and four HIPVs ((*Z*)-3-hexenyl acetate, linalool, (*Z*)-3-hexenyl butyrate, and (*E,E*)-α-farnesene). Linking previous reported electrophysiological responses (Chapter II) to behavioral observations, results revealed key differences in behavioral responses of both parasitoid species to the tested host-related plant volatiles. The specialist parasitoid (*M. croceipes*) was more responsive to most of the HIPVs, whereas, *C. marginiventris* (generalist) showed greater responses to the GLVs. Females of both parasitoid species showed greater behavioral responses than conspecific males. These results

advance our understanding of mechanisms of olfaction, semiochemical-mediated responses, and foraging strategies in parasitoids with different degrees of host specificity.

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Dedication

This work is dedicated to my parents, Mr. and Mrs. Harrison Ngumbi

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Host Location in Insect Parasitoids

Host location in insect parasitoids has received significant attention due to the importance of parasitoids in controlling insect pest populations (Cortesero et al. 1993, Steidle and Scholler 1997, Steidle et al. 2003). Doutt (1964) and Vinson (1976, 1998) divided the process resulting in successful parasitism by insect parasitoids into five steps: host habitat location, host location, host acceptance, host suitability, and host regulation. The first three steps constitute the host selection process. In each of these steps, the female parasitoid often uses chemical stimuli to guide her in the search of a suitable host. Host location and host acceptance are active fields of research, mostly centered around identification of stimuli involved and characterization of behavioral responses of parasitoids to the stimuli (Godfray 1994, Quicke 1997). To succeed, parasitoids must develop efficient strategies for locating hosts in complicated heterogenous environments and for overcoming host defenses and competitors (De Moraes et al. 2000). Such strategies will likely involve exploitation of numerous cues and foraging tactics at multiple scales as well as the development of behavioral and physiological adaptations to the internal host environment. The ability of parasitoids to succesfully locate their hosts is influenced by the interaction of many sources of variation including (1) genetic variation between individuals adapted to different foraging environments (Drost et al. 1988, Prevost and Lewis 1990), (2) phenotypic plasticity of individuals allowing behavioral adaptation to different host habitats (Lewis and Tumlinson 1988, Vet et al. 1990, Lewis et al. 1991), and (3) the parasitoids' physiological state with regard to non-host resources such as food, egg load, or mating

opportunities (Takasu and Lewis 1993, Sirot and Bernstein 1996). Other additional factors that can contribute to succesful host location by parasitoids include climatic conditions, habitat type, and host density (Godfray 1994). Elucidating the processes that lead to succesful host location by parasitoids and identifying the semiochemicals involved increase our understanding and provide potential for the manipulation of these communication systems (Whitman 1988).

1.1.1 Semiochemicals Used by Parasitoids for Foraging and Host Location

The success of parasitoids in suppressing pest populations depends on their ability to locate hosts in a complex olfactory and visual environment. Thus, understanding the mechanisms guiding parasitoids in selecting and distinguishing between different hosts is very critical. Parasitoids use semiochemicals (volatile signals mediating communication) for foraging and location of herbivore hosts (Dicke and Sabelis 1988, Turlings et al. 1990, McCall et al. 1993, De Moraes et al. 1998). These volatile signals can be plant-based, originate from the herbivore host, or produced from an interaction between herbivores and their plant hosts (Turlings et al. 1990, McCall et al. 1993, De Moraes et al. 1998). Volatile signals originating from plants can further be sub-divided into two major groups: constitutive compounds and inducible compounds. Constitutive compounds are constantly present in the plants and are released immediately in response to mechanical plant damage or at the beginning of herbivore feeding damage. Constitutive compounds in cotton plants include *cis*-3-hexenal, hexenal, *trans*-2-hexenal, and *cis*-3-hexenol (Loughrin et al. 1994, McCall et al. 1994). On the other hand, inducible compounds are emitted as a delayed response to herbivore feeding damage. These volatiles are the most reliable cues for the foraging parasitoid if the released compounds are specific for the herbivore species or when the cues can be learned by a parasitoid (Dicke and Vet 1999).

Herbivore-induced chemicals have been described for several plant species, including Lima bean plants (*Phaseolus lunatus* L.) that produce volatiles that attract the predatory mite *Phytoseiulus persimilis* Athias-Henriot (Acari: Phytoseiidae) when damaged by the spidermite *Tetranychus urticae* Koch (Acari: Tetranychidae) (Dicke et al. 1993, Geervliet et al. 1994) or maize plants (*Zea mays* L.) that produce volatiles that attract the hymenopteran larval parasitoid *Cotesia marginiventris* (Cresson) (Hymenoptera: Braconidae) when under attack by *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) caterpillars (Turlings et al. 1991). Cotton (*Gossypium hirsutum* L.) plants have also been shown to produce herbivore-induced volatile compounds (HIPVs) that include *cis*-3-hexenyl acetate, linalool, indole, *cis*-3-hexenyl butyrate, *trans*-2-hexenyl butyrate, (E,E)-α-farnesene, and (E)-β-caryophyllene (Loughrin et al. 1994, McCall et al. 1994, De Moraes et al. 1998). These cotton HIPVs have been shown to be attractive to several parasitoids including *Cardiochiles nigriceps* Viereck (Hymenoptera: Braconidae), *C. marginiventris*, Cresson and *Microplitis croceipes* Cresson (Hymenoptera: Braconidae) (Cortesero et al. 1997, Röse et al. 1998, De Moraes et al. 1998, Chen and Fadamiro 2007).

The chemical composition of plant volatiles released in response to herbivore damage varies among plant tissues (Turlings et al. 1993), varieties/cultivars (Takabayashi et al. 1991, Loughrin et al. 1994), and plant development stage (Takabayashi et al. 1994). Several abiotic factors (light intensity, time of year, water stress) (Takabayashi et al. 1994, Gouinguené and Turlings 2002) are also known to influence the chemical composition of plant volatiles. Beyond that, time of day also influences the composition of an emitted volatile blend (De Moraes et al. 2001). Importantly, quantitative and qualitative differences have been recorded in the volatile chemical profile of cotton induced by different herbivores. McCall et al. (1994), compared composition of volatile blends emitted by undamaged cotton, freshly damaged cotton (0-2 hours

after initiation of feeding by the corn earworm *Helicoverpa zea* Boddie (Lepidoptera: Noctuidae larvae)), and old damaged cotton (16-19 after initiation of feeding by *H. zea* larvae). The authors reported nine compounds emitted only by old damaged plants compared with freshly damaged plants, including (Z)-3-hexenyl acetate, (E)-β-ocimene, (3E)-4,8-dimethyl-1,3,7-nonatriene, (Z)-3-hexenyl butyrate, (E)-2-hexenyl butyrate, (Z)-3-hexenyl-2-methylbutyrate, (E)-2-hexenyl 2-methylbutyrate, and indole. Loughrin et al. (1994) conducted a similar study with cotton seedlings damaged by another related herbivore, beet armyworm *S. exigua* larvae, and reported a similar difference in the composition of volatile blends emitted by freshly damaged versus old damaged plants. However, compounds that were produced only (or in significantly greater amounts) by old damaged plants relative to freshly or undamaged plants included (E)-β-ocimene, (E,E)-α-farnesene, (E)-β-farnesene, linalool, and (3E)-4,8-dimethyl-1,3,7-nonatriene, many of which were not reported by McCall et al. (1994) as emitted by old damaged plants infested with *H. zea* larvae. These results suggest that plants may produce distinct volatile blends in response to different herbivores. Additional studies have shown that the volatile blend signature produced by different plants in response to different herbivores may convey herbivore-specific information to parasitoids and may be utilized by specialist parasitoids for host specificity (Du et al. 1996, De Moraes et al. 1998, De Moraes and Lewis 1999). The authors reported that tobacco, (*Nicotiana tabacum* L.), cotton, and maize plants each emit distinct chemical volatile profiles in response to damage by *H. zea* or sympatric *Heliothis virescens*, Fab. (Lepidoptera: Noctuidae), and the specialist parasitoid *Cardiochiles nigriceps* Viereck (Hymenoptera: Braconidae) was able to exploit these differences in volatile blends produced in response to different herbivores to distinguish infestation by its host *H. virescens* from that by *H. zea* (De Moraes et al. 1998). Additionally, composition of volatile blends released by the plants may vary during the light and

dark phases of the photoperiod (Loughrin et al. 1994), with important ramifications on host location by herbivores and their natural enemies (De Moraes et al. 2001).

Since successful foraging is directly linked with reproductive success, natural selection will favor animals that make optimal use of foraging cues. This implies that parasitoids should respond to the most reliable cues that can be detected (Geervliet et al. 1994). An efficient strategy would be to respond to HIPVs. In addition to being highly detectable, these HIPVs may also be reliable, through the specific interaction of the plant with the herbivore (Dicke et al. 1990, Vet and Dicke 1992).

1.1.2 Specificity of Semiochemicals Used by Parasitoids for Foraging and Host Location

The relationship between the degree of specialization of parasitoids and the specificity of signals needed for successful foraging and host location is an important and current evolutionary question (Vet and Dicke 1992, Vet et al. 1993, Geervliet et al. 1997, Bernays 2001, Steidle and van Loon 2003, Chen and Fadamiro 2007). It is predicted that specialist parasitoid species, utilizing a relatively few number of host species, are expected to possess a more highly sensitive (high olfactory sensitivity to host-related volatiles) and narrowly tuned (selective) host detection olfactory system than generalist parasitoids (Vet and Dicke 1992, Cortesero et al. 1997, Smid et al. 2002, Chen and Fadamiro 2007, Das et al. 2011). This highly efficient host detection system is likely to be expressed in their innate electrophysiological or behavioral responses to host-derived cues, such as herbivore products (feces, silk) and the herbivore itself, or to specific herbivore-induced plant volatiles (Geervliet et al. 1994). For generalist parasitoids, such fixed responses to specific stimuli do not seem functional or may be impossible due to more

physiological constraints compared to specialists. Instead, they are hypothesized to be guided to their hosts by more general stimuli and subsequently learn to respond to more specific stimuli (Vet and Dicke 1992, Geervliet et al. 1994). Comparative olfactory and behavioral responses of generalist and specialist parasitoids to host-related volatiles are rare, and the few studies have produced contrasting reports. On the one hand, some studies reported relatively greater response for specialists compared to generalists (Elzen et al. 1987, Vet et al. 1993, Chen and Fadamiro 2007). Chen and Fadamiro (2007) reported key differences in the electroantennogram (EAG) responses of *C. marginiventris* (a generalist) and *M. croceipes* (a specialist) to different types of host-related volatiles. *Microplitis croceipes*, the specialist parasitoid, was reported to show greater responses to HIPVs, compounds that are specifically linked to their hosts. On the contrary, *C. marginiventris* a (generalist) showed greater responses to green leaf volatiles (GLVs), which are continuously present in the plant and released by freshly damaged plants. In contrast, Geervliet et al. (1996) recorded no difference in the behavioral responses of the specialist *Cotesia rubecula* Marshall (Hymenoptera: Braconidae) and the generalist *C. marginiventris* to host-related volatiles, and both species were unable to distinguish between plant volatiles induced by their hosts versus plant volatiles induced by non-host species. Similarly, Smid et al. (2002) reported no difference in the receptive range of specialist *C. rubecula* and the generalist *Cotesia glomerata* Apanteles (Hymenoptera: Braconidae) to a wide range of host-plant odor compounds. Such discrepancies suggest that diverse species of specialist or generalist parasitoids may respond differently to different types of host-related volatiles. Furthermore, even within a broad category of specialist or generalist parasitoids, differences may exist among species based on the degree of specialization (De Moraes et al. 1998, Tamò et al. 2006).

1.2 Techniques Used to Characterize Responses of Parasitoids to Host-Related Odors

Electrophysiological experiments on insect antennae are performed both as physiological studies on the function of olfactory sense and as a tool for identifying behaviorally-active odors. The most important techniques include the electroantennogram (EAG) and coupled gas chromatography-electroantennographic detection (GC-EAD) (Bjostad 1998).

Electroantennogram (EAG), a technique which measures the electrophysiological responses in the insect antennae, provides a general measure of odorant reception at the peripheral level (Roelofs 1977, Van der Pers and Minks 1998, Park et al. 2002). EAG responses of parasitoids to host-related chemicals have been demonstrated (Li et al. 1992, Salom et al. 1992, Whitman and Eller 1992, Ochieng et al. 2000, Gouinguené et al. 2005, Chen and Fadamiro 2007). Similarly, EAG responses of moths (Lepidoptera) to plant volatiles and pheromone components have also been reported (Jönsson and Anderson 1999, Burguiere et al. 2001, Rajapakse et al. 2006). Research has shown that EAG response spectra to a range of odorants are somewhat species specific (Smith and Menzel 1989, Visser et al. 1996, Visser and Piron 1997). Differences in EAG recordings have been used as a diagnostic tool to relate differences in pheromone detection to genetic differences between *H. virescens* and *Heliothis subflexa* Guenée (Lepidoptera: Noctuidae) (Groot et al. 2005). The EAG technique was also recently used to test for sexual and species differences in the olfactory responses of a specialist (*M. croceipes*) and a generalist (*C. marginiventris*) to different host-related compounds (Chen and Fadamiro 2007). Females of both species exhibited significantly greater EAG responses than conspecific males to GLVs (*cis*-3-hexenol and hexanal), which are released immediately after initiation of herbivore feeding damage. In contrast, males showed greater EAG responses than conspecific females to inducible compounds (*cis*-3-hexenyl acetate, linalool, and (E,E)-α-farnesene) that are released much later

after the initial damage. However, it remains unclear whether EAG response actually correlates to olfactory sensitivity and could be used as a measure of host specificity among insects, and this is one of the questions being asked in this study.

Coupled gas chromatography-electroantennographic detection (GC-EAD) is a precise and reliable technique for the detection of physiologically-active components present in volatile emissions and has been employed successfully to determine electrophysiologically-active compounds in several plant-herbivore-parasitoid systems (Ngi-song and Overholt 1997, Ngi-Song et al. 2000, Smid et al. 2002, Gouinguené et al. 2005). Smid et al. (2002) used the GC-EAD technique to compare the responses of a specialist (*C. rubecula*) and generalist (*C. glomerata*) parasitoid of *Pieris* caterpillars on brussels sprouts and showed that the two parasitoid species responded similarly to a large number of compounds present in the odor of damaged brussel sprout plants. More recently, Gouinguené et al. (2005) used this technique to study electrophysiological responses of three parasitoids (*C. marginiventris* Cresson, *Microplitis rufiventris*, Kok. (Hymenoptera: Braconidae), and *Campoletis sonorensis* Cameron (Hymenoptera: Ichneumonidae) to caterpillar-induced volatiles from maize, cotton, and cowpea (*Vigna unguiculata* L.). Their findings showed that wasps responded to many, but not all compounds, present at physiologically relevant levels. Interestingly, some minor compounds elicited strong responses from the wasps (Gouinguené et al. 2005).

Electrophysiological techniques have the advantage of online identification of the electrophysiologically-active components of volatile blends; however these compounds are not always behaviorally-active to insects (Bjostad 1998). The behavioral significance of these compounds therefore needs to be evaluated in behavioral experiments. Olfactometers (in particular Y-tube and four-choice types) have been used to test for behavioral responses of

parasitoids to host-related chemicals (Jones 1986, Whitman and Eller 1990, Cortesero et al. 1997, Röse et al. 1998, Guerrieri et al. 1999, Potting et al. 1999), host-specific kairomones (e.g. frass, host pheromones) (Loke and Ashley 1984, Röse et al. 1998), and conspecific sex pheromones (Udayagiri and Jones 1992, Suckling et al. 2002). Many of these studies have demonstrated that parasitoids are attracted to a wide range of odor, in particular host related chemicals. For instance, Eller et al. (1988), using the four-choice olfactometer to study the behavioral responses of both sexes of *M. croceipes* to volatiles from the plant-host complex, showed that *M. croceipes* was strongly attracted to volatiles from the plant-host complex placed on filter paper.

1.3 Plant Growth-Promoting Rhizobacteria (PGPR)

Recent quests for effective, safe and lasting pest management programs have targeted the development of new and better products with which to replace conventional pesticides. This includes application of biocontrol agents and products for management of plant pests. Among the different biocontrol agents, many authors have described the potential use of plant growth-promoting rhizobacteria (PGPR) (Kloepper and Schroth 1978). PGPR represent a wide range of root-colonizing bacteria whose application is often associated with increased rates of plant growth (Kloepper 1992, Zehnder et al. 1997), suppression of soil pathogens (Schippers et al. 1987), and the induction of systemic resistance against insect pests (van Loon et al. 1998, Kloepper et al. 1999, Ramamoorthy et al. 2001, Ryu et al. 2004). Induction of resistance by PGPR strains significantly reduced the populations of striped cucumber beetle *Acalyma vittatum* Fabricius (Coleoptera: Chrysomelidae) and spotted cucumber beetle *Diabrotica undecimpunctata* Howardi (Coleoptera: Chrysomelidae) on cucumber (*Cucumis sativus* L.)

(Zehnder et al. 1997). The effects of application of PGPR on induction of volatile organic compounds (VOCs) in treated plants have gone virtually unexamined, despite evidence that induction of plant volatiles is dependent on the interactions of biotic factors, such as plant hormones (de Bruxelles and Roberts 2001, Thaler et al. 2002, Farmer et al. 2003, Ament et al. 2004), herbivore-derived elicitors (Mattiaci et al. 1995, Alborn et al. 1997, Spiteller and Boland 2003), and associated microorganisms including pathogens (Preston et al. 1999, Cardoza et al. 2002) and abiotic factors, such as wounding (Mithofer et al. 2005), heavy metals (Mithofer et al. 2004), and temperature and light (Takabayashi et al. 1994, Gouinguené and Turlings 2002). The lack of research on the role of VOCs in host plant-PGPR-herbivore-parasitoid interactions is surprising considering the explosive growth in research in herbivory-induced plant volatiles and their effects on arthropod herbivores, predators, and parasitoids (De Moraes et al. 1998, Dicke 1999, Pare et al. 1999, Pare and Tumlinson 1999, Kessler and Baldwin 2001, Pichersky and Gershenzon 2002). Additionally, lack of research on effects of PGPR treatment on induction of VOCs is surprising considering the continuing rise in the application of PGPR in several field crops, including cotton plants in the U.S.A. (Glick 1995, Backman et al. 1997, Cleyet-Marcel et al. 2001). Backman et al. (1997) reported that 60-75% of the U.S. cotton crop is treated with Kodiak®, the *Bacillus subtilis* product used for suppression of *Fusarium* and *Rhizoctonia* soil pathogens. Therefore, the effect of PGPR treatment on induction of cotton plant volatiles is worth investigating. Knowledge of the effect of PGPR in influencing chemical-mediated tritrophic interactions will likely contribute to the increased adoption of PGPR products and development of better products while mitigating against potential negative impacts of these products.

1.4 Model System

This study uses a tritrophic model system consisting of cotton plant, *H. virescens* and *S. exigua* (as herbivores), and *M. croceipes* and *C. marginiventris* (as specialist and generalist parasitoids, respectively). Both parasitoids belong to the same family (Hymenoptera: Braconidae). *Microplitis croceipes* is a relatively specialist parasitoid specific to the caterpillars of corn earworm, *H. zea* and *H. virescens*, while *C. marginiventris* is a generalist parasitoid of caterpillars of a wide range of lepidopteran species, including *H. zea*, *H. virescens*, *S. exigua* and several other moth species (Jalali et al. 1987, Turlings et al. 1990, Röse et al. 1998). Both parasitoids were selected as experimental models for this comparative study because they have served as models in previous studies of parasitoid olfaction, and because several aspects of their responses to host-related volatiles have been characterized (e.g., Dmoch et al. 1985, Li et al. 1992, Cortesero et al. 1997, Röse et al. 1998, Park et al. 2002, Gouinguené et al. 2005, Chen and Fadamiro 2007).

1.5 Justification of Study

Despite the intense interest in host-parasitoid interactions, certain aspects of olfactory communication in this group of insects are not well understood. For instance, there are only a few systematic comparative studies of olfaction and behavioral responses in specialist and generalist parasitoids. Additionally, the few studies conducted so far have produced contrasting results (Geervliet et al. 1994, 1996, Cortesero et al. 1997, Smid et al. 2002, Chen and Fadamiro 2007).

A current paradigm regarding the evolution of parasitoid foraging and host location is that the degree of specificity of the signals needed by a parasitoid to successfully locate its host

correlates with its level of specialization (Vet and Dicke 1992, Cortesero et al. 1997, Smid et al. 2002). It is hypothesized that specialist parasitoids utilizing relatively few numbers of hosts have a highly efficient host detection system (high olfactory sensitivity to host related chemical cues) compared to generalist parasitoids. There are, however, very few comparative studies in the literature that have examined olfaction in specialist and generalist parasitoids; fewer have been designed to test the above hypothesis and these have produced contrasting results. Some studies showed that specialist parasitoids exhibit greater electrophysiological and behavioral responses than generalist parasitoids to host-related odor (Elzen et al. 1987, Vet et al. 1993, Chen and Fadamiro 2007). On the other hand, Geerveliet et al. (1996) reported no differences in the response of the specialist *C. rubecula* and the generalist *C. marginiventris* to host related odor. Similarly, Smid et al. (2002) reported no difference in the receptive range of the specialist *C. rubecula* and the generalist *C. glomerata* to a wide range of host-plant odor compounds. Even within a broad category of specialist or generalist parasitoids, differences may exist among species based on the degree of specialization (De Moraes et al. 1998, Tamò et al. 2006). Such discrepancies suggest that diverse species of specialist or generalist parasitoids may respond differently to different types of host-related volatiles and therefore provide relevant justification for additional studies investigating mechanisms of olfaction in parasitic wasps with different degrees of host specificity.

Another research topic that merits further investigation is the potential effect of soil microorganisms on chemical mediated multitrophic interactions. One group of soil microorganisms that warrants study is PGPR. PGPR represent a wide range of root-colonizing bacteria whose application is often associated with increased rates of plant growth (Kloepper 1992, Zehnder et al. 1997), suppression of soil pathogens (Schippers et al. 1987), and the

induction of systemic resistance against insect pests (van Loon et al. 1998, Kloepper et al. 1999, Ramamoorthy et al. 2001, Ryu et al. 2004). The effects of application of PGPR on induction of volatile organic compounds (VOCs) in treated plants have gone virtually unexamined.

The lack of research on the effect of PGPR on induction of plant volatiles is surprising given that it is increasingly being applied in the production of several field crops including cotton (*Gossypium hirsutum* L.), tomato (*Solanum lycopersicum* L.), watermelon (*Citrullus lanatus* Thunb.), and pearl millet (*Pennisetum glaucum*) in the USA or India (Glick 1995, Backman et al. 1997, Cleyet-Marcel et al. 2001, Kokalis-Burelle et al. 2003, Niranjan Raj et al. 2003, Burkett-Cadena et al. 2008). Backman et al. (1997) reported that 60-75% of the US cotton crop is treated with the PGPR product Kodiak®, a *Bacillus subtilis* product used for suppression of *Fusarium* and *Rhizoctonia* soil pathogens. The current lack of knowledge of the effect of PGPR on the induction of volatiles in cotton plant provides a justification for this study. Knowledge of the effect of PGPR in influencing chemical-mediated tritrophic interactions will likely contribute to the increased adoption of PGPR products and development of better products, while mitigating against potential negative impacts of these products.

1.6 Dissertation Outline, Goals and Objectives

The goal of this research is to study chemically-mediated, multitrophic interactions among plants, microorganisms, herbivores, and parasitoids. Specifically, my research seeks to characterize mechanisms of olfaction and response to host-related odor in two parasitic wasps: *C. marginiventris* Cresson and *M. croceipes* Cresson (Hymenoptera: Braconidae) with different degrees of host specificity using an integration of analytical, behavioral, and electrophysiological techniques. My research utilizes a tritrophic model system consisting of cotton, its two key

caterpillar (Lepidoptera: Noctuidae) pests (*H. virescens* and *S. exigua*) and their parasitoids (*M. croceipes*-specialist and *C. marginiventris*-generalist) to answer the following key questions: i) is there a correlation between the degree of specialization of parasitoids and their olfactory sensitivity to host-related odor? ii) do female and male parasitoids show similar olfactory and behavioral preference to different host-related odors? and iii) do PGPR have any effects on the induction of cotton volatiles and what consequences might this have on response of parasitoids?

My dissertation is divided into two major themes. Theme 1 (Chapters II, III, and IV) focuses on characterizing and identifying qualitative and quantitative differences in responses of both parasitoids *M. croceipes* (specialist) and *C. marginiventris* (generalist) to various types of host-related odors using electroantennogram (EAG), coupled gas chromatography electroantennogram (GC-EAD), and coupled gas chromatography mass spectroscopy (GC-MS) techniques (Chapters II and III). Chapter IV focuses on the effects of PGPR on the induction of cotton volatiles and consequences for response of parasitoids. Theme II (Chapter V) focuses on comparing behavioral responses of both sexes of parasitoids (*M. croceipes* (specialist) and *C. marginiventris* (generalist)) to various types of host-related odors.

In Chapter II, I recorded electroantennogram (EAG) responses of two parasitoid species (*M. croceipes* and *C. marginiventris*) and their lepidopteran hosts (*H. virescens* and *S. exigua*) to a wide array of odor stimuli in order to determine whether the EAG response spectrum of an insect correlates to its degree of host specificity. The compounds evaluated included seven host-related plant volatiles, representing green leaf volatiles (GLVs) (*cis*-3-hexenal, *trans*-2-hexenal, hexanal, and β-pinene), and herbivore-induced plant volatiles (HIPV) (*cis*-3-hexenyl acetate, linalool, and *cis*-3-hexenyl butyrate), seven ecologically irrelevant plant volatiles (dimethyl disulfide, benzaldehyde, phenyl acetonitrile, phenyl isothiocyanate, geraniol, *trans*-

cinammaldehyde, and pentyl hexanoate), and several types of host specific odor stimuli, including synthetic host sex pheromones and extracts of host caterpillar and body frass. I also tested the EAG responses of female moths of the caterpillar hosts of the parasitoids, *H. virescens* and *S. exigua*, to some of the odor stimuli. Results showed that the specialist parasitoid showed greater EAG responses than the generalist to host-specific odor and one HIPV (*cis*-3-hexenyl butyrate), whereas the generalist showed relatively greater EAG responses to the GLVs and unrelated plant volatiles. I detected no difference in the EAG responses of *H. virescens* and *S. exigua* to any of the tested odors. The goal of Chapter III was to determine if there was a correlation between the degree of specialization of parasitoids and their olfactory sensitivity to host-related odors. The objective was to determine similarities and differences in antennal responses of both parasitoid species to headspace volatiles of cotton plants. I compared GC-EAD (coupled gas chromatography electroantennogram detection) responses of a specialist (*M. croceipes*) and a generalist (*C. marginiventris*) parasitoid to headspace volatiles of cotton plants damaged by *H. virescens* (a host species for both parasitoids) vs. *S. exigua* (a host species of *C. marginiventris*). Thirty volatile components were emitted by cotton plants in response to feeding by either of the two caterpillars; but 18 components were significantly elevated in the headspace of *H. virescens* damaged plants. Sixteen consistently elicited GC-EAD responses in both parasitoids. *Cotesia marginiventris* showed significantly greater GC-EAD responses than *M. croceipes* to most green leaf volatile components, whereas several herbivore-induced volatile components elicited comparatively greater responses in *M. croceipes*. Results suggest that differences in the ratios of identical volatile compounds between similar volatile blends may be used by specialist parasitoids to discriminate between host-plant and non-host plant complexes.

In Chapter IV, I evaluated the potential of PGPR on the induction of cotton volatiles and consequences for response of parasitoids. The goal of this study was to test effects of application of PGPR on induction of VOCs in cotton plants and further test the consequences of induced volatiles for the attraction to parasitoids of cotton herbivores. Three PGPR strains were evaluated: i) *Bacillus pumilis* strain INR-7, ii) Blend 8, and iii) Blend 9. A water control was also tested. PGPR strains were applied as spore preparations. I used gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) to analyze and identify peaks resulting from extracts of headspace volatiles from PGPR treated and untreated cotton. Noticeable quantitative and qualitative differences were recorded between extracts from PGPR treated and untreated cotton plants. A total of 11 peaks were detected from the headspace of PGPR treated cotton plants and, out of the eleven, only three peaks were slightly detectable in untreated cotton plants. Differences in root growth between PGPR treated vs. untreated plants were recorded, with Blend 9 recording the highest root growth. These PGPR treated plants were also highly attractive to parasitoids with Blend 9 showing the highest attraction to *M. croceipes*.

In Chapter V, studies were conducted to determine if there were sexual and species differences in behavioral responses of a specialist and generalist parasitoid to host-related volatiles. Y-tube olfactometer bioassays were conducted to compare responses of naïve females and males of both parasitoid species to select synthetic plant-based host-related volatiles: two GLVs (hexanal and (Z)-3-hexen-1-ol) and four HIPVs ((Z)-3-hexenyl acetate, linalool, (Z)-3-hexenyl butyrate, and (*E,E*)-α-farnesene). Linking previous reported electrophysiological responses to behavioral observations, results revealed key differences in behavioral responses of both parasitoid species to the tested host-related plant volatiles. The specialist parasitoid (*M. croceipes*) was more responsive to most of the HIPVs, whereas *C. marginiventris* (generalist)

showed greater responses to the GLVs. Females of both parasitoid species showed greater behavioral responses than conspecific males. My dissertation provides the first systematic comparative study of GC-EAD responses of *C. marginiventris* (generalist), and *M. croceipes* (specialist), to herbivore-induced cotton volatiles and also reports the first evidence in the literature for the potential of PGPR in eliciting induction of VOCs in cotton. Results from my dissertation advance our understanding of olfaction, semiochemical-mediated responses, and foraging strategies in parasitoids with different degrees of host specificity.

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CHAPTER 2

ELECTROANTENNOGRAM (EAG) RESPONSES OF *MICROPLITIS CROCEIPES* AND *COTESIA MARGINIVENTRIS* AND THEIR LEPIDOPTERAN HOSTS TO A WIDE ARRAY OF ODOR STIMULI: CORRELATION BETWEEN EAG RESPONSE AND DEGREE OF HOST SPECIFICITY?

2.1 Introduction

Electroantennogram (EAG), a technique which measures the electrophysiological responses in the insect antennae, provides a general measure of odorant reception at the peripheral level (Roelofs 1977, Van der Pers and Minks, 1998, Park et al. 2002). EAG responses represent the summed, direct current (DC) potential response of several different and narrowly tuned olfactory receptor neurons on an insect antenna (Schneider 1957). The EAG technique has been used in pheromone identification studies, and more recently for identification of plant volatiles that mediate insect-plant or tritrophic interactions (Cossé et al. 1995, Blight et al. 1997, Honda et al. 1999). However, EAG activity may not necessarily indicate behavioral activity (Park et al., 2001). Thus, the biological role of EAG active compounds must be determined in behavioral bioassays.

There is ample evidence that EAG response of insects to many pheromones can be species-specific (Smith and Menzel 1989, Visser and Piron 1997, Park et al. 2002, Groot et al. 2005) or race-specific (Linn et al. 1999, El-Sayed et al. 2003). Because the specificity of EAG responses of male moth antennae to conspecific pheromone has been instrumental in pheromone identifications, EAG recordings have been used as a diagnostic tool to correlate differences in pheromone detection to genetic differences in several moth species (El-Sayed et al. 2003, Groot

et al. 2005). However, EAG response of insects to plant volatiles or other kairomones is not species-specific, and it remains unclear if there is a correlation between EAG response spectra of insects to plant volatiles or host-related volatiles and their diet breadth or host specificity. The existence of such a correlation may imply the potential use of EAG recordings to provide an indication of the diet breadth or host specificity of insects. In that case, one would predict that specialist monophagous or oligophagous insect herbivores should show narrower EAG response spectra than polyphagous generalist herbivores. Similarly, since they utilize fewer hosts and thus are likely to possess a relatively more narrowly-tuned (selective) host detection olfactory system, specialist parasitoids should show relatively narrower EAG response spectra to plant volatiles than generalists, which have a broader host range. The need for generalist parasitoids to locate different hosts on a wide variety of plants further suggests that they may have evolved the ability to respond to a wider array of plant volatiles than specialists.

Here, we tested the above prediction by recording EAG responses of females of two caterpillar parasitoid species (Hymenoptera: Braconidae) with different degree of host specificity, *Microplitis croceipes* (Cresson) and *Cotesia marginiventris* (Cresson), to a wide array of odor stimuli including green leaf volatiles (GLVs), herbivore-induced plant volatiles (HIPVs), host-specific volatiles (i.e. host sex pheromones, extracts of host caterpillar body and host frass), and ecologically irrelevant plant volatiles (i.e. volatiles not known to be produced by the hosts of the tested insects). *M. croceipes* is a specialist parasitoid of caterpillars of *Heliothis* spp., whereas *C. marginiventris* is a generalist parasitoid of caterpillars in several genera including *Heliothis* spp. and *Spodoptera* spp. The two parasitoid species were selected for this study because they have served as models in previous studies of parasitoid olfaction, and several aspects of their responses to host-related volatiles have been characterized (Loughrin et al. 1994,

Cortesero et al. 1997, Röse et al. 1998, Park et al. 2002, Shalit et al. 2003, Gouinguené et al. 2005, Chen and Fadamiro 2007, Ngumbi et al. 2009). Additionally we tested EAG responses of adult females of the caterpillar hosts of the parasitoids, *Heliothis virescens* Fab. and *Spodoptera exigua* (Hubner) (Lepidoptera: Noctuidae) to most of the above odorants. Both *H. virescens* and *S. exigua* are naturally distributed throughout USA and are important pests of key agricultural crops such as corn and cotton (Pearson 1982, Stadelbacher et al. 1986). *Heliothis virescens* is a preferred host of *M. croceipes* (Stadelbacher et al. 1984, King et al. 1985), whereas *S. exigua* is a preferred host of *C. marginiventris* (Jalali et al. 1987) but not a known host for *M. croceipes*. Based on the results of a preliminary study in which differences in the EAG responses of both parasitoid species to various synthetic host-related volatile compounds were recorded (Chen and Fadamiro 2007) and assuming a correlation between EAG response spectra and host specificity of parasitoids or diet breath, we hypothesized that the specialist parasitoid, *M. croceipes* will have a narrower EAG response spectrum than the generalist, *C. marginiventris* by showing relatively greater EAG responses to host-specific odor but lower responses to GLVs and ecologically irrelevant plant volatiles. Because the two lepidopteran host species (*H. virescens* and *S. exigua*) differ little in their diet breadth and host plant use, we hypothesized that both will show similar EAG response spectra to plant volatiles.

2.2 Materials and Methods

2.2.1. Insects. The parent cultures of *M. croceipes* and *C. marginiventris* were provided by the USDA-ARS, Insect Biology and Population Management Research Laboratory (Tifton, Georgia, USA) and the Department of Entomology, University of Georgia (Tifton campus, contact: John Ruberson), respectively. *Microplitis croceipes* was reared on caterpillars of *H.*

virescens, whereas *C. marginiventris* was reared on *S. exigua*. The rearing procedures of both parasitoids were similar to those of Lewis and Burton (1970). Eggs purchased from Benzone Research (Carlisle, PA, USA) were used to start laboratory colonies of the two lepidopteran host species, *H. virescens*, and *S. exigua*. Caterpillars of both species were reared on a laboratory-prepared pinto bean diet (Shorey and Hale 1965) at 25 ± 1 °C, 75 ± 5% r.h. and 14:10 L:D photoperiod. For each parasitoid species, newly emerged adults were collected prior to mating, sexed, and placed in pairs of individuals of opposite sex (mated individuals) in a 6-cm diameter plastic Petri dish supplied with water and sugar sources. Water was provided by filling a 0.5 ml microcentrifuge tube with distilled water and threading a cotton string through a hole in the cap of the tube. About 5 drops (2 µl per drop) of 10% sugar solution were smeared on the inside of the Petri dish cover with a cotton-tipped applicator. For each lepidopteran species, newly emerged female moths were collected and placed in clear plastic rectangular cages (30 × 30 × 13 cm tall) supplied with water and sugar sources. Water and sugar solution (10%) were provided by filling a 25 ml glass cylinder with distilled water and placing an 8 cm long cotton absorbal wick (Wheat Ridge, CO, USA) at the center. The cylinder was then sealed with Parafilm. Mated female moths and parasitoids (aged 3-5 days) were used for EAG recordings.

2.2.2. Test Odor Stimuli. Three major categories of odor stimuli were tested for the parasitoids: synthetic host-related plant volatiles, synthetic ecologically irrelevant plant volatiles, and host-specific odor stimuli (host sex pheromones, and extracts of host caterpillar body and frass). Seven host-related plant volatiles were tested in this study: *cis*-3-hexenal, *trans*-2-hexenal, hexanal, β-pinene, *cis*-3-hexenyl acetate, linalool (racemic), and *cis*-3-hexenyl butyrate. The first three compounds (*cis*-3-hexenal, *trans*-2-hexenal and hexanal) are components of green leaf

volatiles (GLVs) of most plants, while the remaining four compounds are herbivore-inducible plant volatiles (HIPV) in cotton (*Gossypium hirsutum* L) and several other plant species (Loughrin et al. 1994, McCall et al. 1994, De Moraes et al. 1998, Hoballah et al. 2002). *Cis*-3-Hexenyl acetate, a compound from the lipoxygenase pathway, was classified as a herbivore–inducible compound in our study because it has been shown to be induced by caterpillar feeding in cotton plants (Loughrin et al. 1994, McCall et al. 1994, Ngumbi et al. 2009). All selected compounds have previously been reported to elicit antennal and/or behavioral responses in both parasitoids (Li et al. 1992, Park et al. 2001, 2002, Chen and Fadamiro 2007) and their lepidopteran hosts (Burguiere et al. 2001, Rajapakse et al. 2006). Seven ecologically irrelevant plant volatiles were tested, including (arranged in the order of molecular weight) dimethyl disulfide, benzaldehyde, phenyl acetonitrile, phenyl isothiocyanate, geraniol, *trans*-cinammaldehyde, and pentyl hexanoate. Phenyl acetonitrile and phenyl isothiocyanate are isothiocyanates typically produced by plants in Brassicaceae family, while geraniol (an acyclic monoterpene alcohol found in lemongrass and aromatic herb oils) and *trans*-cinnamaldehyde (a pale yellow viscous liquid occurs naturally in the bark of cinnamon trees and other species of the genus *Cinnamomum*) are essential oils. These compounds were classified as ecologically irrelevant volatiles because they are not known to be produced by the plant hosts of the tested lepidopteran species (*H. virescens* and *S. exigua*), or used by the tested insects for host location. The ecologically irrelevant plant volatiles were evaluated simply to determine the range of antennal perception in both parasitoids and their moth hosts. All synthetic test compounds were purchased from Sigma® Chemical Co. (St. Louis, Missouri) with purity >97% as indicated on the labels. Each compound was diluted in hexane (HPLC grade) to give 100 µg/µl solutions.

Further dilutions were made to give 0.1, 1 and 10 µg/µl solutions. The solutions were kept in a freezer at -20 °C until used.

Several types of host-specific odor stimuli were also tested for the parasitoids including synthetic host sex pheromones and extracts of host caterpillar body and frass. The sex pheromone of both *H. virescens* and *S. exigua* were tested as single components and blends. For *H. virescens*, we tested the major (Z11-16 Ald) and minor (Z9-14 Ald) sex pheromone components, and a blend of the two components in a ratio of 16:1. We also tested *S. exigua* major (Z9E12-14 Ac) and minor (Z9-14 OH) sex pheromone components, and a blend of the two components at a ratio of 10:1. The sex pheromone blend tested for each moth species has been shown to elicit behavioral responses in conspecific males and could be considered as an optimal pheromone blend for each moth species (Mitchell et al. 1978, Mitchell et al. 1983). Pheromones were purchased from Bedoukian Research (Danbury, CT) and ISCA Technologies, Inc. (Riverside, CA) with 98% purity. Solutions of synthetic test pheromone components were dissolved in hexane to obtain 100 µg/µl solutions. All synthetic plant volatiles and pheromones were tested at two doses (1 and 100 µg), which represented low and high doses, respectively. EAG responses of both parasitoids to extracts of host caterpillar body and frass were also determined. Frass extracts (either with hexane or water) were obtained following the procedures of Mattiaci and Dicke (1995) with some modifications. Briefly, 10 g of fresh frass obtained from caterpillars (*H. virescens* or *S. exigua*) feeding on artificial diet was extracted with 5 ml of hexane or water for 24 hr at room temperature. Collected extracts were stored in a freezer (-20 °C) until use. Body extracts of host caterpillars were obtained following the procedures described by Yasuda and Wakamura (1996) with some modifications. Briefly, caterpillars (2^{nd}-3^{rd} instar) of *H. virescens* or *S. exigua* weighing ~ 1 g were extracted with 2 ml of 1:2 mixture of

hexane and acetone for one hour at room temperature. Second and third instar caterpillars were used since they are the stages normally attacked by the parasitoids. The extract was filtered with anhydrous sodium sulphate and silica gel. The filtrate was concentrated to 200 µl under a gentle stream of nitrogen, and was stored in a freezer (-20 °C) until use. Extracts of host caterpillar frass and body were tested at only one dose (10 µl).

2.2.3. EAG Recordings. The EAG technique and protocols were similar to those previously described by Chen and Fadamiro (2007). The reference electrode, consisting of a glass capillary (1.1 mm ID) filled with 0.1 M KCL solution, was connected either to the neck of an isolated head of an adult female parasitoid or to the base of an excised female moth antenna. The recording electrode consisted of a similar glass capillary connected to the antennal tip (with the last segment of the antenna cut off). Chlorinated silver-silver junctions were used to maintain electrical conduct between the electrode and input of the preamplifier. The analog signal was detected through a probe (INR-II. Syntech®, The Netherlands), and was captured and processed with a data acquisition controller (IDAC-4, Syntech®, The Netherlands) and analyzed using EAG 2000 software (Syntech®, the Netherlands) on a personal computer (PC). Test compounds diluted in hexane were delivered as 10-µl samples placed on a filter paper (7 × 40 mm, Whatman® No. 1). The solvent was allowed to evaporate and the impregnated filter paper was placed into a glass Pasteur pipette (~14 cm in length, Fisher Scientific, Pittsburgh, PA, USA) constituting an odor cartridge. The control stimulus consisted of a similar pipette containing a filter paper impregnated with 10 µl of hexane. The tip of the pipette was placed about 3 mm into a small hole in the wall of a glass tube (13 cm long, 8 mm diameter) oriented towards the antennal preparation (~0.5 cm away from the preparation). The stimuli were provided as 0.2 s puffs of air

(2 ml) into a continuous humidified air stream at 1000 ml min $^{-1}$ generated by an air stimulus controller (CS-55, Syntech®, The Netherlands). At least a 2 min interval was allowed between successive stimulations for antenna recovery. Parasitoids and moths aged 3-5 days were tested. Preliminary tests showed that isolated parasitoid head and excised moth antenna preparations lasted up to 40 min with no noticeable decreases in EAG responses observed over this time period at room temperature. Thus, for each category of test compounds, a test series of the same dose (1 µg/µl or 100 µg/µl) was applied to ten antenna preparations of each parasitoid or moth species in the following order: hexane control, standard stimulus, odorant compounds, hexane control and standard stimulus. One hundred micrograms of *cis*-3-hexenol was used as the standard stimulus (Chen and Fadamiro 2007) and presented to an antenna at the beginning and end of a recording series to confirm activity of an antennal preparation. Test compounds were presented in a random sequence. Experiments were carried out in batches replicated in time by testing an equal number of individuals of both parasitoids and their lepidopteran hosts daily in a random order.

2.2.4. Statistical Analyses. For analysis, EAG response to the solvent control was deducted from the EAG amplitudes elicited by the test odor stimuli. Absolute EAG data met the key assumptions of parametric tests and thus were not transformed prior to analysis. Absolute EAG responses to each odorant were compared between the two parasitoid species or the two moth species using the Student's *t*-test ($P < 0.05$; JMP Version 7.01, SAS Institute 2007). For each parasitoid or moth species, EAG responses to compounds within each odor stimulus category at each dose were analyzed by using Analysis of Variance (ANOVA) followed by the

Tukey-Kramer HSD multiple comparison test ($P < 0.05$; JMP® 7.0.1, SAS Institute 2007) to establish significant differences among the compounds tested.

2.3 Results

2.3.1. EAG Responses of Parasitoids to Host-Related Plant Volatiles. Table 1 shows the results of Student's *t* test comparison of the two parasitoid species to the different odor stimuli. The three GLVs, *cis*-3-hexenal, *trans*-2-hexenal and hexanal, and two HIPVs, β-pinene and linalool at both doses (1 and 100 µg) elicited significantly greater EAG responses in the generalist, *C. marginiventris* than in the specialist, *M. croceipes* (Fig. 1a). In contrast, *M. croceipes* showed significantly greater EAG responses to the HIPV, *cis*-3-hexenyl butyrate at both doses, compared to *C. marginiventris* (Fig. 1a). Figure 1a also shows significant differences in the responses of *M. croceipes* (1 µg: $F = 11.63$, df = 6, $P < 0.0001$; 100 µg: $F = 16.72$, df = 6, $P < 0.0001$) and *C. marginiventris* (1 µg: $F = 4.77$, df = 6, $P = 0.0005$; 100 µg: $F = 14.27$, df = 6, $P < 0.0001$) to the seven tested host-related volatiles at both doses. *Cis*-3-Hexenyl butyrate and *trans*-2-hexenal elicited the highest EAG response in *M. croceipes,* whereas *trans*-2-hexenal elicited the highest EAG response in *C. marginiventris*. β-pinene and linalool elicited relatively lower EAG responses in both parasitoid species.

2.3.2. EAG Responses of Parasitoids to Ecologically Irrelevant Plant Volatiles. Significant differences were also recorded in the EAG responses of both parasitoid species to the tested ecologically irrelevant plant volatiles (Table 1). All but one of the tested unrelated volatiles elicited significantly greater EAG responses in the generalist, *C. marginiventris* compared to *M. croceipes,* irrespective of dose (Fig. 1b). The lone exception was phenyl

acetonitrile, which elicited only a numerically greater response in *C. marginiventris*. The seven ecologically irrelevant plant volatiles elicited significantly different EAG responses in *M. croceipes* (1 µg: $F = 4.66$, df = 6, $P = 0.0006$; 100 µg: $F = 9.90$, df = 6, $P < 0.0001$) and *C. marginiventris* (1 µg: $F = 5.48$, df = 6, $P < 0.0001$; 100 µg: $F = 5.88$, df = 6, $P < 0.0001$; Fig. 1b). At the 100 µg dose, benzaldehyde elicited the highest EAG response in *M. croceipes*, significantly greater than the remaining compounds. Similarly, EAG response of *C. marginiventris* to benzaldehyde was greater than EAG responses to the other compounds. Dimethyl disulfide elicited the lowest EAG response in both parasitoid species (Fig. 1b).

2.3.3. EAG Responses of Parasitoids to Host Sex Pheromones. Student's *t* test also revealed significant differences between the two parasitoid species in their EAG responses to host sex pheromones (Table 1). The specialist, *M. croceipes* had significantly greater EAG responses to Z11-16 Ald, the major sex pheromone of its main host, *H. virescens*, at both doses than the generalist, *C. marginiventris* (Fig. 2a). However, EAG responses of both species to Z9-14 Ald (minor sex pheromone component of *H. virescens*) were not significantly different. *M. croceipes* also had greater EAG responses than *C. marginiventris* to the pheromone blend of *H. virescens* (a 16:1 blend of Z11-16 Ald and Z9-14 Ald), but this was significant only at the 100 µg dose. In contrast, Z9E12-14 Ac, the major sex pheromone component of *S. exigua*, elicited significantly greater EAG responses in *C. marginiventris* at both doses than in *M. croceipes* (Fig. 2a). *Cotesia marginiventris* also showed numerically (but not significantly) higher EAG responses than *M. croceipes* to Z9-14 OH, a minor component of *S. exigua* pheromone and to the tested *S. exigua* pheromone blend (a 10:1 blend of Z9E12-14 Ac and Z9-14 OH). Significant differences were also recorded in the response of *M. croceipes* (1 µg: $F = 5.42$, df = 5, $P =$

0.0004; 100 µg: $F = 15.91$, df = 5, $P < 0.0001$) and *C. marginiventris* (1 µg: $F = 2.28$, df = 5, $P = 0.0595$; 100 µg: $F = 1.69$, df = 5, $P = 0.0495$) to the six different sex pheromone stimuli (i.e. *H. virescens* and *S. exigua* sex pheromone components and blends) (Fig. 2a). For *M. croceipes,* the highest EAG response was elicited by *H. virescens* pheromone blend followed by Z11-16 Ald (*H. virescens* major pheromone component), while the lowest EAG response was elicited by Z9-14 OH (*S. exigua* minor pheromone component). In contrast, *S. exigua* pheromone blend elicited significantly greater EAG response in *C. marginiventris* than either of the major pheromone components of *H. virescens* at both doses and to Z9-14 Ald (minor pheromone component of *H. virescens*) at the 100 µg dose. EAG response of *C. marginiventris* to *S. exigua* pheromone blend was also significantly greater than to Z9-14 OH (*S. exigua* minor pheromone component) at the 1 µg dose, and numerically greater than EAG response to *H. virescens* pheromone blend.

2.3.4. EAG Responses of Parasitoids to Host Frass and Caterpillar Body Extracts.

Student's *t*-test also revealed significant differences in the responses of both parasitoid species to the different host-specific stimuli (Table 1), with each species showing relatively greater EAG responses to odor stimuli of its preferred host. The specialist, *M. croceipes* showed significantly greater EAG responses than *C. marginiventris* to *H. virescens* caterpillar frass hexane extract ($t = 2.50$, df = 1, $P = 0.012$), frass water extract ($t = 4.31$, df = 1, $P = 0.0003$), and body extract ($t = 2.84$, df = 1, $P = 0.0056$; Fig. 2b). In contrast, *C. marginiventris* showed significantly greater EAG responses than *M. croceipes* to *S. exigua* caterpillar frass hexane extract ($t = 2.57$, df = 1, $P = 0.0248$), and body extract ($t = 2.70$, df = 1, $P = 0.0148$; Fig. 2b). However, no significant difference was recorded in the response of both species to *S. exigua* caterpillar frass water extract ($t = 0.15$, df = 1, $P = 0.8804$). Comparing the response of each species to the six host-specific

stimuli, *H. virescens* frass hexane extract elicited the highest EAG response in *M. croceipes* followed by *H. virescens* caterpillar body extract ($F = 21.09$, df = 5, $P < 0.0001$). For *C. marginiventris*, *S. exigua* frass hexane extract elicited significantly greater EAG response compared to the other five stimuli ($F = 14.68$, df = 5, $P < 0.0001$). In general, frass water extracts of *H. virescens* and *S. exigua* elicited the lowest EAG responses in both parasitoid species (Fig. 2b).

2.3.5. EAG Responses of *H. virescens* and *S. exigua* to Host-Related Plant Volatiles.

Table 2 shows the results of Student's *t* test comparison of the two moth species to the different odor stimuli. All of the tested plant volatiles elicited EAG responses in females of *H. virescens* and *S. exigua* at the two tested doses (1 and 100 µg) (Fig. 3a). In general, higher EAG responses were recorded at the 100 µg dose than at the 1 µg dose. However, no significant differences were recorded in the EAG responses of *H. virescens* and *S. exigua* to any of the tested volatiles, irrespective of dose (Fig. 3a). At the 1 µg dose, all seven host-related plant volatiles elicited similar EAG responses in *H. virescens* ($F = 1.42$, df = 6, $P = 0.2202$), and *S. exigua* ($F = 1.49$, df = 6, $P = 0.1944$). At the higher 100 µg dose, however, *trans*-2-hexanal and hexanal elicited significantly greater EAG responses than the remaining compounds in *H. virescens* ($F = 11.89$, df = 6, $P < 0.0001$), and *S. exigua* ($F = 10.92$, df = 6, $P < 0.0001$; Fig. 3a).

2.3.6. EAG Responses of *H. virescens* and *S. exigua* to Ecologically Irrelevant Plant Volatiles.

Both *H. virescens* and *S. exigua* showed significant EAG responses to the ecologically irrelevant plant volatiles but the responses were not clearly dose dependent (Fig. 3b). Furthermore, no significant differences were recorded in EAG responses of both moth species to

any of the ecologically irrelevant plant volatiles, irrespective of dose (Fig. 3b). Significant differences were recorded in the response of *H. virescens* to the seven ecologically irrelevant plant volatiles (1 µg: $F = 2.53$, df = 6, $P = 0.0296$; 100 µg: $F = 4.99$, df = 6, $P = 0.0003$). Benzaldehyde elicited the highest EAG response in *H. virescens* at both doses, while dimethyl disulfide elicited the lowest EAG responses. *Spodoptera exigua* also showed significantly different responses to the ecologically irrelevant plant volatiles (1 µg: $F = 2.99$, df = 6, $P = 0.0125$; 100 µg: $F = 4.49$, df = 6, $P = 0.0008$). Pentyl hexanoate elicited the highest EAG response at the 1 µg dose, while benzaldehyde elicited the highest EAG response at the 100 µg dose. Dimethyl disulfide also elicited the lowest EAG response in *S. exigua* at both doses (Fig. 3b).

2.4 Discussion

Our results revealed intriguing differences in the EAG responses of both parasitoid species to the tested odor stimuli. As predicted, the generalist parasitoid, *C. marginiventris* showed a wider EAG response spectrum to odor than the specialist, *M. croceipes*. While the generalist showed greater EAG response than the specialist to most green leaf volatiles (GLVs) and unrelated plant volatiles, the specialist showed relatively greater responses to host-specific odor stimuli such as host sex pheromones and host caterpillar frass and body extracts, and to *cis*-3-hexenyl butyrate, a herbivore-induced plant volatile (HIPV). These results are fairly consistent with those reported in a preliminary study in which Chen and Fadamiro (2007) compared the EAG responses of *M. croceipes* and *C. marginiventris* to two GLVs (*cis*-3-hexenol and hexanal) and three HIPVs (*cis*-3-hexenyl acetate, linalool, and (*E,E*)-α-farnesene). In that study, *C.*

marginiventris also showed relatively greater EAG responses than *M. croceipes* to the two GLVs (Chen and Fadamiro (2007).

I am not aware of any published studies which compare the behavioral response of both parasitoid species to a wide range of volatiles, as evaluated in the present EAG study. However, my data are consistent with those which demonstrated higher behavioral response of *C. marginiventris* to GLVs and volatiles from freshly damaged plants than to volatiles from plants with old damage (Cortesero et al. 1997, Hoballah et al. 2002, D'Alessandro and Turlings 2005, Hoballah and Turlings 2005). It would seem adaptive for generalist parasitoids to show greater response than specialists to GLVs and a wider array of plant volatiles since they attack numerous hosts on different host plants. In contrast, specialist parasitoids are likely to have evolved the ability to respond to a narrower range of volatiles, while showing greater olfactory response to the volatiles which are specifically linked to their hosts, such as host frass, body odor, and host sex pheromones. *Cis*-3-Hexenyl butyrate was the only tested HIPV which elicited significantly greater EAG response in *M. croceipes* compared to *C. marginiventris*. This compound is a major HIPV component emitted by cotton plants damaged by both *H. virescens* and *S. exigua* caterpillars (Loughrin et al. 1994, McCall et al. 1994, Ngumbi et al. 2009), and has been shown to elicit behavioral response in *M. croceipes* (Whitman and Eller 1992). A recent study showed that *cis*-3-hexenyl butyrate is emitted in greater amounts by plants damaged by *H. virescens* compared to plants damaged by *S. exigua* (Ngumbi et al. 2009), suggesting that this compound could play an important role in host location behavior of *M. croceipes*. In contradiction to our hypothesis and previous GC-EAD results (Ngumbi et al. 2009), the HIPV, linalool elicited significantly greater EAG response in *C. marginiventris* compared to *M. croceipes*, as was reported earlier by Chen and Fadamiro (2007). These contradictory results with racemic linalool,

composed of both (+) and (-) enantiomers, may be related to differences in the concentration of linalool reaching the antenna in the EAG versus GC-EAD tests. A recent study showed that the two enantiomers of linalool were perceived in different parts of the brain of *Manduca sexta* (L.) (Lepidoptera: Sphingidae) (Reisenman et al. 2004). Thus, it is possible that the observed differential electrophysiological responses of our test parasitoids to racemic linalool may be related to concentration. Future studies will test this hypothesis and attempt to resolve our contradictory EAG and GC-EAD results with linalool.

Compared to *C. marginiventris*, *M. croceipes* showed relatively greater EAG response to frass hexane and water extracts and body extract of caterpillars of *H. virescens*, its preferred host, whereas *C. marginiventris* showed comparatively greater EAG response to frass hexane extract and body extracts of caterpillars of *S. exigua*, one of its key hosts. These results imply the ability of parasitoids to use host-specific odor stimuli such as frass and host body odor to discriminate between preferred and non-preferred host species, and are consistent with previous findings by other authors. For instance, *M. croceipes* has been reported to use host frass as a host location cue (Jones et al. 1971, Eller et al. 1988, Lewis and Tumlinson 1988). Frass volatiles represent a source of host-specific information which allows specialist parasitoids such as *M. croceipes* to discriminate host and non-host species from a distance (Alborn et al. 1995, Cortesero et al. 1997). Previous studies also demonstrated attraction of *C. marginiventris* to frass hexane extract of *Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae) and the actual (unextracted) frass of *S. exigua* (Loke and Ashley 1984). More recently, *C. marginiventris* was found to be attracted to chemical footprints of its host, *S. frugiperda* on infested plants (Rostás and Wölfling 2009).

Both parasitoids showed EAG response to the sex pheromones of the adult form (moth) of their hosts even though caterpillars are their actual hosts. Furthermore, *M. croceipes* showed

significantly greater EAG response than *C. marginiventris* to host sex pheromones, suggesting that specialist parasitoids may have evolved a greater ability (than generalists) to associate host (adult moth) sex pheromones with host (caterpillar) availability. These results are not surprising, given that host sex pheromones have been shown to attract many parasitoid species, mainly egg parasitoids (Nordlund et al. 1983, Noldulus and van Lenteren 1985, Colazza et al. 1997, Powell 1999). The larval parasitoid, *Cotesia plutellae* (Kurdjumov) (Hymenoptera: Braconidae) was also shown to be attracted to the sex pheromone of its host *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) (Reddy et al. 2002).

In contrast to the results obtained for the parasitoids, we found no major differences in the EAG response spectra of the two moth species, *H. virescens* and *S. exigua*, to all tested plant volatiles. This finding is not surprising given that caterpillars of both species are generalist herbivores with similar diet breadth. The GLVs (*trans*-2-hexenal, and hexanal) elicited the highest EAG responses in both moth species, as has previously been reported for *S. exigua* (Dickens et al. 1993) and *S. frugiperda* (Malo et al. 2004). Similarities in the response of parasitoids and their hosts (moths) to plant volatiles have also been reported (Salkeld 1959, Guerin and Visser 1980). Similar results were also obtained in the present study, in which *trans*-2-hexenal and hexanal elicited the largest EAG responses in the parasitoids as well as their host moths. My data, which showed EAG responses of both moth species to the tested unrelated plant volatiles, are also not astounding. Using GC-EAD (coupled gas chromatography electroantennographic-detection) and single cell recordings, Jönsson and Anderson (1999) showed that *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae) responded to cotton plant volatiles with the ability to discriminate between damaged and undamaged host plants. Rajapakse et al. (2006) reported that *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae)

showed EAG responses to its common host plants, pigeon pea (*Cajanus cajan* L), tobacco (*Nicotiana tabacum* L), cotton, and bean (*Phaseolus vulgaris* L), as well as to non-host plants such as lantana (*Lantana camara* L) and oleander (*Nerium oleander* L). Results from a related study that investigated receptor neurons in three heliothine moth species (Lepidoptera: Noctuidae) with different degrees of host specificity, *H. virescens* (oligophagous), *Heliothis armigera* (Hübner) (polyphagous), and *Helicoverpa assulta* (Guenée) (oligophagous), revealed the presence of similar types of plant odor receptor neurons in all three species, suggesting that functionally similar olfactory receptors are conserved in related species despite the evolution of polyphagy and oligophagy (Stranden et al. 2003). Taken together, the above findings and my data suggest that polyphagous/oligophagous herbivorous insects such as the moth species tested in the present study likely use a broad suite of volatiles common to many plants for host location. Therefore, the EAG technique, which measures gross olfactory response to odor, may not be robust enough to provide an indication of the diet breadth of moths. Further studies with moth models of different diet breaths (monophagous versus polyphagous) are necessary to confirm this prediction.

The generalist parasitoid, *C. marginiventris* showed greater EAG responses than *M. croceipes* to all tested ecologically irrelevant plant volatiles, whereas both moth species showed similar EAG responses to the ecologically irrelevant plant volatiles. Among the ecologically irrelevant plant volatiles, benzaldehyde elicited the highest EAG response while dimethyl disulfide elicited the least EAG response in both parasitoid species. Benzaldehyde also elicited the highest and dimethyl disulfide the lowest EAG responses in both moth species, although the differences between the compounds were not as clear as those recorded for the parasitoids. Benzaldehyde has also been reported to elicit EAG response in *M. croceipes* (Li et al. 1992, Park

et al. 2002), and in the moths *H. armigera* (Burguiere et al. 2001) and *Choristoneura rosaceana* (Harris) (Stelinski et al. 2003). The ability of parasitoids and moths to show notable EAG responses to ecologically irrelevant plant volatiles could be because they encounter these volatiles in their habitats (Vinson 1976, Powell and Poppy 2001). Dimethyl disulfide is a component of larval frass of the diamond back moth, *P. xylostella*, and is among the three disulfides reported to play a role in the host searching behavior of the parasitoid, *Diadromus pulchellus* (Auger et al. 1989). Thus, my data in which both *M. croceipes* and *C. marginiventris* showed very low EAG responses to dimethyl disulfide are not surprising, given that both parasitoids are not known to attack *P. xylostella*.

In summary, my results demonstrated a correlation between EAG response spectra of parasitoids and their degree of host specificity, supporting my hypothesis that specialist parasitoids will have a narrower EAG response spectrum than generalists. This EAG study represents an initial attempt to test if the EAG response spectrum of an insect can give an indication of its degree of host specificity or diet breadth. Future studies with other appropriate parasitoid and moth models are needed to confirm the present results.

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Table 1. Results of Student's *t* test analysis to compare EAG responses of *Microplitis croceipes* and *Cotesia marginiventris* to host-related plant volatiles, ecologically irrelevant plant volatiles, and host sex pheromones at two doses

Compound	Dose (µg)	*t*	*P*
Host-related plant volatiles			
cis-3-Hexenal	1	2.33	0.016
	100	2.46	0.002
trans-2-Hexenal	1	2.74	0.008
	100	2.97	0.014
Hexanal	1	3.10	0.004
	100	3.35	0.002
β-Pinene	1	3.19	0.003
	100	3.08	0.008
cis-3-Hexenyl acetate	1	0.33	0.371
	100	1.76	0.442
Linalool	1	1.90	0.029
	100	1.98	0.003
cis-3-Hexenyl butyrate	1	3.32	0.004
	100	3.21	0.004
Ecologically irrelevant plant volatiles			
Dimethyl disulfide	1	1.95	0.041
	100	1.88	0.032
Benzaldehyde	1	4.11	0.003
	100	2.09	0.021
Phenyl acetonitrile	1	1.12	0.141
	100	1.13	0.152
trans-Cinnamaldehyde	1	1.99	0.031
	100	2.46	0.032
Phenyl isothiocyanate	1	3.40	0.002
	100	2.49	0.012
Geraniol	1	3.34	0.003
	100	3.25	0.004
Pentyl hexanoate	1	3.80	0.005
	100	3.45	0.005
Sex pheromones			
Z11-16 Ald (*H. virescens*)	1	2.90	0.001
	100	2.97	0.010
Z9-14 Ald (*H. virescens*)	1	1.79	0.997
	100	1.99	0.062
Blend (Z11-16 Ald/Z9-14 Ald)	1	2.01	0.970
	100	6.30	<0.0001
Z9E12-14 Ac (*S. exigua*)	1	3.22	0.004
	100	2.23	0.045
Z9-14 OH (*S. exigua*)	1	1.23	0.072
	100	1.04	0.322
Blend (Z9E12-14 Ac/ Z9-14 OH)	1	1.95	0.963
	100	1.73	0.055

Table 2. Results of Student's *t* test analysis to compare EAG responses of *Heliothis virescens* and *Spodoptera exigua* to host-related plant volatiles and ecologically irrelevant plant volatiles at two doses

Compound	Dose (µg)	*t*	*P*
Plant volatiles			
cis-3-Hexenal	1	0.39	0.649
	100	0.38	0.723
trans-2-Hexenal	1	0.70	0.754
	100	1.54	0.796
Hexanal	1	1.15	0.868
	100	2.20	0.973
β-Pinene	1	0.87	0.802
	100	1.12	0.745
cis-3-Hexenyl acetate	1	0.53	0.700
	100	0.87	0.882
Linalool	1	2.64	0.991
	100	0.80	0.757
cis-3-Hexenyl butyrate	1	3.14	0.996
	100	3.24	0.765
Ecologically irrelevant plant volatiles			
Dimethyl disulfide	1	0.54	0.700
	100	0.79	0.781
Benzaldehyde	1	0.63	0.745
	100	0.43	0.300
Phenyl acetonitrile	1	0.37	0.682
	100	1.24	0.884
trans-Cinnamaldehyde	1	0.48	0.798
	100	0.53	0.681
Phenyl isothiocyanate	1	0.28	0.700
	100	0.36	0.608
Geraniol	1	1.35	0.659
	100	0.78	0.225
Pentyl hexanoate	1	0.88	0.765
	100	0.80	0.781

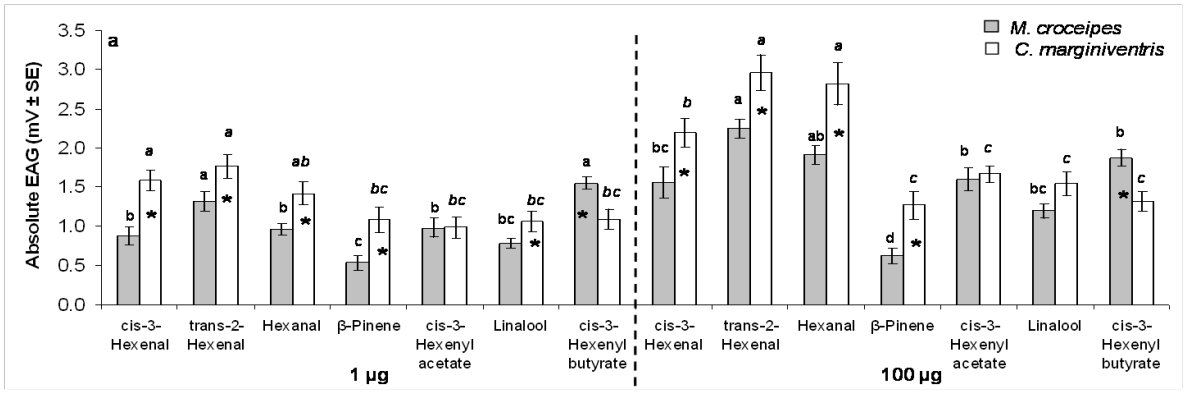
Figure Legend

Figure 1. Absolute EAG responses (mV ± SE, n = 10) of *Microplitis croceipes* and *Cotesia marginiventris* to **(a)** host-related plant volatiles, and **(b)** ecologically irrelevant plant volatiles at two doses (1 µg and 100 µg). *denotes significant difference between the two species (*t* test, $P < 0.05$). Means for the same species and dose having no letter in common are significantly different among compounds (ANOVA, Tukey HSD test, $P < 0.05$). Letters in italics are for *C. marginiventris*.

Figure 2. Absolute EAG responses (mV ± SE, n = 10) of *Microplitis croceipes* and *Cotesia marginiventris* to **(a)** host sex pheromones, and **(b)** host-specific stimuli (caterpillar body and frass extracts) at two doses (1 µg and 100 µg). Blend (H) = *H. virescens* pheromone blend (Z11-16 Ald + Z9-14 Ald in the ratio of 16:1), Blend (S) = *S. exigua* pheromone blend (Z9E12-14 Ac + Z9-14 OH in the ratio of 10:1). *denotes significant difference between the two species (*t* test, $P < 0.05$). Means for the same species and dose having no letter in common are significantly different among odor stimuli (ANOVA, Tukey HSD test, $P < 0.05$). Letters in italics are for *C. marginiventris*.

Figure 3. Absolute EAG responses (mV ± SE, n = 10) of *Heliothis virescens* and *Spodoptera exigua* to **(a)** host-related plant volatiles, and **(b)** ecologically irrelevant plant volatiles at two doses (1 µg and 100 µg). *denotes significant difference between the two species (t test, $P < 0.05$). Means for the same species and dose having no letter in common are significantly different among compounds (ANOVA, Tukey HSD test, $P < 0.05$). Letters in italics are for *S. exigua*.

Figure1


a
M. croceipes
C. marginiventris
Absolute EAG (mV ± SE)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
cis-3-Hexenal
trans-2-Hexenal
Hexanal
β-Pinene
cis-3-Hexenyl acetate
Linalool
cis-3-Hexenyl butyrate
1 µg
100 µg

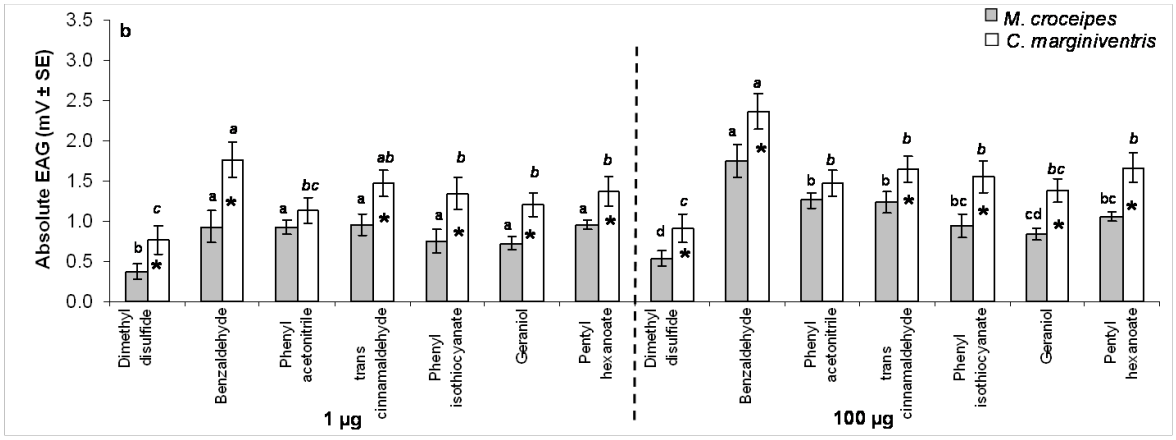

b
M. croceipes
C. marginiventris
Absolute EAG (mV ± SE)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Dimethyl disulfide
Benzaldehyde
Phenyl acetonitrile
trans cinnamaldehyde
Phenyl isothiocyanate
Geraniol
Pentyl hexanoate
1 µg
100 µg

Figure2

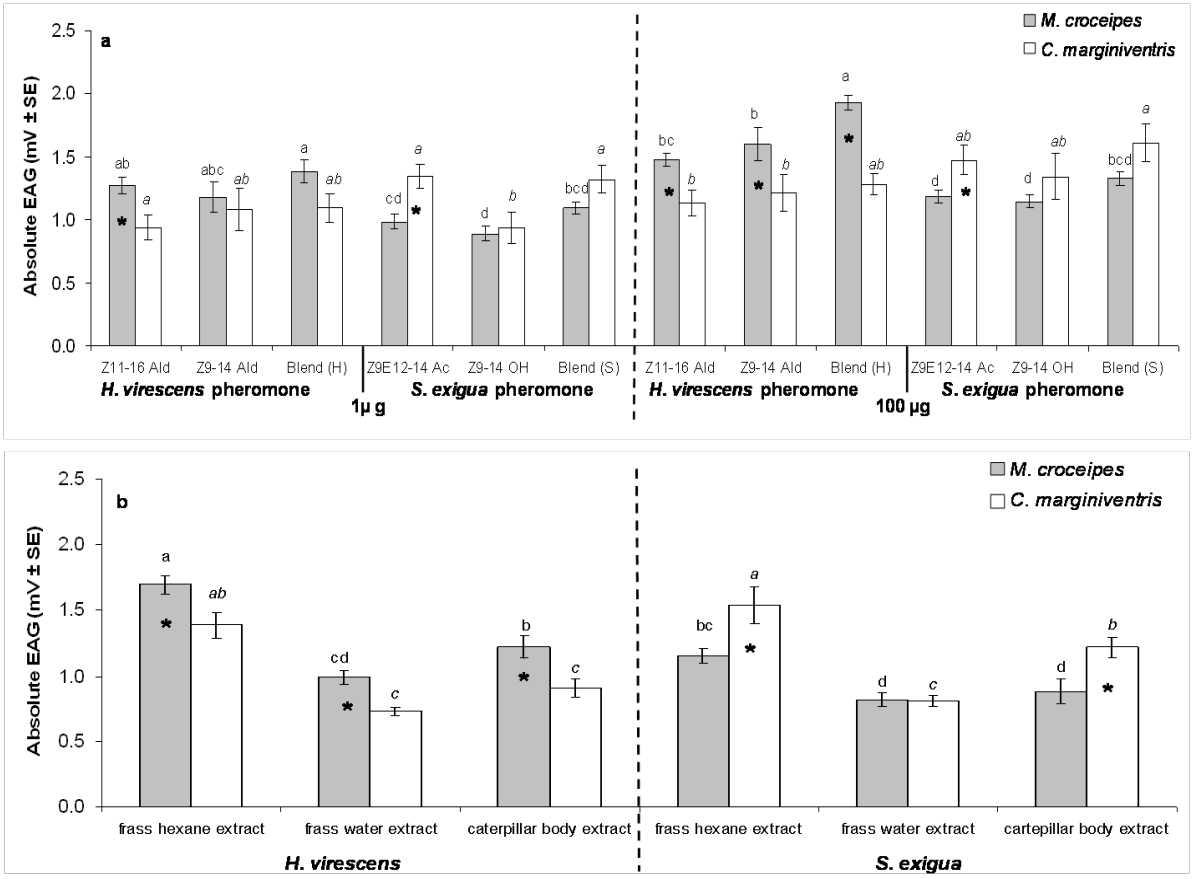
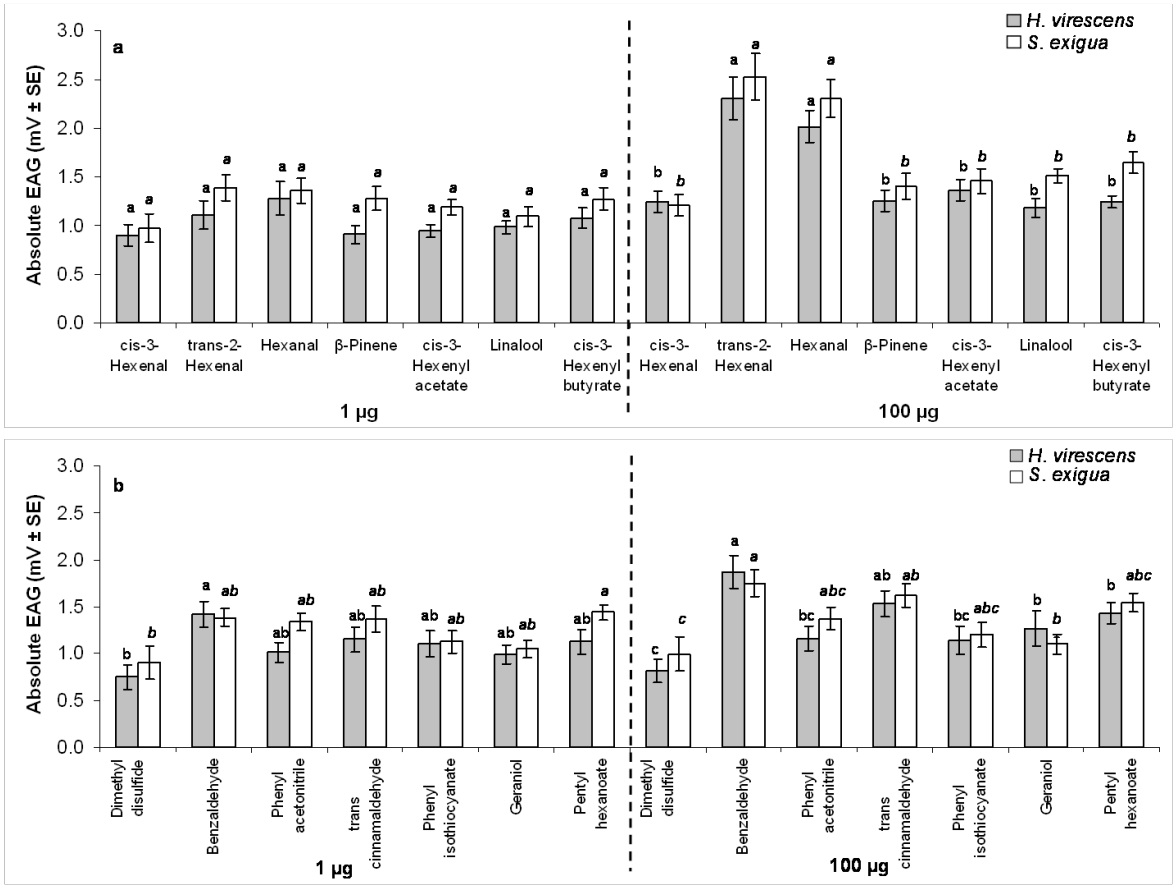

a
M. croceipes
C. marginiventris
Absolute EAG (mV ± SE)
Z11-16 Ald
Z9-14 Ald
Blend (H)
Z9E12-14 Ac
Z9-14 OH
Blend (S)
H. virescens pheromone
S. exigua pheromone
1μ g
100 μg
b
frass hexane extract
frass water extract
caterpillar body extract
cartepillar body extract
H. virescens
S. exigua

Figure3


a
b
H. virescens
S. exigua
Absolute EAG (mV ± SE)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
cis-3-Hexenal
trans-2-Hexenal
Hexanal
β-Pinene
cis-3-Hexenyl acetate
Linalool
cis-3-Hexenyl butyrate
Dimethyl disulfide
Benzaldehyde
Phenyl acetonitrile
trans cinnamaldehyde
Phenyl isothiocyanate
Geraniol
Pentyl hexanoate
1 µg
100 µg

CHAPTER 3

COMPARATIVE GC-EAD RESPONSES OF A SPECIALIST (MICROPLITIS CROCEIPES) AND A GENERALIST (COTESIA MARGINIVENTRIS) PARASITOID TO COTTON VOLATILES INDUCED BY TWO CATERPILLAR SPECIES

3.1 Introduction

Plants emit blends of volatile compounds in response to insect herbivory (Turlings et al. 1990, McCall et al. 1994, Loughrin et al. 1994, De Moraes et al. 1998). This production of volatile compounds is triggered by substances present in the oral secretion of herbivores (Dicke et al. 1993, Turlings et al. 1993). The volatile compounds released from herbivore-damaged plants can be sub-divided into two major groups: constitutive compounds and inducible or herbivore-induced plant volatiles (HIPVs). Constitutive compounds are constantly present in plants and are released immediately in response to mechanical damage or at the beginning of herbivore feeding, and include in many plants green leaf volatiles (GLVs) such as *cis*-3-hexenal, hexanal, and *cis*-3-hexen-1-ol (Turlings et al. 1990, Dicke et al. 1993, Loughrin et al. 1994, McCall et al. 1994, Cortesero et al. 1997, Smid et al. 2002, Gouinguené et al. 2005). On the other hand, HIPVs are emitted as a delayed response to herbivore feeding damage. HIPVs in cotton (*Gossypium hirsutum* L) and some other plant species include *cis*-3-hexenyl acetate, *cis*-3-hexenyl butyrate, indole, and various terpenoids such as (*E,E*)-α-farnesene, (*E*)-β-farnesene, (*E*)-β-ocimene, and linalool (Dicke 1994, Loughrin et al. 1994, McCall et al. 1994, Cortesero et al. 1997).

Although the emission of volatiles is assumed to represent a generalized response to herbivore damage, the blends of volatile compounds released from herbivore damaged plants differ qualitatively and quantitatively depending on plant species and variety (Dicke et al. 1990, Loughrin et al. 1994, Hoballah et al. 2002), herbivore species (De Moraes et al. 1998, Loughrin et al. 1994, McCall et al. 1994), and the developmental stage of an herbivore (Takabayashi et al. 1991, Du et al. 1996). For instance, corn (*Zea mays* L.) plants infested by beet armyworm *Spodoptera exigua* (Hübner) caterpillars emitted linalool, (*3E*)-4,8-dimethyl-1,3,7-nonatriene, (*trans*)-α-bergamotene and (*E*)-β-farnesene as major compounds, all of which were not detected in the headspace of soybean (*Glycine max* L.) plants infested by the same herbivore species (Turlings et al. 1993). In cotton plants, feeding by corn earworm *Helicoverpa zea* (Boddie) or *S. exigua* caterpillars induced the production of distinctive volatile blends that were qualitatively and quantitatively different (Loughrin et al. 1994, McCall et al. 1994). McCall et al. (1994) reported that cotton plants damaged by *H. zea* emitted several compounds including (*Z*)-3-hexenyl acetate, (*E*)-β-ocimene, (*3E*)-4,8-dimethyl-1,3,7-nonatriene, (*Z*)-3-hexenyl butyrate, (*E*)-2-hexenyl butyrate, (*Z*)-3-hexenyl-2-methylbutyrate, (*E*)-2-hexenyl 2-methylbutyrate, and indole. Loughrin et al. (1994) conducted a similar study with cotton plants damaged by *S. exigua* and reported several compounds, including some of the above compounds, and many which were not reported by McCall et al. (1994) such as (*Z*)-jasmone, (*E*)-β-farnesene, and (*E,E*)-α-farnesene. Such differences in the composition of volatiles induced by different herbivore species may convey herbivore-specific information to parasitoids, and thus shape their foraging strategies (Dicke and Sabelis 1988, Turlings et al. 1990, McCall et al. 1993, Turlings et al. 1995). In particular, the volatile blend signature produced by plants in response to different herbivores may be used by specialist parasitoids as signals for host specificity (Du et al. 1996, De Moraes et al.

1998). For instance, the specialist parasitoid *Cardiochiles nigriceps* Viereck was able to exploit the differences in volatile blends produced by cotton or corn plants in response to different herbivores to distinguish infestation by its host, *H. virescens* from that by the closely related *H. zea* (De Moraes et al. 1998).

The question of whether specialist and generalist parasitoids show differential response to different suites of host-related volatiles has been a major focus of evolutionary ecology in recent years (Vet et al. 1993, Geervliet et al. 1996, Bernays 2001, Chen and Fadamiro 2007, Stilmant et al. 2008). It is predicted that specialist parasitoids utilizing fewer number of hosts are likely to possess a relatively more highly sensitive (high olfactory sensitivity to host-related chemical cues) and narrowly-tuned (selective) host detection olfactory system than generalist parasitoids (Vet and Dicke 1992, Cortesero et al. 1997, Smid et al. 2002, Chen and Fadamiro 2007). However, only a few studies have compared olfactory response and sensitivity to host-related volatiles in specialist and generalist parasitoids to date, and these have produced contrasting results (Elzen et al. 1987, Vet et al. 1993, Geerveliet et al. 1996, Chen and Fadamiro 2007). On the one hand, some studies reported relatively greater response for specialists compared to generalists (Elzen et al. 1987, Vet et al. 1993). In contrast, Geervliet et al. (1996) recorded no differences in the behavioral responses of the specialist, *Cotesia rubecula* Marshall and the generalist, *Cotesia marginiventris* (Cresson) to host-related volatiles, and both species were unable to distinguish between plant volatiles induced by their hosts versus plant volatiles induced by nonhost species. Similarly, Smid et al. (2002) reported no differences in the receptive range of the specialist, *C. rubecula* and the generalist, *Cotesia glomerata* L. to a wide range of host-related odor compounds. Such discrepancies in the above studies suggest that diverse species of specialist and generalist parasitoids may respond differently to different types of host-related

volatiles. Furthermore, even within a broad category of specialist or generalist parasitoids, differences may still exist among species based on the degree of specialization (De Moraes et al. 1998, Tamo et al. 2006).

In this study, we tested the above prediction using a tritrophic model system consisting of cotton (plant), *H. zea* and *S. exigua* (herbivores), and two parasitoids (Hymenoptera: Braconidae) with different degrees of host specificity, *Microplitis croceipes* (Cresson) and *C. marginiventris*. *M. croceipes* is a relatively specialist parasitoid specific to the caterpillars of *H. zea* and *H. virescens*, while *C. marginiventris* is a generalist parasitoid of caterpillars of a wide range of lepidopteran species, including *S. exigua*, *H. zea*, and *H. virescens* (Jalali et al. 1987, Turlings et al. 1990, Lewis et al. 1991, Röse et al. 1998). Both parasitoid species were selected as experimental models for this comparative study because they have served as models in previous studies of parasitoid olfaction, and several aspects of their responses to host-related volatiles have been characterized (e.g., Dmoch et al. 1985, Li et al. 1992, Cortesero et al. 1997, Röse et al. 1998, Park et al. 2002, Gouinguené et al. 2005). For the first time, we used the coupled gas chromatography electroantennogram detection (GC-EAD) technique to test for similarities and differences in the antennal responses of both parasitoid species to headspace volatiles of cotton plants infested with *H. virescens* (a host species for both parasitoids) versus *S. exigua* (a host species for *C. marginiventris* but not for *M. croceipes*). Based on the results of a recent study that recorded differences in the electroantennogram (EAG) responses of both parasitoid species to various synthetic host-related volatile compounds (Chen and Fadamiro, 2007), I hypothesized that *M. croceipes* will show relatively greater GC-EAD responses than *C. marginiventris* (generalist) to the HIPV components of cotton headspaces, whereas the GLV components, which

are emitted passively by plants and as a generalized response to herbivore damage will elicit relatively greater GC-EAD activity in the generalist.

3.2 Materials and Methods

3.2.1 Plants. Cotton (*G. hirsutum*, var. max 9) plants were grown in individual pots (9 cm high, 11 cm diameter) in a greenhouse (Auburn University Plant Science Greenhouse Facility) at 25 °C ± 10, 15:9 h (L/D) photoperiod and 50 ± 10% relative humidity. Seeds were planted in a top soil/vermiculite/peat moss mixture. Plants used for headspace volatile collections were 4-6 weeks old.

3.2.2 Caterpillars (Parasitoid Hosts). Two lepidopteran species, *H. virescens* and *S. exigua*, were used as parasitoid hosts in this study. Both species are distributed throughout the United States and are important pests of important agricultural crops including corn and cotton. Eggs purchased from Benzon Research (Carlisle, PA) were used to start laboratory colonies of both species. Caterpillars of both species were reared on a laboratory-prepared pinto bean diet (Shorey and Hale, 1965) at 25 ± 1^{o}C, 75 ± 5% relative humidity and 14:10-h (L/D) photoperiod.

3.2.3 Parasitoids. The parent cultures of *M. croceipes* and *C. marginiventris* were provided by the USDA-ARS, Insect Biology and Population Management Research Laboratory (Tifton, Georgia) and the University of Georgia, Tifton campus (contact: John Ruberson), respectively. *M. croceipes* was reared on caterpillars of *H. virescens*, its preferred host (Stadelbacher et al. 1984, King et al. 1985), whereas *C. marginiventris* was reared on caterpillars of its main host *S. exigua* (Jalali et al. 1987). Rearing procedures were similar to those of Lewis

and Burton (1970) and the rearing conditions were the same as described above for the caterpillar hosts. For each species, newly emerged adults were collected prior to mating, sexed, and placed in pairs of individuals of opposite sex (mated individuals) in a 6-cm diameter plastic Petri dish supplied with water and sugar sources. Water was provided by filling a 0.5 ml microcentrifuge tube with distilled water and threading a cotton string through a hole in the cap of the tube. About 4-6 drops (2 µl per drop) of 10% sugar solution were smeared on the inside of the Petri dish cover with a cotton-tipped applicator. Female parasitoids (aged 3-5 days old) of both species were used for the experiments.

3.2.4 Collection and GC Analysis of Headspace Volatiles. The methodology and protocols used for volatile collection were similar to those reported by Gouingiene et al. (2005), but with some modifications. Headspace volatiles were collected both from caterpillar damaged and undamaged cotton plants. To induce the production of HIPVs from cotton plants, 30 second instar caterpillars of *H. virescens* or *S. exigua* were allowed to feed on a potted cotton plant for 12 hr prior to volatile collection. The pot with the potting soil was wrapped with aluminum foil to minimize evaporation of water and volatiles from the soil. The plant (with the feeding caterpillars) was then placed in a volatile collection chamber (Analytical Research Systems, Inc., Gainesville, FL.) consisting of a 5 L glass jar. A purified (using activated charcoal) air stream of 500 ml/min was passed through the jar at room temperature for 24 hr. Headspace volatiles were trapped using a trap containing 50 mg of Super-Q (Alltech Associates, Deerfield, IL) and eluted with 200 µl of methylene chloride. Resulting extracts (200 µl) were stored in a freezer (at -20 °C) until use. Another container with potting soil but without a plant was used to check for miscellaneous impurities and background noise. The collection system was checked and

controlled for breakthrough of the trap during sampling. One µl of each headspace volatile extract was injected into a Shimadzu gas chromatography, GC-17A equipped with a flame ionization detector (FID). The dimension of the capillary column used was as follows: Rtx-1MS, 0.25 µm ID, 0.25m film thickness (Restek, Bellefonte, PA). Helium was used as carrier gas at a flow rate of 1 ml/min. The GC oven was programmed as follows: inject at 40 °C, hold at 40 °C for 2 minute, and then increase by 5 °C/min to 200 °C for a total of 40 minutes. The temperature of both injector and detector was set at 200 °C.

3.2.5 GC-EAD Recordings. The extracts were subjected to coupled gas chromatography-electroantennogram detection (GC-EAD) analyses with females of both parasitoid species to detect biologically active peaks (components). GC-EAD analyses were conducted with samples of headspace volatiles from cotton plants infested with *H. virescens* or *S. exigua* caterpillars and detected with antennae of *M. croceipes* or *C. marginiventris* females (total of 4 combinations or treatments). The GC-EAD techniques used were similar to those described by Smid et al. (2002). Briefly, the system was based on the above Shimadzu GC-17A equipped with a FID and coupled to an electroantennogram (EAG) detector. The dimension of the GC capillary column was same as described above. The column effluent was mixed with 30 ml/min make-up helium and split at a ratio of 1:2 (v/v), with one part going to the FID and the other through a heated (220 °C) transfer line (Syntech, Hilversum, Netherlands) into a charcoal filtered, humidified airstream (1000 ml/min) directed at the antenna preparation (EAG detector). The GC oven was programmed as above. The antenna preparation and EAG techniques were the same as previously described by Chen and Fadamiro (2007). A Glass capillary (1.1 mm I.D.) filled with Ringer solution was used as an electrode. Parasitoids were first anaesthetized by

chilling and the head isolated. The reference electrode was connected to the neck of the isolated head, while the recording electrode was connected to the antennal tip (with the last segment of antenna cut off). Chlorinated silver-silver chloride junctions were used to maintain electrical contact between the electrodes and input of a 1 × preamplifier (Syntech®, the Netherlands). The analog signal was detected through a probe (INR-II, Syntech®, The Netherlands), captured and processed with a data acquisition controller (IDAC-4, Syntech®, The Netherlands), and later analyzed with software (EAG 2000, Syntech®, The Netherlands) on a personal computer. A 3-µl aliquot of each sample was injected for a GC-EAD run. Five successful GC-EAD recordings were obtained for each treatment. GC-EAD traces were overlaid on the computer monitor and inspected for statistically and consistent qualitative and quantitative differences among the treatments.

3.2.6 GC-MS Analyses. The GC-EAD active peaks in each treatment were later identified by gas chromatography-mass spectrometry (GC-MS) using an Agilent 7890A GC coupled to a 5975C Mass Selective Detector, with a HP-5ms capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness). One µl of each headspace extract was injected into the GC-MS in splitless mode and using the GC conditions described above for GC-EAD. The chromatographic profiles were similar to those obtained from GC-EAD recordings making it possible to match the peaks. Mass spectra were obtained using electron impact (EI, 70 eV). Identification of EAD-active peaks was done by using NIST 98 library (National Institute of Standards and Technology, Gaithersburg, Maryland) and by comparing with published GC profiles of cotton head space volatiles (Thompson et al. 1971, Loughrin et al. 1994, McCall et al. 1994).

The structures of the identified compounds were confirmed using commercially available synthetic standards with purity > 97% (as indicated on the labels) obtained from Sigma® Chemical Co. (St. Louis, Missouri). Significant differences in the amounts of each volatile component emitted by *H. virescens* damaged versus *S. exigua* damaged cotton plants were established by using the Student's t-test ($P < 0.05$: SAS Institute 1998).

3.2.7 GC-EAD Analyses with Synthetic Blend. In order to confirm the observed differences in the GC-EAD responses of both parasitoids to the headspace extracts, a synthetic blend mimicking the headspace of caterpillar-infested cotton plants was prepared. This blend was formulated to mimic closely the active components of the headspace of cotton plants infested with *H. virescens*, although the same compounds were detected also in the headspace of cotton plants infested with *S. exigua.* It consisted of 13 synthetic volatile compounds which were identified as key biologically active components in the headspace volatiles of cotton plants infested with *H. virescens*, and blended at an approximate ratio in which they were detected in the headspace. The compounds were purchased from Sigma® Chemical Co. with purity > 97% and included *cis*-3-hexenal, *trans*-2-hexenal, *cis*-3-hexen-1-ol, *cis*-3-hexenyl acetate, *trans* -2-hexenyl acetate, linalool, *cis*-3-hexenyl butyrate, *trans*-2-hexenyl butyrate, indole, *cis*-jasmone, α-farnesene, α-humulene, and *trans*-nerolidol, blended in the ratio of 4.8, 7.8, 1.9, 19.8, 12.2, 2.2, 13.3, 11.1, 7.2, 0.4, 4.6, 4.3, and 10.2, respectively. Each compound was diluted in hexane to give 100 µg/µl solutions. GC-EAD responses of both parasitoid species were tested to the synthetic blend as described above. A 3-µl aliquot of the blend was injected for a GC-EAD run.

Five successful GC-EAD recordings were obtained for each treatment and compared as described above.

3.3 Results

3.3.1 GC and GC-MS Analysis of Headspace Volatiles. The GC profiles of the extracts of headspace volatiles from cotton plants infested with *H. virescens* or *S. exigua* versus uninfested (undamaged) plants are shown in Figure 1. A total of 30 peaks (volatile components) were detected in the headspace of plants infested with *H. virescens* or *S. exigua* (Fig. 1A, B). The same compounds were detected in both extracts, meaning that no qualitative differences were recorded. However, noticeable quantitative differences were recorded between the two extracts. In particular, 18 peaks were significantly elevated in the headspace of plants infested with *H. virescens* compared to plants infested with *S. exigua* (Table 1). These elevated peaks, as identified by GC-MS, included *cis*-3-hexenal, *cis*-3-hexen-1-ol, α-pinene, β-myrcene, *cis*-3-hexenyl butyrate, *cis*-3-hexenyl-2-methyl butyrate, *cis*-jasmone, α-farnesene, *trans*-nerolidol, and several other HIPV components. No peaks were obviously elevated in the headspace of plants infested with *S. exigua*, relative to those infested with *H. virescens*. Most of the above peaks were not detected or were detected in insignificant amounts in the headspace of undamaged cotton plants (Fig. 1C). Only five peaks (components) were slightly detectable in undamaged plants and were identified by GC-MS as α-pinene, *trans*-2-hexenyl butyrate, linalool, *n*-decanal, and caryophyllene. However, all five components were detected in much greater amounts in the headspace of caterpillar-infested plants.

3.3.2 GC-EAD and GC-MS Analyses. Similarities were recorded in the GC-EAD responses of *M. croceipes* and *C. marginiventris* females to volatiles from cotton plants infested with the two caterpillar species. Sixteen components of the headspace of caterpillar-infested plants elicited consistent GC-EAD responses in both parasitoid species (Figs. 2 and 3). As identified by GC-MS, these volatiles included several GLVs (*cis*-3-hexenal, *trans*-2-hexenal, *cis*-3-hexen-1-ol, and *trans*-2- hexen-1-ol) and HIPVs ((*E*)-4,8-dimethyl-1,3,7-nonatriene, *cis*-3-hexenyl butyrate, *trans*-2-hexenyl butyrate, *n*-decanal, *cis*-3-hexenyl-2-methyl butyrate, *trans*-2-hexenyl-2-methyl butyrate, indole, isobutyl tiglate, (*E*)-2-hexenyl tiglate, *cis*-jasmone, caryophyllene, α-*trans* bergamotene, α-farnesene, α-humulene, β-farnesene, β-hemachalene, and *trans*-nerolidol). More importantly, key differences were recorded in the response patterns of both parasitoids to the different components of the headspace extracts. Quantitatively, *C. marginiventris* (generalist) showed greater GC-EAD responses to the GLV (e.g., *cis*-3-hexenal, *trans*-2-hexenal and *cis*-3-hexen-1-ol) components of the two extracts, compared to *M. croceipes* (specialist) (Figs. 2 and 3). In contrast, several HIPV components of both extracts (e.g., *cis*-3-hexenyl acetate, linalool, *cis*-3-hexenyl butyrate and *trans*-2-hexenyl butyrate) elicited relatively greater responses in *M. croceipes*, compared to *C. marginiventris*. Note that responses of *C. marginiventris* to some of the HIPV components were very low and barely detectable in Figures 1 and 2. In general, the GC-EAD responses of both parasitoid species to the synthetic blend mimicked their responses to the headspace volatiles of caterpillar-infested plants (Fig. 4).

3.4 Discussion

This study showed that *M. croceipes* and *C. marginiventris* females were capable of responding antennally to many but not all of the caterpillar-induced cotton volatiles, with both

parasitoid species showing differential electrophysiological responses to the different components of the volatile blends. Compared to undamaged plants, cotton plants emitted detectable amounts of a wide range of volatiles, specifically 30 volatile compounds, in response to damage by *H. virescens* or *S. exigua*. In general, our results are in agreement with those previously reported by other authors on the induction of cotton volatiles by caterpillar species (Loughrin et al. 1994, McCall et al. 1994), but with some important differences. Loughrin et al. (1994) and McCall et al. (1994) reported 23 and 22 compounds, respectively from the headspace of caterpillar-damaged cotton plants, most of which were identified also in the present study. These compounds included GLVs such as *cis*-3-hexenal, *trans*-2-hexenal, and *cis*-3-hexen-1-ol, and HIPVs such as *cis*-3-hexenyl acetate, linalool, (*E,E*)-4,8-dimethyl-1,3,7-nonatriene, *cis*-3-hexenyl butyrate, *trans*-2-hexenyl butyrate, *trans*-2-hexenyl-2-methyl butyrate, indole, *cis*-jasmone, (*E,E*)-α-farnesene, α-humulene, and *trans*-nerolidol. However, we also detected additional volatile compounds which were not reported by Loughrin et al. (1994) and McCall et al. (1994), including *n*-decanal, (*E*)-2-hexenyl tiglate, and β-hemachelene. The difference between our results and those reported by Loughrin et al. (1994) and McCall et al. (1994) may be due to many factors including differences in headspace volatile collection methodology, sensitivity of the analytical system, and cotton cultivar. For instance, we collected cotton volatiles continuously for 24 hr beginning 12 hr after the plants were infested with caterpillars. Loughrin et al. (1994) collected volatiles for a 3-hr duration in each trap continuously for 60 hr, beginning 1 hr after plants were infested with caterpillars, while McCall et al. (1994) collected volatiles continuously for 2 hr beginning 16-19 hr after caterpillar feeding began. Furthermore, differences in the species/strains and stages of caterpillars tested may play a role. Loughrin et al.

(1994) used *S. exigua* caterpillars, while *H. zea* caterpillars were used by McCall et al. (1994). In the present study, we tested *H. virescens* and *S. exigua* caterpillars.

We recorded obvious differences in the amounts of the volatile compounds induced by *H. virescens* versus *S. exigua*. Of the total 30 components identified, 18 were detected in significantly higher amounts in the headspace of *H. virescens* damaged plants, compared to *S. exigua* damaged plants. These results suggest that the essential difference between the volatile blends induced by both caterpillar species is quantitative, rather than qualitative. Similar differences in the headspace volatile composition of plants infested by different herbivore species have been reported in cotton (McCall et al. 1994, Loughrin et al. 1994, De Moraes et al. 1998), corn (Turlings et al. 1998, De Moraes et al. 1998), cabbage (Agelopoulous and Keller 1994, Geervliet et al. 1997), and tobacco (De Moraes et al. 1998). It has been proposed that herbivore-specific volatile blends that differ significantly and consistently may provide reliable, information-rich signals to foraging parasitoids (De Moraes et al. 1998). Thus, the elevated volatiles in the headspace of *H. virescens* damaged cotton plants, compared to *S. exigua* damaged plants, may convey herbivore-specific information to specialist parasitoids, such as *M. croceipes*. On the other hand, generalist parasitoids, such as *C. marginiventris*, which have a wide host range, may not necessarily use herbivore-specific signals for host location.

Only 16 of the 30 volatile components consistently elicited GC-EAD responses in *M. croceipes* and *C. marginiventris*, suggesting that not all the volatile components are perceived by both parasitoid species, a finding in concert with those previously reported for some other parasitoid wasp species (Li et al. 1992, Light et al. 1992, Park et al. 2001, Smid et al. 2002, Gouinguené et al. 2005). It is noteworthy that most of the 16 GC-EAD active volatile compounds were among those elevated in *H. virescens* damaged plants. Our results showed no

obvious qualitative differences in the range of compounds detected by both parasitoid species. This is the first comparative study of GC-EAD responses of both parasitoid species to herbivore-induced cotton volatiles. In one of the few similar studies on other tritrophic systems, Smid et al. (2002) reported no differences in the GC-EAD responses of the specialist parasitoid, *C. rubecula* and the generalist, *C. glomerulata* to a wide range of volatiles from Brussels sprouts damaged by two species of *Pieris* caterpillars. In contrast, Gouinguené et al. (2005) reported some key differences in the GC-EAD responses of three parasitoid wasps to maize volatiles damaged by *Spodoptera littoralis* Boisduval caterpillars. Relatively more compounds elicited GC-EAD responses in the generalists, *C. marginiventris* and *Campoletis sonorensis* (Cameron), compared to *Microplitis rufiventris* Kok., which is found more often on *S. littoralis* (Gouinguené et al. 2005).

The major difference recorded in my study was in the intensity of GC-EAD response of both parasitoids to several compounds. The generalist, *C. marginiventris* showed relatively greater GC-EAD responses than the specialist, *M. croceipes* to some GLV components, whereas several HIPV components elicited comparatively greater responses in *M. croceipes*. Although I was unable to quantify these differences statistically because my software had no such capability, the differential GC-EAD responses of both parasitoid species to the GLV versus HIPV components of the headspace extracts are obvious in Figures 2-4. Similar differences in the intensity of response of parasitoids to host-related compounds were also reported by Gouinguené et al. (2005). Those authors reported that the generalist parasitoids, *C. marginiventris* and *C. sonorensis* showed a greater sensitivity to cotton GLVs than the more restricted *M. rufiventris.* My results, in which females of the generalist *C. marginiventris* showed comparatively greater GC-EAD responses to GLVs, which are continuously present in the plant and released in freshly

damaged plants, support my hypothesis, and are somewhat in agreement with previous electrophysiological (Chen and Fadamiro 2007) and behavioral studies (Cortesero et al. 1997, Hoballah et al. 2002, D'Alessandro and Turlings 2005, Hoballah and Turlings 2005). In contrast, the specialist *M. croceipes* showed greater GC-EAD responses to the HIPVs, which are more specifically linked to its host. These findings were verified by the results of the GC-EAD tests with the synthetic blend, which also showed the same differences in the intensity of response of both parasitoid species.

In general, *M. croceipes* showed slightly greater GC-EAD responses to headspace volatiles collected from cotton plants damaged by its host species (*H. virescens*) than to headspace volatiles collected from cotton plants that were damaged by the non-host species (*S. exigua*). My GC data showed that the essential difference between the volatile blends of cotton plants induced by *H. virescens* versus *S. exigua* was in the amounts and consequently ratios of the same identical compounds. De Moraes et al. (1998) reported also that the main difference in the volatile blends of plants damaged by *H. virescens* versus *H. zea* was in the ratios of identical compounds. Those authors further reported that the specialist parasitoid *C. nigriceps* could distinguish behaviorally plants damaged by its host, *H. virescens* from those damaged by *H. zea* (a non-host species), possibly by exploring the differences in the ratios of identical compounds in the volatile blends. Thus, the differences recorded in this study in the ratios of the same identical compounds in the volatile blends induced by the two caterpillar species may be exploited by *M. croceipes* to differentiate plants damaged by its host from non-host species. This proposition is supported by my GC-EAD results which showed greater response of *M. croceipes* to volatiles from *H. virescens* damaged plants, compared to *S. exigua* damaged plants. The need to discriminate hosts from related non-hosts based on subtle differences in the ratios of identical

compounds in volatile blends is likely a challenging task for specialist parasitoids, such as *M. croceipes*. In contrast, no obvious differences were observed in the response of *C. marginiventris* to volatile blends induced by both caterpillar species. My data for *C. marginiventris* are in agreement with the report by Geervliet et al. (1996) that a related generalist species, *C. glomerata*, was unable to distinguish between plant volatiles induced by its hosts versus plant volatiles induced by non-host species. These results are not surprising, given that generalist parasitoids do not have to rely on herbivore-specific signals for host location. However, it is possible that associative learning may improve the overall ability of *C. marginiventris* and other generalist parasitoids to respond to the HIPV components of the volatile blends (Turlings et al. 1989, 1993, Vet and Groenewold 1990, Vet 1999, Steidle and van Loon 2003, Tamò et al. 2006).

The recorded differences in the antennal sensitivity of *M. croceipes* and *C. marginiventris* to host-related volatiles may be related to possible differences in the abundance and distribution of olfactory sensilla on the antennae of both parasitoid species. Sensilla placodea have been identified as the main olfactory sensilla responsive to host-related volatiles in *M. croceipes* (Ochieng et al. 2000) and *Cotesia* spp. (Bleeker et al. 2004). A comparative study of antennal morphology of the closely related *C. rubecula* and *C. glomerata* revealed significant differences in the density and distribution of this sensilla type (Bleeker et al. 2004). In an ongoing comparative study of antennal sensilla of *M. croceipes* and *C. marginiventris*, Das (2011) recorded relatively greater numbers of olfactory sensilla placodea on *M. croceipes* than on *C. marginiventris* antennae. This difference in the density of olfactory sensilla may explain the differences in GC-EAD responses of both parasitoid species recorded in this study.

In summary, the results support my hypothesis and may provide insights into how specialist parasitoids can distinguish between plants damaged by their hosts versus plants

damaged by closely related non-hosts, even though the different hosts may induce the emission of qualitatively similar volatile blends. The data suggest that differences between similar volatile blends in the ratios of identical volatile compounds may contribute to host specificity in specialist parasitoids, such as *M. croceipes*. Further discrimination of different host-plant complexes may be mediated at short range by host contact kairomones (which are typically of relatively lower volatility), such as host feces (Loke and Ashley 1984, Dmoch et al. 1985, Afsheen et al. 2008) and caterpillar chemical footprints on infested plants (Rostás and Wölfling 2009). Future behavioral studies are necessary to confirm whether or not the ability of *M. croceipes* to distinguish between plants damaged by its host and non-host caterpillars (Rosé et al. 1997), is in fact mediated by the subtle quantitative differences in volatile blends, as recorded in this study. If confirmed, the neurophysiological mechanisms mediating this fine scale ability for odor discrimination will be addressed in the future using single sensillum and neuroanatomical techniques.

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Table 1. Composition of volatiles collected from cotton plants infested for 24 hr with *H. virescens* or *S. exigua* caterpillars and undamaged control plants

ID	Compound[1]	*H. virescens* damaged		*S. exigua* damaged		Undamaged	
		Amount (ng ± SE) [2]	Relative %	Amount (ng ± SE) [2]	Relative %	Amount (ng ± SE) [2]	Relative %
1	*cis*-3-hexenal	39,350 ± 3212 a	1.9	1,408 ± 238 b	0.09	0	0
2	*trans*-2-hexenal	63,420 ± 1106	3.0	72,438 ± 2520	5.0	0	0
3	*cis*-3-hexen-1-ol	15,740 ± 670 a	0.8	8,200 ± 720 b	0.5	0	0
4	*trans*-2-hexen-1-ol	69,402 ± 2230	3.3	67,120 ± 1340	4.7	0	0
5	α-pinene	98,310 ± 3110 a	4.5	83,120 ± 2620 b	5.8	100 ± 25	18.5
6	β-pinene	58,239 ±1939 a	2.8	42,300 ± 1940 b	2.9	0	0
7	myrcene	120,259 ± 5920 a	5.8	15,465 ± 853 b	1.1	0	0
8	*cis*-3-hexenyl acetate	161,470 ± 2350	7.7	120,475 ± 4860	8.4	0	0
9	*trans*-2-hexenyl acetate	99,214 ± 1074	4.8	111,345 ± 3740	7.8	0	0
10	limonene	110,259 ± 983 a	5.3	84,330 ± 750 b	5.9	0	0
11	β-ocimene	120,257 ± 1506 a	5.8	89,354 ± 2015 b	6.2	0	0
12	linalool	18,343 ± 939	0.9	18,468 ± 542	1.3	150 ± 38	27.7
13	unknown	59,320 ± 1812	2.8	58,458 ± 2040	4.1	0	0
14	4,8-dimethyl-1,3,7-nonatriene	21,320 ± 1003	1.0	78,800 ± 1296	5.5	0	0
15	*cis*-3-hexenyl butyrate	108,345 ± 1690 a	5.2	36,900 ± 1165 b	2.5	0	0
16	*trans*-2-hexenyl butyrate	90,210 ± 4500	4.3	91,356 ± 4300	6.4	135 ± 60	25.0
17	*n*-decanal	5,300 ± 412	0.3	4,800 ± 109	0.3	75 ± 18	13.8
18	*cis*-3-hexenyl-2-methyl butyrate	135,100 ± 3600 a	6.5	2,800 ± 198 b	0.2	0	0
19	*trans*-2-hexenyl-2-methyl butyrate	128,950 ± 5300	6.2	115,220 ± 5200	8.0	0	0
20	indole	58,430 ± 1250 a	2.8	43,200 ± 2700 b	3.0	0	0
21	isobutyl tiglate	15,900 ± 840 a	0.8	2,300 ± 350 b	0.2	0	0
22	2-hexenyl tiglate	6,500 ± 152	0.3	14,999 ± 1650	1.0	0	0
23	*cis*-jasmone	3,200 ± 636 a	0.2	900 ± 330 b	0.1	0	0
24	caryophyllene	170,500 ± 6835	8.2	154,230 ± 5300	10.7	80 ± 40	14.8
25	α-*trans* bergamotene	16,378 ± 910 a	0.8	468 ± 130 b	0.03	0	0
26	α-farnesene	37,745 ± 2470 a	1.8	23,300 ± 3564 b	1.6	0	0
27	α-humulene	35,200 ± 1119 a	1.7	2,300 ± 745 b	0.2	0	0
28	β-farnesene	48,239 ± 636 a	2.3	1,305 ± 248 b	0.09	0	0
29	β-hemachalene	94,600 ± 3830 a	4.5	65,780 ± 3200 b	4.6	0	0
30	*trans*-nerolidol	83,170 ± 868 a	4.0	23,450 ± 1950 b	1.6	0	0

[1] In order of elution during gas chromatography

[2] Values (amount emitted) are mean ± SE of five replicate extractions

Means across the same row followed by different letters are significantly different ($P < 0.05$, t-test).

Table 2. Quantification of GC-EAD responses of *M. croceipes* and *C.marginiventris* to the different components of headspace extracts of cotton plants infested with *H. virescens* or *S. exigua*, and a synthetic blend of GC-EAD active components

ID	Compound[a]	*H. virescens*-infested		*S. exigua*-infested		Synthetic Blend	
		Microplitis croceipes (µV ± SE)[b]	*Cotesia marginiventris* (µV ± SE)[b]	*Microplitis croceipes* (µV ± SE)[b]	*Cotesia marginiventris* (µV ± SE)[b]	*Microplitis croceipes* (µV ± SE)[b]	*Cotesia marginiventris* (µV ± SE)[b]
1	*cis*-3-hexenal	72 ± 6.6 b	192 ± 10 a	56 ± 4.0 b	172 ± 12 a	140 ± 8.9 b	240 ± 11 a
2	*trans*-2-hexanal	64 ± 6.3 b	82 ± 8.4 a	56 ± 4.0 b	88 ± 6.2 a	62 ± 4.8 b	96 ± 6.8 a
3	*cis*-3-hexen-1-ol	44 ± 4.0 b	72 ± 8.0 a	48 ± 8.0 b	80 ± 6.3 a	76 ± 4.5 b	98 ± 6.3 a
4	*cis*-3-hexenyl acetate	144 ± 7.2 a	92 ± 8.0 b	176 ± 6.4 a	72 ± 8.5 b	136 ± 7.4 a	84 ± 4.0 b
5	*trans*-2-hexenyl acetate	52 ± 6.3	48 ± 6.3	54 ± 6.3	46 ± 5.8	96 ± 7.4 a	28 ± 4.8 b
6	linalool	72 ± 6.9 a	24 ± 4.0 b	80 ± 6.3 a	24 ± 4.0 b	80 ± 7.4 a	64 ± 6.2 b
7	4,8-dimethyl nonatriene	92 ± 5.0	88 ± 5.0	100 ± 9.0 a	44 ± 4.0 b		
8	unknown	108 ± 5.0	88 ± 8.0	100 ± 12	72 ± 4.8		
9	*cis*-3-hexenyl butyrate	104 ± 7.5 a	60 ± 6.3 b	172 ± 8.0 a	56 ± 4.2 b	240 ± 10 a	68 ± 4.8 b
10	*trans*-2-hexenyl butyrate	100 ± 6.3 a	60 ± 5.3 b	100 ± 6.3 a	32 ± 4.8 b	62 ± 4.8 a	28 ± 3.6 b
11	*trans*-2-hexenyl-2-methyl butyrate	60 ± 6.3	40 ± 8.9	88 ± 8.0 a	24 ± 4.0 b		
12	indole	24 ± 9.8	36 ± 7.5	80 ± 6.3 a	32 ± 4.8 b	28 ± 4.8	16 ± 4.0
13	*cis*-jasmone	52 ± 4.8	38 ± 4.8	48 ± 5.8 a	12 ± 4.8 b	88 ± 4.8 a	52 ± 4.4 b
14	α-farnesene	60 ± 6.3	48 ± 8.0	42 ± 4.9	12 ± 3.8	88 ± 8.0 a	24 ± 4.0 b
15	α-humulene	60 ± 6.3 a	8 ± 3.8 b	38 ± 3.7	16 ± 4.2	16 ± 4.0	8 ± 4.8
16	*trans*-nerolidol	16 ± 4.0	12 ± 4.8	12 ± 4.8	9 ± 4.8	20 ± 6.3	20 ± 6.3

[a] In order of elution during gas chromatography
[b] Values (µv) are mean ± SE of five replicates
Means across the same row for the same headspace extract or synthetic blend followed by different letters are significantly different ($P<0.05$, t-test).

Figure Legend

Figure 1. Chromatographic profiles of headspace volatiles collected from cotton plants infested with *H. virescens* (A) or *S. exigua* (B) caterpillars, versus undamaged control plants (C). Identified compounds: (1) *cis*-3-hexenal; (2) *trans*-2-hexenal; (3) *cis*-3-hexen-1-ol; (4) trans-2-hexen-1-ol; (5) α-pinene; (6) β-pinene; (7) myrcene; (8) *cis*-3-hexenyl acetate; (9) *trans*-2-hexenyl acetate; (10) limonene; (11) β-ocimene; (12) linalool; (13) unknown; (14) (*E*)-4,8-dimethyl-1,3,7-nonatriene; (15) *cis*-3-hexenyl butyrate; (16) *trans*-2-hexenyl butyrate; (17) *n*-decanal (18) *cis*-3-hexenyl-2-methyl butyrate; (19) *trans*-2-hexenyl-2-methyl butyrate; (20) indole; (21) isobutyl tiglate; (22) (*E*)-2-hexenyl tiglate; (23) *cis*-jasmone; (24) caryophyllene; (25) α-*trans* bergamotene; (26) α-farnesene; (27) α-humulene; (28) β-farnesene; (29) β-hemachalene; (30) *trans*-nerolidol.

Figure 2. GC-EAD responses of *M. croceipes* (A) and *C. marginiventris* (B) to headspace volatiles from *H. virescens* damaged cotton plants. GC-EAD active compounds: (1) *cis*-3-hexenal; (2) *trans*-2-hexenal; (3) *cis*-3-hexen-1-ol; (4) *cis*-3-hexenyl acetate; (5) *trans* -2-hexenyl acetate; (6) linalool; (7) (*E*)-4,8-dimethyl-1,3,7-nonatriene; (8) unknown; (9) *cis*-3-hexenyl butyrate; (10) *trans*-2-hexenyl butyrate ; (11) *trans*-2-hexenyl-2-methylbutyrate; (13) *cis*-jasmone; (14) α-farnesene; (15) α-humulene; (16) *trans*-nerolidol. Note that responses of *C. marginiventris* to some of the HIPV components were almost too low to be detectable in this and the next two figures.

Figure 3. GC-EAD responses of *M. croceipes* (A) and *C. marginiventris* (B) to headspace volatiles from *S. exigua* damaged cotton plants. GC-EAD active compounds: (1) *cis*-3-hexenal; (2) *trans*-2-hexenal; (3) *cis*-3-hexen-1-ol; (4) *cis*-3-hexenyl acetate; (5) *trans* -2-hexenyl acetate; (6) linalool; (7) (*E*)-4,8-dimethyl-1,3,7-nonatriene; (8) unknown; (9) *cis*-3-hexenyl butyrate; (10) *trans*-2-hexenyl butyrate; (11) *trans*-2-hexenyl 2-methylbutyrate; (12) indole; (13) *cis*-jasmone; (14) α-farnesene; (15) α-humulene; (16) *trans*-nerolidol.

Figure 4. GC-EAD responses of *M. croceipes* (A) and *C. marginiventris* (B) to a synthetic blend mimicking the headspace volatiles of caterpillar-infested cotton plants. The blend consisted of 13 compounds (listed below) identified as key biologically active components in the headspace volatiles of cotton plants infested with *H. virescens*, and blended at the approximate ratio in which they were detected in the headspace. Synthetic compounds: (1) *cis*-3-hexenal; (2) *trans*-2-hexenal; (3) *cis*-3-hexen-1-ol; (4) *cis*-3-hexenyl acetate; (5) *trans* -2-hexenyl acetate; (6) linalool; (9) *cis*-3-hexenyl butyrate; (10) *trans*-2-hexenyl butyrate ; (12) indole; (13) *cis*-jasmone; (14) α-farnesene; (15) α-humulene; (16) *trans*-nerolidol.

Figure 1

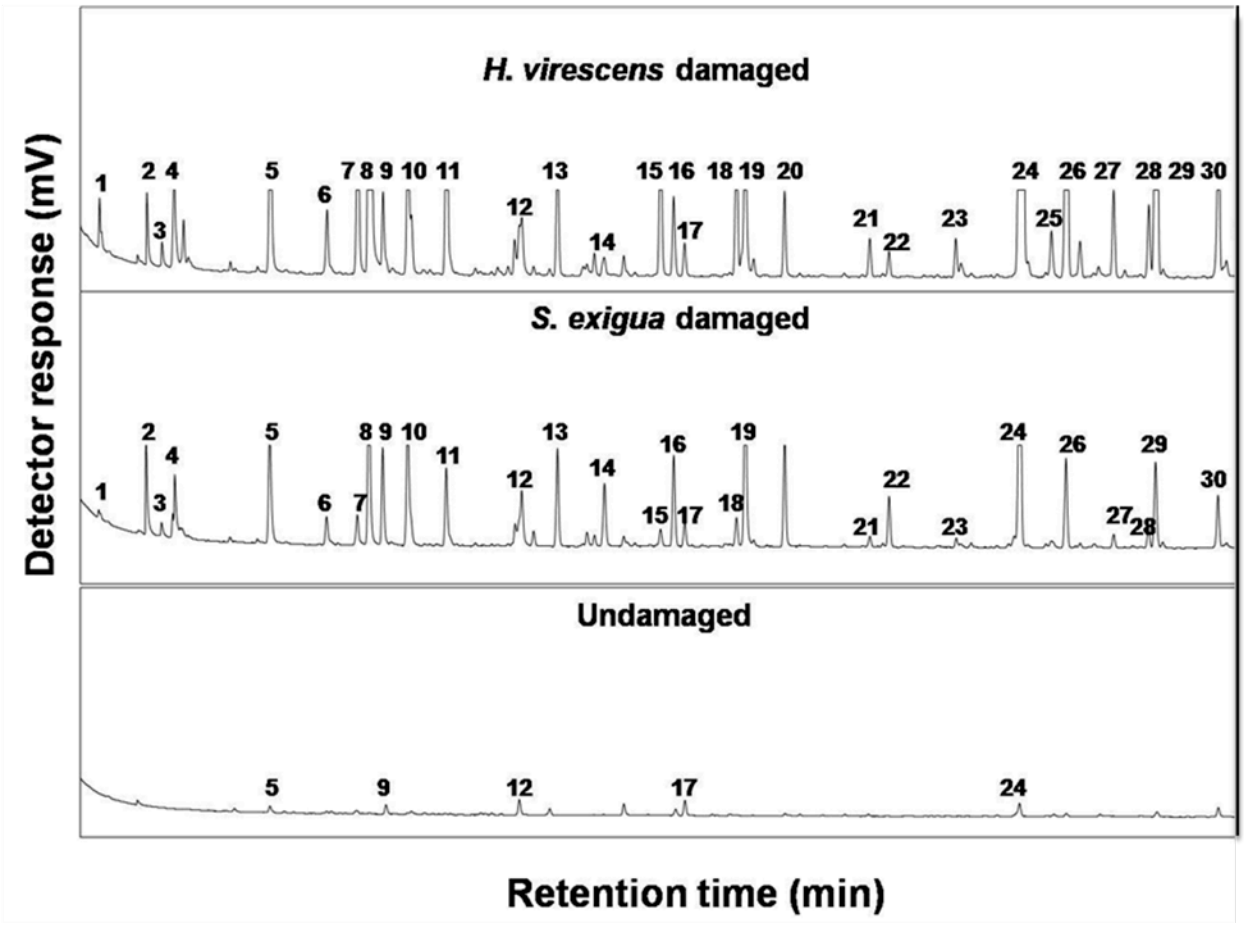
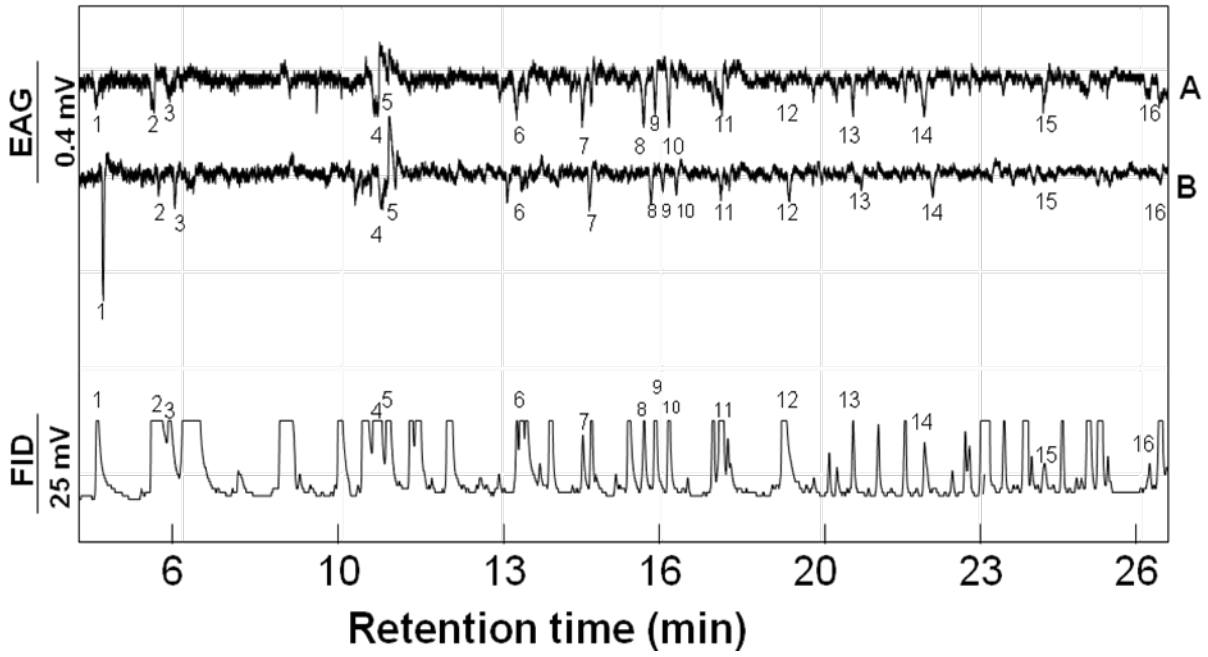

H. virescens damaged
S. exigua damaged
Undamaged
Detector response (mV)
Retention time (min)

Figure 2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A
B
FID
25 mV
1
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3
4
5
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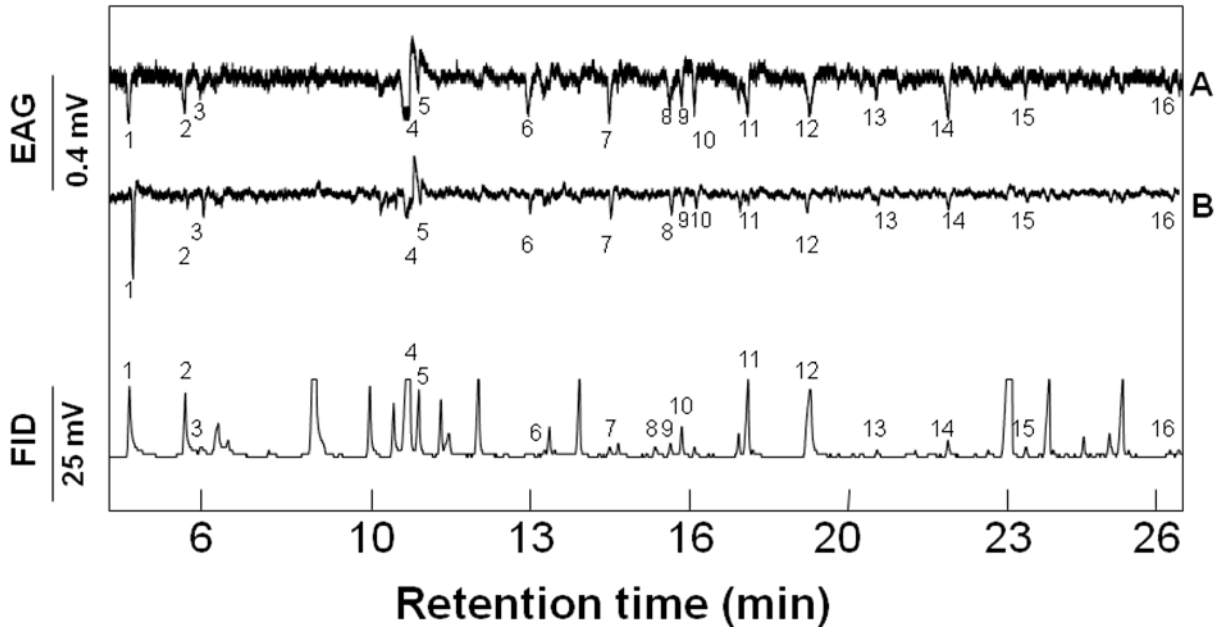
23
26
Retention time (min)

Figure 3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0.4 mV
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FID
25 mV
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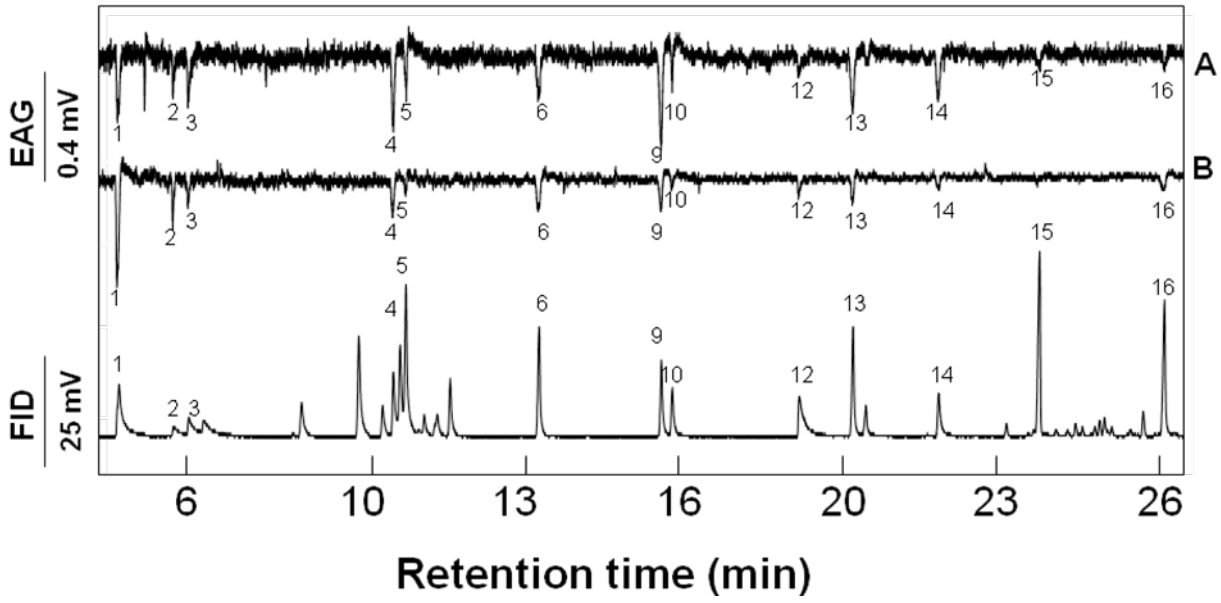
26
Retention time (min)

Figure 4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0.4 mV
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23
26
Retention time (min)

CHAPTER 4

EFFECTS OF PLANT GROWTH-PROMOTING RHIZOBACTERIA ON INDUCTION OF COTTON PLANT VOLATILES AND ATTRACTION OF PARASITOIDS

4.1 Introduction

Plant Growth-Promoting Rhizobacteria (PGPR) represent a wide range of root-colonizing bacteria whose application is often associated with increased rates of plant growth (Kloepper 1992, Zehnder et al. 1997, Kloepper et al. 2004), suppression of soil pathogens (Schippers et al. 1987, Burkett-Cadena et al. 2008) and the induction of systemic resistance against insect pests (van Loon et al. 1998, Kloepper et al. 1999, Ramamoorthy et al. 2001, Zehnder et al. 2001, Ryu et al. 2004, Ji et al. 2006). PGPR-based inoculants include formulations containing a single strain, a mixture of two strains, or complex mixtures of over 10 strains of *Bacillus* spp. (Lucy et al. 2004, Kloepper and Ryu, 2006). The effects of application of PGPR on induction of volatile organic compounds (VOCs) in treated plants are virtually unexamined, despite evidence that induction of plant volatiles is dependent on the interactions of biotic factors, such as plant hormones (de Bruxelles and Roberts, 2001, Thaler et al. 2002, Farmer et al. 2003, Ament et al. 2004), herbivore-derived elicitors (Mattiaci et al. 1995, Alborn et al. 1997, Spiteller and Boland, 2003), associated microorganisms including pathogens (Preston et al. 1999, Cardoza et al. 2002), and abiotic factors, such as wounding (Mithöfer et al. 2005), heavy metals (Mithöfer et al. 2004) and temperature and light (Takabayashi et al. 1994, Gouinguene and Turlings 2002). The lack of research on effects of PGPR on induction of plant volatiles is surprising given that PGPR are increasingly being applied to production of several field crops including cotton (*Gossypium*

hirsutum L.), tomato (*Solanum lycopersicum* L.), watermelon (*Citrullus lanatus* Thunb.), and pearl millet (*Pennisetum glaucum*) in the USA or India (Glick 1995, Backman et al. 1997, Cleyet-Marcel et al. 2001, Kokalis-Burelle et al. 2003, Niranjan Raj et al. 2003, Burkett-Cadena et al. 2008). Backman et al. (1997) reported that 60-75% of the US cotton crop is treated with the PGPR product Kodiak®, a *Bacillus subtilis* product used for suppression of *Fusarium* and *Rhizoctonia* soil pathogens.

Like herbivores that use VOCs in their search for suitable host plants (Dicke et al. 2000), parasitic insects are known to use blends of VOCs for foraging and host location of their herbivore hosts (Turlings et al. 1990, McCall et al. 1993, De Moraes et al. 1998). These VOCs can originate from the plant, herbivore host, or an interaction between herbivores and the plant (McCall et al., 1994, Cortesero et al. 1997). Plant-based VOCs are further categorized into green leaf volatiles (GLVs) (such as *cis*-3-hexenal and *cis*-3-hexen-1-ol) which are released immediately in response to mechanical damage or at the beginning of herbivore feeding, and herbivore-induced plant volatiles (HIPVs) (such as *cis*-3-hexenyl butyrate, (E)-β-ocimene, linalool and (E)-β-farnesene) which are emitted as a delayed response to herbivore feeding damage. These blends of VOCs, which are highly attractive to parasitoids of cotton herbivores including *Microplitis croceipes* Cresson and *Cotesia marginiventris* (Cresson) (Hymenoptera: Braconidae), are triggered by caterpillar feeding (De Moraes et al. 1998, Chen and Fadamiro 2007, Ngumbi et al. 2009, 2010). It is possible that PGPR could trigger VOC production in cotton with important consequences for foraging parasitoids and other chemical mediated insect-plant and tri-trophic interactions.

In this study, I tested the hypothesis that PGPR would elicit changes in cotton plant VOCs and alter the growth of cotton roots. Additionally, I hypothesized that parasitoids of cotton

herbivores would show greater attraction to PGPR treated cotton plants compared to untreated cotton plants via changes in the emission of VOCs. PGPR treated and untreated cotton plants were grown under greenhouse conditions and headspace volatiles collected 4-6 weeks post planting. I used coupled gas chromatography-mass spectrometry (GC-MS) to identify and analyze headspace volatiles from PGPR treated and untreated cotton plants. I used a four- choice olfactometer to study the behavior of *M. croceipes* when presented with PGPR treated plants versus untreated plants. To my knowledge, this is the first report of PGPR eliciting the production of altered changes in VOCs profiles in cotton plants.

4.2 Materials and Methods

4.2.1 PGPR Strains. Three PGPR strains (all from Auburn University) were used in this study: i) *Bacillus pumilis* strain INR-7 (AP 18), ii) Blend 8 containing four strains of spore forming *Bacilli* (AP 188, 209, 217 218), and iii) Blend 9, containing four strains of spore forming *Bacilli* (AP 136, 188, 219, 295).

4.2.2 Plants. Cotton (*G. hirsutum*, var. max 9) seeds were used. To each cotton seed, 1 ml of PGPR at spore concentrations of 10^8 for a population per seed of 10^7 was applied. The seeds were then grown in individual pots (9 cm high, 11 cm diameter) in a greenhouse (Auburn University Plant Science Greenhouse Facility) at 25 °C ± 10, 15:9 h (L/D) photoperiod, and 50 ± 10% relative humidity. Seeds were planted in a top soil/vermiculite/peat moss mixture. Additionally, every week, 1 ml of aqueous bacterial suspension (10^9 colony forming units) (cfu/ml) was applied to the growing cotton plants. Plants used for headspace volatile collections were 4 to 6 weeks old.

4.2.3 Insects. Parent cultures of *M. croceipes* were provided by the USDA-ARS, Insect Biology and Population Management Research Laboratory (Tifton, Georgia). *Microplitis croceipes* was reared on caterpillars of *Heliothis virescens* (Fab.) Lepidoptera: Noctuidae, its preferred host (Stadelbacher et al. 1984, King et al. 1985), using a procedure similar to that of Lewis and Burton (1970). Eggs purchased from Benzone Research (Carlisle, PA, USA) were used to start laboratory colonies of *H. virescens*, which was reared on a laboratory-prepared pinto bean diet (Shorey and Hale 1965). All colonies were maintained at 25 ± 1°C, 75 ± 5% RH, and under a L14:D10 photoperiod. Newly emerged *M. croceipes* adults were collected prior to mating, sexed, and placed in pairs of individuals of opposite sex (mated individuals) in a 6-cm diameter plastic Petri dish supplied with water and sugar sources. Water was provided by filling a 0.5 ml microcentrifuge tube with distilled water and threading a cotton string through a hole in the cap of the tube. About 5 drops (2 µl per drop) of 10% sugar solution were smeared on the inside of the Petri dish cover with a cotton-tipped applicator. Naïve parasitoids (aged 3-5 days) were used for the bioassays.

4.2.4 Collection and GC Analysis of Headspace Volatiles. The methodology and protocols used for volatile collection were similar to those reported by Gouinguené et al. (2005), but with some modifications. Headspace volatiles were collected both from PGPR treated and untreated cotton plants as well as PGPR treated and untreated caterpillar damaged cotton plants. To induce the production of herbivore induced plant volatiles (HIPVs) from PGPR treated and untreated plants, 30 2nd instar caterpillars of *Heliothis virescens* Fab. (Lepidoptera: Noctuidae) were allowed to feed on a potted cotton plant for 12 h prior to volatile collection. The pot with the potting soil was wrapped with aluminum foil to minimize evaporation of water and volatiles

from the soil. The plant was then placed in a volatile collection chamber (Analytical Research Systems, Inc., Gainesville, FL) consisting of a 5 L glass jar. A purified (using activated charcoal) air stream of 500 ml/min was passed through the jar at room temperature for 24 hr. Headspace volatiles were collected using a trap containing 50 mg of Super-Q (Alltech Associates, Deerfield, IL) and eluted with 200 µl of methylene chloride. The resulting extracts (200 µl) were stored in a freezer (at -20 °C) until use. Another container with potting soil without a plant was used to check for miscellaneous impurities and background noise. The collection system was checked and controlled for breakthrough of the trap during sampling. One µl of each headspace volatile extract was injected into a Shimadzu GC-17A equipped with a flame ionization detector (FID). The dimension of the capillary column used was as follows: Rtx-1MS, 0.25 mm I.D., 0.25 µm film thickness (Restek, Bellefonte, PA). Helium was used as carrier gas at a flow rate of 1 ml/min. The GC oven was programmed as follows: inject at 40 °C, hold at 40 °C for 2 minutes, and then increase by 5 °C/min to 200 °C for a total of 40 minutes. The temperatures of both injector and detector were set at 200 °C. Five replicates were carried out.

4.2.5 GC-MS Analysis. GC profiles of each plant treatment were later identified by GC-MS using an Agilent 7890A GC coupled to a 5975C Mass Selective Detector, with a HP-5ms capillary column (30 m × 0.25 mm I.D., 0.25 µm film thickness). One µl of each headspace extract was injected into the GC in splitless mode, using the GC conditions described above. Mass spectra were obtained using electron impact (EI, 70 eV). Identification of peaks was done by using NIST 98 library (National Institute of Standards and Technology, Gaithersburg, Maryland) and by comparing with published GC profiles of cotton head space volatiles (Thompson et al. 1971, Loughrin et al. 1994, McCall et al. 1994). The structures of the identified

compounds were confirmed using commercially available synthetic standards with purity > 97% (as indicated on the labels) obtained from Sigma® Chemical Co. (St. Louis, Missouri).

4.2.6 Analysis of Cotton Root Growth. A separate experiment was carried out to determine if treatment of cotton with PGPR would lead to differences in cotton root growth. To each cotton seed, 1 ml of PGPR (INR-7, Blend 8, and Blend 9) at spore concentrations of 10^8 for a population per seed of 10^7 was applied. The seeds were then grown in individual pots (15 cm high, 21 cm diameter) in a greenhouse (Auburn University Plant Science Greenhouse Facility) at 25 °C ± 10, 15:9 h (L/D) photoperiod, and 50 ± 10% relative humidity. Seeds were planted in a top soil/vermiculite/peat moss mixture. Additionally, every week, 1 ml of aqueous bacterial suspension (10^9) colony forming units (cfu/ml) was applied. Plants used for cotton root growth analysis were two weeks old. After washing roots, an analysis of root architecture was made on each plant's rooting system using the system of Regent Instruments, Inc. (Sainte-Foy, Quebec), which consists of scanner model LA 1600+ and WinRhizo software (version 2004a). Data from the resulting analyses were collected for two root parameters: root surface area and root volume (0-0.5 and 0.5-1.0 mm). Data on root dry weight were also collected. Eight replicates were done.

4.2.7 Four-Choice Olfactometer Bioassays with Parasitoids. Attraction of *M. croceipes* to odors of PGPR treated vs. untreated plants, as well as PGPR treated caterpillar damaged vs. undamaged plants, was assessed in four-choice olfactometer bioassays (Analytical Research Systems, Gainesville, FL). The apparatus was similar to the system described by Pettersson (1970) and Kalule and Wright (2004). It consists of a central chamber with orifices at the four corners through which purified and humidified air was drawn in, creating four potential

odor fields, and a central orifice where mixing of the airflow from the arms occurred. A constant airflow of 500 ml/min was maintained through each of the four orifices at the corners of the olfactometer. Mixtures of air from the control arms and volatile odors from the treatment arms were sucked out from the olfactometer with a constant airflow of 2.5 l/min, through the central orifice. Volatile odors emanated from plants that were 4-6 weeks old post-planting. The pot with the potting soil was wrapped with aluminum foil to minimize evaporation of water and volatiles. The plants were then placed in 5 L glass jar (32 cm high, 14.5 cm diameter) volatile collection chambers (Analytical Research Systems, Inc., Gainesville, FL USA) and purified air (500 ml/min) was passed through the chambers and into each of the 4 orifices at the corners of the olfactometer.

Naïve 3-5-d-old female *M. croceipes* were used in all experiments. A wasp was removed from the cage with an aspirator and introduced singly into a glass tube (1.5 cm). The glass tube was connected to the central orifice of the olfactometer to expose the wasp to the volatile odors/air mixtures. Once in the chamber, a parasitoid was given 15 min to make a choice among the four air fields. If the parasitoid had not made a choice within this duration, it was removed, discarded, and not included in the analyses. In order to remove any directional bias in the chamber, the olfactometer and the position of plants, were rotated after eight parasitoids had been tested. A total of 32 parasitoids were tested each day (8 parasitoids per rotation). Three sets of four-choice olfactometer experiments were conducted to test the ability of females *M. croceipes* to differentiate between uninfested or infested PGPR treated versus untreated cotton plants. In the first experiment the following two treatments and two controls were compared: (1) PGPR strain INR7 treated plant (2) PGPR Blend 9 treated plant (3) Untreated (control) plant, (4) blank control (empty chamber). Based on the results of the first experiment, which showed

significant attraction of the parasitoid to PGPR Blend 9 treated plants compared to untreated (control) plants (Fig. 6), a second experiment was conducted to determine if PGPR treatment is as potent as caterpillar infestation/damage in attracting parasitoids to plants. For this experiment the best PGPR treatment (Blend 9) as determined in the first experiment was selected, and the following two treatments and two controls were compared: (1) PGPR Blend 9 treated plant infested, (2) PGPR Blend 9 treated plant uninfested, (3) Untreated (control) plant infested, and (4) control (empty chamber). Each plant was infested with 30 *H. virescens* caterpillars. A third experiment was conducted based on the result of the second experiment which showed that untreated (control) plants infested with 30 caterpillars were equally as attractive to parasitoids as PGPR Blend 9 treated plants infested with 30 caterpillars. I reasoned that PGPR treatment may be signaling a lower level of caterpillar damage than the level tested in the second experiment. To test this hypothesis and determine if PGPR treatment is as good as low level of caterpillar damage in attracting parasitoids to plants, the same treatments and controls tested in experiment 2 were compared but each infested plant was infested with two *H. virescens* caterpillars.

Four-choice olfactometer bioassays were carried out between 10:00 and 18:00 hrs each day at 25 ± 1 ^{o}C, 60 ± 5% r.h. and 250 lux. The first experiment was replicated five times, while experiments 2 and 3 were replicated four times. Each replicate used a new set of plants.

4.2.8 Statistical Analysis. Data met the key assumptions of Analysis of Variance and thus were not transformed prior to analysis. Significant differences in the amounts of each volatile component emitted by PGPR treated (*Bacillus pumilis* strain INR-7, Blend 8, and Blend 9) treated and untreated plants were established using Analysis of Variance (ANOVA) followed by the Tukey-Kramer HSD multiple comparison test (P<0.05, JMP 7.0.1, SAS Institute 2007).

Significant differences in cotton root growth were established by ANOVA followed by the Tukey-Kramer HSD multiple comparison test ($P<0.05$, JMP 7.0.1, SAS Institute 2007). Four-choice olfactometer data were analyzed by one-way ANOVA followed by the Tukey-Kramer HSD multiple comparison test ($P<0.05$, JMP 7.0.1, SAS Institute 2007).

4.3 Results

4.3.1 GC and GC-MS Analyses of Headspace Volatiles. The GC profiles of the extracts of headspace volatiles from PGPR treated and untreated cotton plants are shown in Fig. 1. A total of 11 peaks (volatile components) were detected in the headspace of PGPR treated (INR-7, Blend 8, and Blend 9) cotton plants (Fig. 1). These peaks, as identified by GC-MS, included α-pinene, β-pinene, β-myrcene, *cis*-3-hexenyl acetate, limonene, (β)-ocimene, linalool, caryophyllene, α-humulene, and β-farnesene. Most of these peaks were not detected or were detected in insignificant amounts in the headspace of untreated cotton plants (Fig. 1). Only three peaks (components) were detectable in untreated cotton plants and were identified by GC-MS as α-pinene, *cis*-3-hexenyl acetate, and caryophyllene. However, all three components were detected in much greater amounts in the headspace of PGPR treated plants. Additionally, significant differences were recorded between the PGPR treatments. For instance, PGPR strain INR-7 treated cotton plants released significantly more α-pinene, β-pinene, β-myrcene, *cis*-3-hexenyl acetate, and β-ocimene than Blend 8 or Blend 9 treated plants (Table 1, Fig. 1). Additionally, β-ocimene was not detected in Blend 9 (Table 1, Fig. 1). Figure 2 shows the GC profiles of the headspace volatiles emitted by the following four treatments: untreated (control) uninfested plants, untreated (control) *H. virescens* infested plants, PGPR Blend 9 treated uninfested plants, and PGPR Blend 9 treated *H. virescens* infested plants. Identical peaks (28)

were detected in extracts of untreated (control) *H. virescens* infested plants and PGPR Blend 9 treated *H. virescens* infested plants (Table 2, Fig. 2). However, 10 peaks (components) were detected in PGPR Blend 9 treated uninfested plants compared with only 3 peaks detected in untreated (control) uninfested plants (Fig. 2).

4.3.2 Analysis of Cotton Root Growth. Cotton root growth promotion resulting after PGPR treatment is shown in Figs. 2, 3, and 4. Inoculation of cotton seeds with PGPR strains INR-7, Blend 8, and Blend 9, significantly promoted growth compared with the untreated control. Significant differences were recorded among the treatments in root surface area ($F_{3,7}$ = 74.78, P <0.0001; Fig. 3), root volume ($F_{3,7}$ = 50.42, P <0.0001; Fig. 4), and root dry weight ($F_{3,7}$ = 28.07, P<0.0001; Fig. 5). In all cases, Blend 9 treated plants had the highest root surface area, root volume, and root dry weight. INR-7 and Blend 8 treated plants also had significantly higher root growth parameters than untreated plants (Figs. 3, 4 and 5).

4.3.3 Four-Choice Olfactometer Bioassays with Parasitoids. In the first experiment, significant differences were recorded in the response of female *M. croceipes* to the two treatments and two controls. Parasitoids were significantly ($F_{3,16}$ = 106.64, P < 0.0001) more attracted to Blend 9 treated plants (69 %) compared with INR-7 treated plants (29 %), untreated (control) plants (0 %), or blank control (empty chamber) (0 %) (Fig. 6). Significant differences were also recorded among the treatments ($F_{3,12}$ = 35.92, P <0.0001) in experiment 2, which was designed to determine if PGPR treatment is as potent as caterpillar infestation/damage (30 *H. virescens* caterpillars) in attracting parasitoids to plants. As expected, PGPR Blend 9 treated plants infested with 30 caterpillars (46%) and untreated (control) plants infested with 30

caterpillars (41%) were highly attractive to parasitoids. However, parasitoids were more attracted to untreated (control) plants infested with 30 caterpillars (41%) than to uninfested PGPR Blend 9 treated plants (13%) (Fig. 7), suggesting that PGPR treatment was not as potent as infestation with 30 caterpillars in attracting parasitoids. The results of the third experiment, in which a lower level of infestation (2 *H. virescens* caterpillars per plant) was tested, also showed significant differences among the treatments and controls ($F_{3,12} = 7.12$, $P = 0.0053$). The most attractive treatment was PGPR Blend 9 treated plants infested with two caterpillars (58%). However, significantly more parasitoids were attracted to uninfested PGPR Blend 9 treated plant (25%) compared with untreated (control) plants infested with two caterpillars (15%) (Fig. 8). These results showed that PGPR treatment was at least as effective as low levels of caterpillar damage in attracting parasitoids to plants.

4.4 Discussion

My results show that plant growth-promoting rhizobacteria (PGPR) alter volatile organic compounds (VOCs) production in cotton plants. The discovery that PGPR alters the production of VOCs in cotton constitutes an unreported mechanism for the elicitation of plant volatile production by rhizobacteria. All tested PGPR treatments (INR7, Blend 8 and Blend 9) elicited the emission of VOCs that were not detected in untreated cotton plants. Eleven components were detected in the headspace of PGPR treated plants. In the headspace of untreated plants, most of these compounds were not detected or were detected in insignificant amounts (only three were detected). In addition to altering VOC production, PGPR treatments also led to cotton plant root growth promotion, with Blend 9 showing the highest root growth promotion. PGPR have previously been reported to promote plant growth (including roots) in several plant species. Most

intriguingly, results from the four-choice olfactometer experiments show that parasitoids were able to distinguish between PGPR treated and untreated plants, preferring the former over the latter.

The major components detected in headspace collections of PGPR treated plants were: α-pinene, β-pinene, β-myrcene, *cis*-3-hexenyl acetate, limonene, β-ocimene, linalool, caryophyllene, α-humulene, and β-farnesene (Table 1, Figure 1). These compounds have been reported before to be constituents of blends of VOCs emitted from caterpillar damaged cotton plants (Loughrin et al. 1994, De Moraes et al. 1998, Ngumbi et al. 2009). However, unlike previous reports, the PGPR induced blend of VOCs is qualitatively different from VOCs emitted by caterpillar damaged plants (Table 2, Figure 2). I define differences in the quality of the blend of VOCs as differences in the presence of specific compounds in the blend and/or ratio of the components. My results suggest that some VOC, such as α-pinene, β-pinene, *cis*-3-hexenyl acetate, limonene, β-ocimene, linalool, caryophyllene, α-humulene, and β-farnesene may be elicited by PGPR. Previous studies have reported that VOC production in plants may be triggered by plant hormones (de Bruxelles and Roberts, 2001, Thaler et al. 2002, Farmer et al. 2003, Ament et al. 2004), herbivore-derived elicitors (Mattiaci et al. 1995, Alborn et al. 1997, Spiteller and Boland, 2003), pathogens (Cardoza et al. 2002), wounding (Mithöfer et al. 2005), and heavy metals (Mithöfer et al. 2004). My findings demonstrate that PGPR elicit the induction of VOCs and further studies are warranted to understand the mechanisms by which treatment of cotton plants with PGPR led to the production of VOCs that differ from untreated plants.

My data on cotton root analysis suggest that PGPR treatment enhanced cotton root growth. Increase in root weight growth as a result of PGPR treatment has been recorded for other crops, including sweet basil (*Ocimum basilicum* L.) and tomato (*Solanum lycopersicum* L.)

(Kloepper 1992, Zehnder et al. 1997, Kloepper et al. 2004, Burkett-Cadena et al. 2008, Banchio et al. 2009, Humberto et al. 2010). PGPR have been applied to different crops for the purposes of growth enhancement and other positive effects in plants, such as seed emergence, tolerance to drought, and increase in weight of plant shoots and roots (Glick 1995, Kloepper et al. 2004, Kokalis-Burelle et al. 2006, Yildirim et al. 2006; van Loon, 2007). Humberto et al. (2010) showed that inoculation of tomato plants with growth promoting *Bacillus subtilis* led to tomato root growth promotion and this was evident 3 weeks after inoculation. These findings corroborate my results in which growth promotion of cotton roots was evident 2 weeks after inoculation. In addition to promoting root growth, PGPR treated plants enhance a plant's ability to defend itself from insects and pathogens by eliciting defensive responses, also known as induced systemic resistance (ISR) (Kloepper et al. 2004) or by antibiosis (Zehnder et al. 2001). Some of the reported examples include reduced insect herbivory in cucumber *Cucumis sativa* (L.) (Zehnder et al. 1997) and resistance to whitefly *Bemicia tabaci* (Hanafi et al. 2007).

The results of the behavioral experiments clearly show the ability of the specialist parasitic wasp, *M. croceipes*, to detect, distinguish and exploit the differences between PGPR treated versus untreated plants. Specifically, PGPR treated plants were highly attractive to parasitoids, with Blend 9 treated plants being the most attractive. Further evaluation demonstrated that Blend 9 treated but uninfested plants were even more attractive to parasitoids than untreated plants with low levels of caterpillar infestations (2 *H. virescens* caterpillars per plant). Volatile organic compounds (VOCs) emitted systematically by plants can act as host location cues for foraging parasitoids (Röse et al. 1998, De Moraes et al. 1998, Ngumbi et al. 2009). My results showed that PGPR treated plants were highly attractive to parasitoids as compared to untreated plants. These findings could be attributed to the blend of VOCs being

produced by the PGPR treated plants that is absent in untreated plants. These PGPR induced compounds have been implicated in natural enemy attraction through behavioral studies and antennal electrophysiological studies (Röse et al. 1998, Chen and Fadamiro 2007, Ngumbi et al. 2009). These data clearly showed the ability of the specialist parasitic wasp, *M. croceipes*, to detect, distinguish and exploit the differences between PGPR treated versus untreated plants.

Among the tested PGPR treatments, Blend 9 treated plants were the most attractive to parasitoids. Interestingly, Blend 9 treated plants consistently did not release β-ocimene. Thus, could the absence of β-ocimene in the blend of VOCs emitted by Blend 9 treated plants responsible for the enhanced attraction of *M. croceipes* to PGPR Blend 9 treated plants? Previous studies have reported that parasitoids like *M. croceipes* can detect and exploit qualitative and quantitative differences in blends of VOCs when searching for their herbivore hosts (De Moraes et al. 1998). In a related study investigating the impact of PGPR on natural enemies of *Myzus persicae* (Hemiptera: Aphididae), Boutard-Hunt et al. (2009) reported that densities of natural enemies were significantly higher in plots treated with PGPR as compared to untreated plots. By providing specific and reliable chemical signals, plants may acquire a competitive advantage in the recruitment of herbivore natural enemies.

In summary, my results show that treatment of cotton plants with single strains or blends of several strains of PGPR (plant growth-promoting rhizobacteria) elicits changes in cotton plant VOCs with important consequences for foraging parasitoids. Together, the results suggest that PGPR treatment could signal low levels of caterpillar damage necessary for attraction of parasitoids to plants, most likely via increased emission of HIPVs. These findings establish a new function for PGPR in mediating insect-plant and tri-trophic interactions.

Further studies are needed to investigate if increased emission and induction of VOCs by PGPR is a common phenomenon in multiple crops under different ecological conditions. Additional studies are necessary to test if key natural enemy species in other cropping systems show similar response to PGPR treated plants. If confirmed, results from such studies will demonstrate that treatment of plants with PGPR may be a viable component of integrated pest management of pests in many agro-ecosystems.

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Table 1 Composition of headspace volatiles emitted by untreated (control) cotton plants vs. cotton plants treated with PGPR strain INR-7, PGPR Blend 8, or PGPR Blend 9

ID	Compound[a]	Untreated (control) cotton plants	Cotton plants treated with PGPR strain INR-7	Cotton plants treated with PGPR Blend 8	Cotton plants treated with PGPR Blend 9
1	α-pinene	58 ± 12[d]	12,960 ± 2288[a]	9,766 ± 1011[b]	5,714 ± 519[c]
2	β-pinene	0[d]	2,739 ± 1782[a]	2,298 ± 280[b]	786 ± 132[c]
3	β-myrcene	0[d]	4,084 ± 105[a]	3,044 ± 94[b]	864 ± 148[c]
4	*cis*-3-hexenyl acetate	62 ± 5[d]	3,730 ± 79[a]	1,884 ± 107[b]	700 ± 143[c]
5	limonene	0[b]	2,266 ± 146[a]	2,230 ± 122[a]	2,188 ± 137[a]
6	β-ocimene	0[c]	4,000 ± 79[a]	3,036 ± 116[b]	0[c]
7	linalool	0[c]	456 ± 59[b]	2,050 ± 73[a]	1,964 ± 94[a]
8	unknown	0[c]	2,962 ± 123[a]	2,352 ± 210[b]	2,962 ± 45[a]
9	caryophyllene	75 ± 10[d]	6,928 ± 787[b]	8,380 ± 842[a]	3,182 ± 200[c]
10	α-humulene	0[c]	1,844 ± 136[a]	1,811 ± 120[a]	288 ± 42[b]
11	β-farnesene	0[c]	1,836 ± 96[a]	1,830 ± 52[a]	284 ± 56[b]

Note: Volatiles were collected for 24 h.

[a]In order of elution during gas chromatography

[b]Values (amount emitted) are mean ± SE of five replicates

Means across the same row followed by different letters are significantly different ($P < 0.05$, ANOVA)

Table 2 Composition of headspace volatiles emitted by untreated (control) uninfested cotton plants vs. untreated (control) *H. virescens* infested plants, PGPR Blend 9 treated uninfested plants, or PGPR Blend 9 treated *H. virescens* infested plants

ID	Compound	Untreated (control) uninfested cotton plants	Untreated (control) *H. virescens* infested plants	PGPR Blend 9 treated uninfested plants	PGPR Blend 9 treated *H. virescens* infested plants
1	*cis*-3-hexenal	0	39,740 ± 2985[a]	0	38,844 ± 3397[a]
2	*trans*-2-hexenal	0	63,131 ± 2653[a]	0	63,020 ± 2527[a]
3	*cis*-3-hexen-1-ol	0	15,720 ± 916[a]	0	15,340 ± 1262[a]
4	*trans*-2-hexen-1-ol	0	68,602 ± 2774[a]	0	68,802 ± 2451[a]
5	α-pinene	58 ± 12[c]	93,110 ± 1345[a]	5,714 ± 519[b]	95,110 ± 1081[a]
6	β-pinene	0	58,039 ± 4522[a]	786 ± 132[b]	57,839 ± 1606[a]
7	myrcene	0	120,239 ± 6930[a]	864 ± 148[b]	119,979 ± 6500[a]
8	*cis*-3-hexenyl acetate	0	161,450 ± 5000[a]	700 ± 143[b]	163,510 ± 4300[a]
9	*trans*-2-hexenyl acetate	0	98,814 ± 1892[a]	0	99,270 ± 1504[a]
10	limonene	0	110,272 ± 3614[a]	2,188 ± 137[b]	110,059 ± 3460[a]
11	β-ocimene	0	120,177 ± 3147[a]	0	120,466 ± 4200[a]
12	linalool	62 ± 16[c]	18,343 ± 1704[a]	1,964 ± 94[b]	18,863 ± 1660[a]
13	unknown	0	57,320 ± 2531[a]	2,962 ± 45[b]	60,720 ± 2100[a]
14	4,8-dimethyl-1,3,7-nonatriene	0	20,920 ± 2166[a]	0	20,736 ± 2109[a]
15	*cis*-3-hexenyl butyrate	0	106,285 ± 2136[a]	0	108,725 ± 4628[a]
16	*trans*-2-hexenyl butyrate	0	88,170 ± 2420[a]	0	90,730 ± 3256[a]
17	*n*-decanal	0	4,700 ± 541[a]	0	4,900 ± 877[a]
18	*cis*-3-hexenyl-2-methyl butyrate	0	135,100 ± 6607[a]	0	135,695 ± 6779[a]
19	*trans*-2-hexenyl-2-methyl butyrate	0	128,350 ± 5055[a]	0	126,950 ± 6136[a]
20	indole	0	58,430 ± 2051[a]	0	68,430 ± 1934[a]
21	isobutyl tiglate	0	15,700 ± 1139[a]	0	15,500 ± 1028[a]
22	2-hexenyl tiglate	0	6,700 ± 190[a]	0	6,620 ± 97[a]
23	*cis*-jasmone	0	55,811 ± 928[a]	0	69,200 ± 1484[a]
24	caryophyllene	75 ± 10	172,500 ± 6461[a]	3,182 ± 200[c]	186,500 ± 6825[b]
25	α-*trans* bergamotene	0	15,778 ± 832[b]	0	17,578 ± 817[a]
26	α-farnesene	0	38,145 ± 1754[a]	288 ± 42[b]	39,345 ± 1500[a]
27	α-humulene	0	32,400 ± 1023[a]	0	34,800 ± 994[a]
28	β-farnesene	0	47,979 ± 870[a]	0	52,439 ± 1072[a]

Note: Volatiles were collected for 24 h.

[1] In order of elution during gas chromatography

[2] Values (amount emitted) are mean ± SE of five replicate extractions

Means across the same row followed by different letters are significantly different ($P < 0.05$, ANOVA).

Figure Legend

Figure 1. Chromatographic profiles of headspace volatiles from untreated (control) cotton plants vs. cotton plants treated with PGPR strain INR-7, PGPR Blend 8, or PGPR Blend 9. Identified compounds: (1) α-pinene; (2) β-pinene; (3) β-myrcene; (4) *cis*-3-hexenyl acetate; (5) Limonene; (6) β-ocimene; (7) linalool; (8) unknown; (9) caryophyllene; (10) α-humulene; (11) β-farnesene

Figure 2. Chromatographic profiles of headspace volatiles collected from untreated (control 1) cotton plants uninfested with caterpillars, untreated (control 2) cotton plants infested with caterpillars, PGPR Blend 9 treated cotton plants uninfested with caterpillars, and PGPR Blend 9 treated cotton plants infested with caterpillars. Identified compounds: (1) *cis*-3-hexenal; (2) *trans*-2-hexenal; (3) *cis*-3-hexen-1-ol; (4) trans-2-hexen-1-ol; (5) α-pinene; (6) β-pinene; (7) myrcene; (8) *cis*-3-hexenyl acetate; (9) *trans*-2-hexenyl acetate; (10) limonene; (11) β-ocimene; (12) linalool; (13) unknown; (14) (*E*)-4,8-dimethyl-1,3,7-nonatriene; (15) *cis*-3-hexenyl butyrate; (16) *trans*-2-hexenyl butyrate; (17) *n*-decanal (18) *cis*-3-hexenyl-2-methyl butyrate; (19) *trans*-2-hexenyl-2-methyl butyrate; (20) indole; (21) isobutyl tiglate; (22) (*E*)-2-hexenyl tiglate; (23) *cis*-jasmone; (24) caryophyllene; (25) α-*trans* bergamotene; (26) α-farnesene; (27) α-humulene; (28) β-farnesene.

Figure 3. Root surface area (cm^2) of untreated (control) cotton plants vs. cotton plants treated with PGPR strain INR-7, PGPR Blend 8, or PGPR Blend 9. Means followed by different letters are significantly different ($P < 0.05$, ANOVA, Tukey-Kramer HSD multiple comparison test, n = 8)

Figure 4. Root volume (cm^3) of untreated (control) cotton plants vs. cotton plants treated with PGPR strain INR-7, PGPR Blend 8, or PGPR Blend 9. Means followed by different letters are significantly different ($P < 0.05$, ANOVA, Tukey-Kramer HSD multiple comparison test, n = 8).

Figure 5. Root dry weight (g) of untreated (control) cotton plants vs. cotton plants treated with PGPR strain INR-7, PGPR Blend 8, or PGPR Blend 9. Means followed by different letters are significantly different ($P < 0.05$, ANOVA, Tukey-Kramer HSD multiple comparison test, n = 8)

Figure 6. Response of naïve female *M. croceipes* in a four-choice olfactometer to untreated (control) cotton plants vs. cotton plants treated with PGPR strain INR-7, PGPR Blend 9, or blank control (empty chamber). Thirty-two parasitoids were tested each day and replicated five times. Means followed by different letters are significantly different ($P < 0.05$, ANOVA, Tukey-Kramer HSD multiple comparison test, n = 5)

Figure 7. Responses of naïve female *M. croceipes* in a four-choice olfactometer to untreated (control) cotton plants infested vs. cotton plants treated with PGPR Blend 9 infested, PGPR Blend 9 uninfested, or blank control (empty chamber). Plants were infested with 30 *H. virescens* caterpillars. Thirty-two parasitoids were tested each day and replicated four times. Means followed by different letters are significantly different ($P < 0.05$, ANOVA, Tukey-Kramer HSD multiple comparison test, n = 4)

Figure 8. Responses of naïve female *M. croceipes* in a four-choice olfactometer to untreated (control) cotton plants infested vs. cotton plants treated with PGPR Blend 9 infested, PGPR Blend 9 uninfested, or blank control (empty chamber). Plants were infested with two *H. virescens* caterpillars. Thirty-two parasitoids were tested each day and replicated four times. Means followed by different letters are significantly different ($P < 0.05$, ANOVA, Tukey-Kramer HSD multiple comparison test, n = 4)

Figure 1

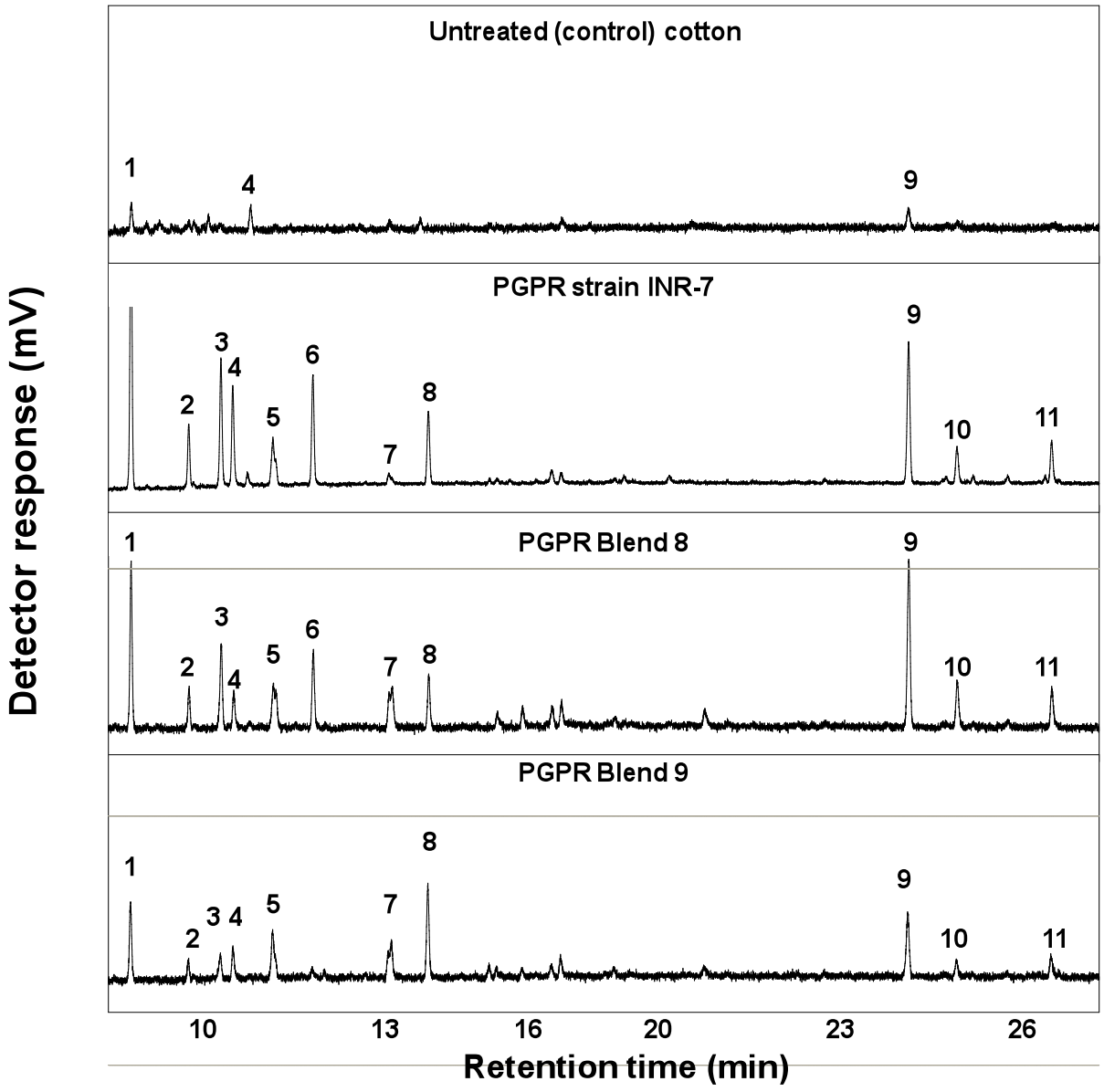

Untreated (control) cotton
PGPR strain INR-7
PGPR Blend 8
PGPR Blend 9
Detector response (mV)
Retention time (min)
10
13
16
20
23
26

Figure 2

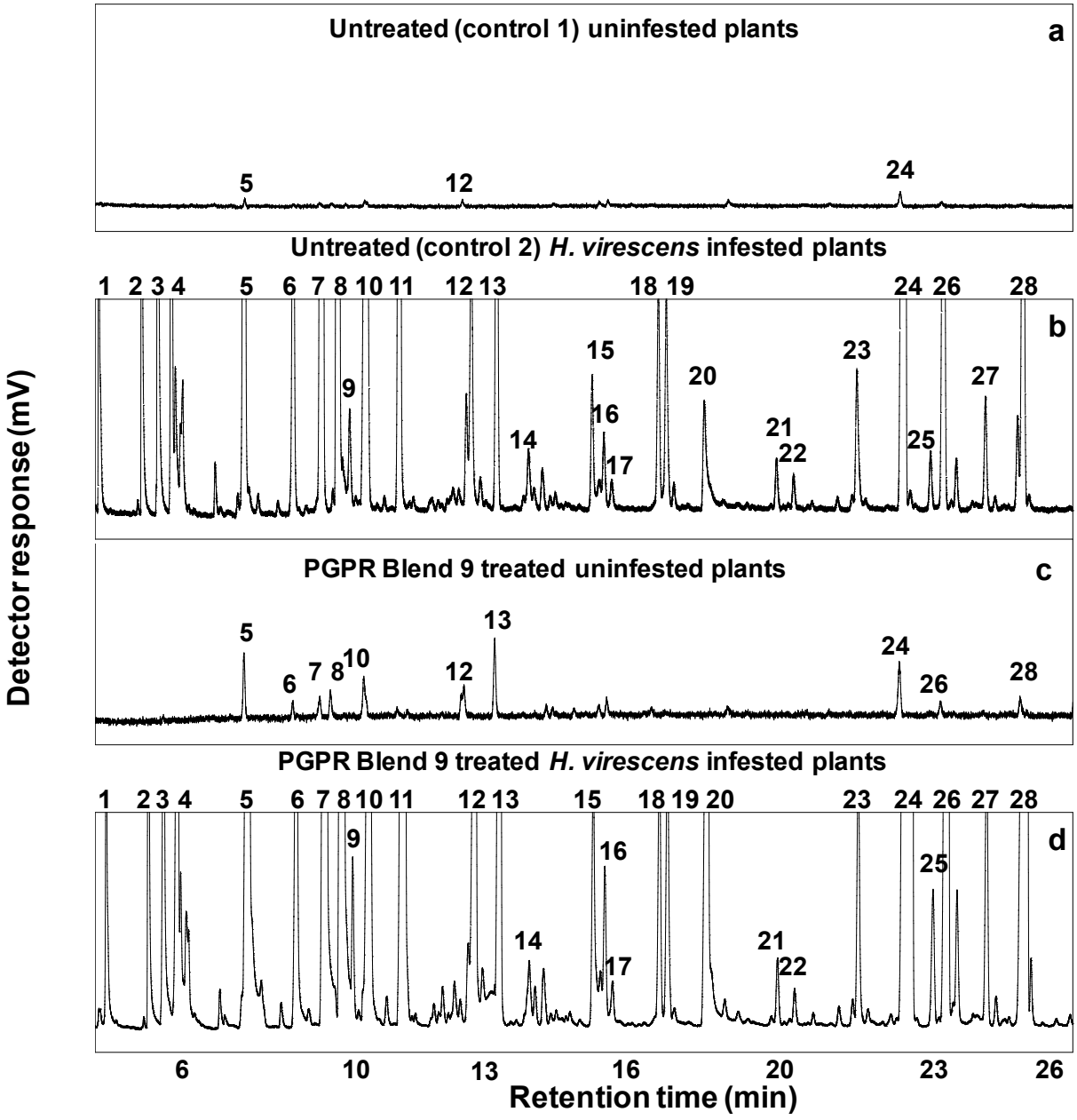

Untreated (control 1) uninfested plants
a
5
12
24
Untreated (control 2) H. virescens infested plants
1 2 3 4 5 6 7 8 10 11 12 13 18 19 24 26 28
b
9
14
15
16
17
20
21
22
23
25
27
PGPR Blend 9 treated uninfested plants
c
5
6 7 8 10
12
13
24
26
28
PGPR Blend 9 treated H. virescens infested plants
1 2 3 4 5 6 7 8 10 11 12 13 15 18 19 20 23 24 26 27 28
d
9
14
16
17
21
22
25
Detector response (mV)
6 10 13 16 20 23 26
Retention time (min)

Figure 3

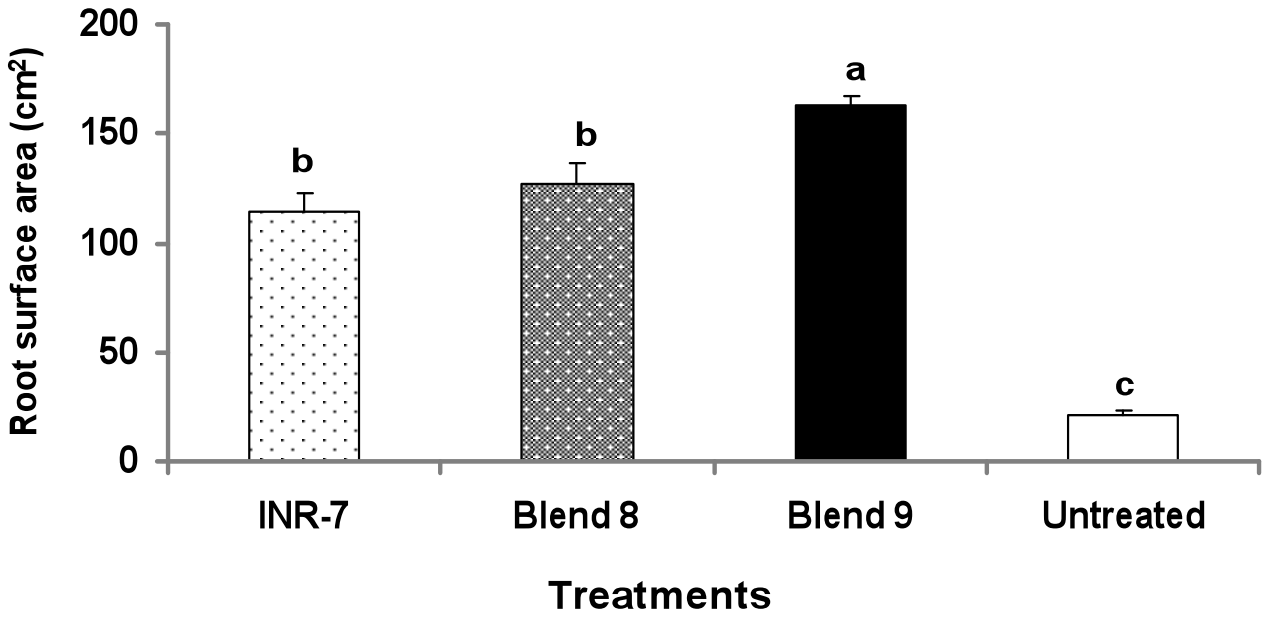

200
150
100
50
0
Root surface area (cm²)
b
b
a
c
INR-7
Blend 8
Blend 9
Untreated
Treatments

Figure 4

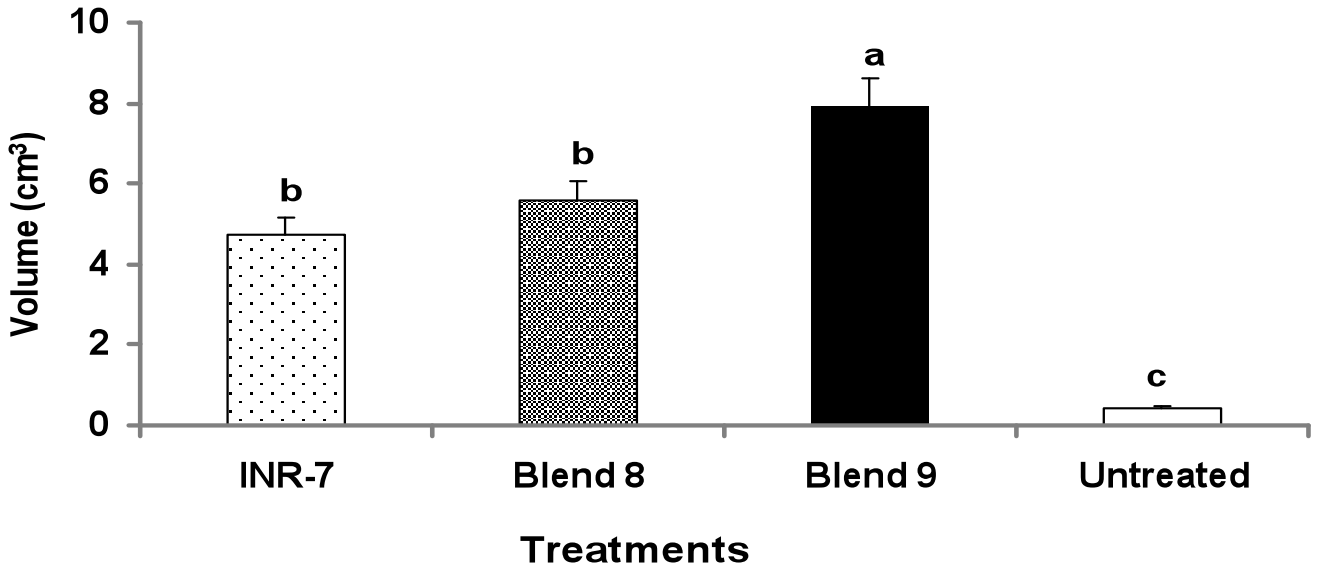

10
8
6
4
2
0
Volume (cm³)
b
b
a
c
INR-7
Blend 8
Blend 9
Untreated
Treatments

Figure 5

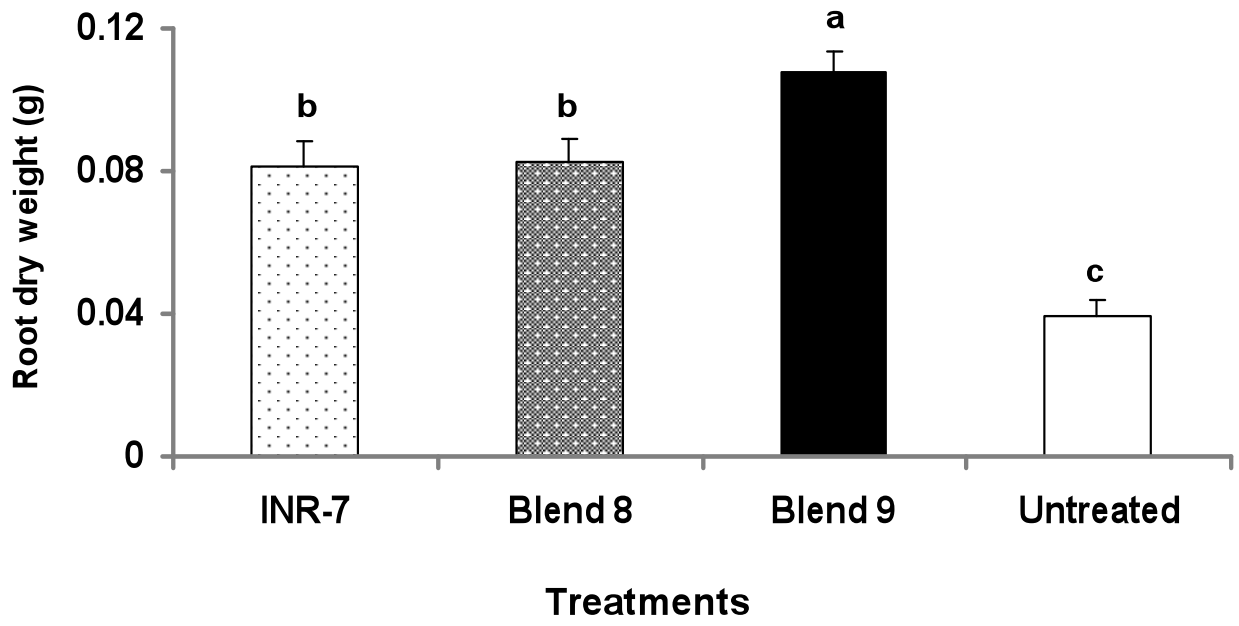

0.12
0.08
0.04
0
Root dry weight (g)
b
b
a
c
INR-7
Blend 8
Blend 9
Untreated
Treatments

Figure 6

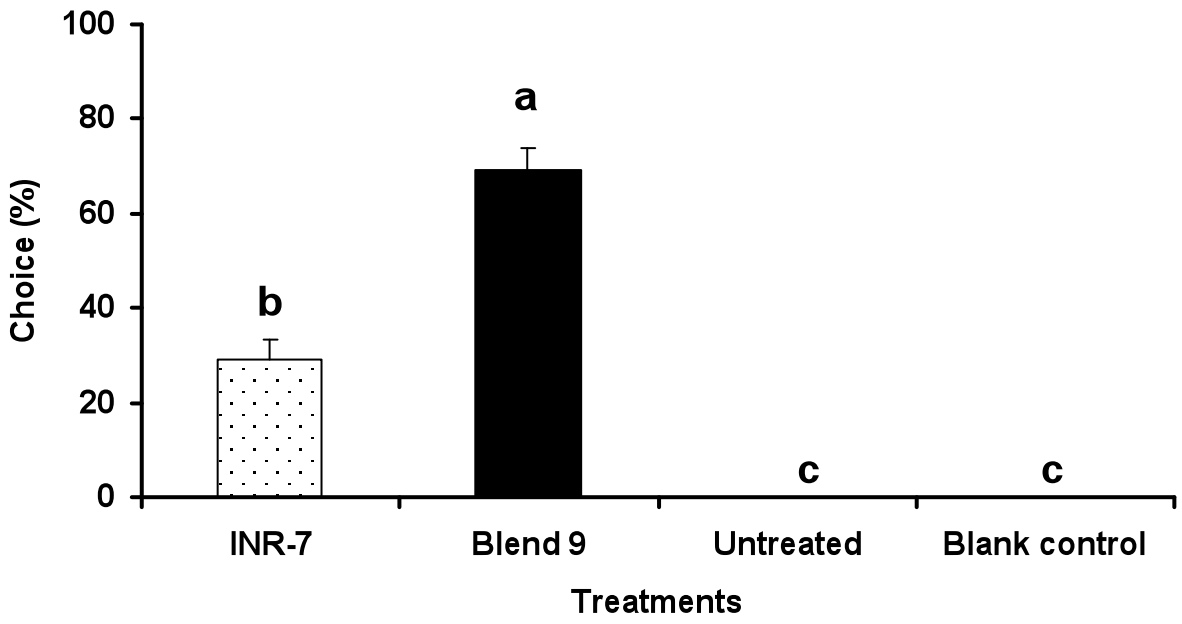

100
80
60
40
20
0
Choice (%)
a
b
c
c
INR-7
Blend 9
Untreated
Blank control
Treatments

Figure 7

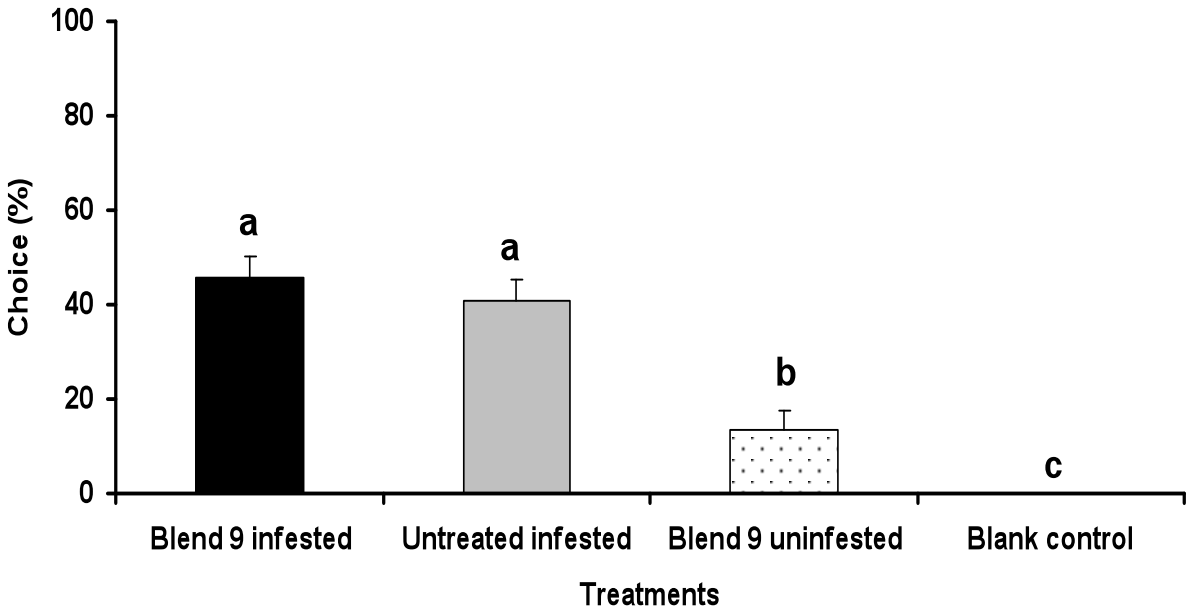
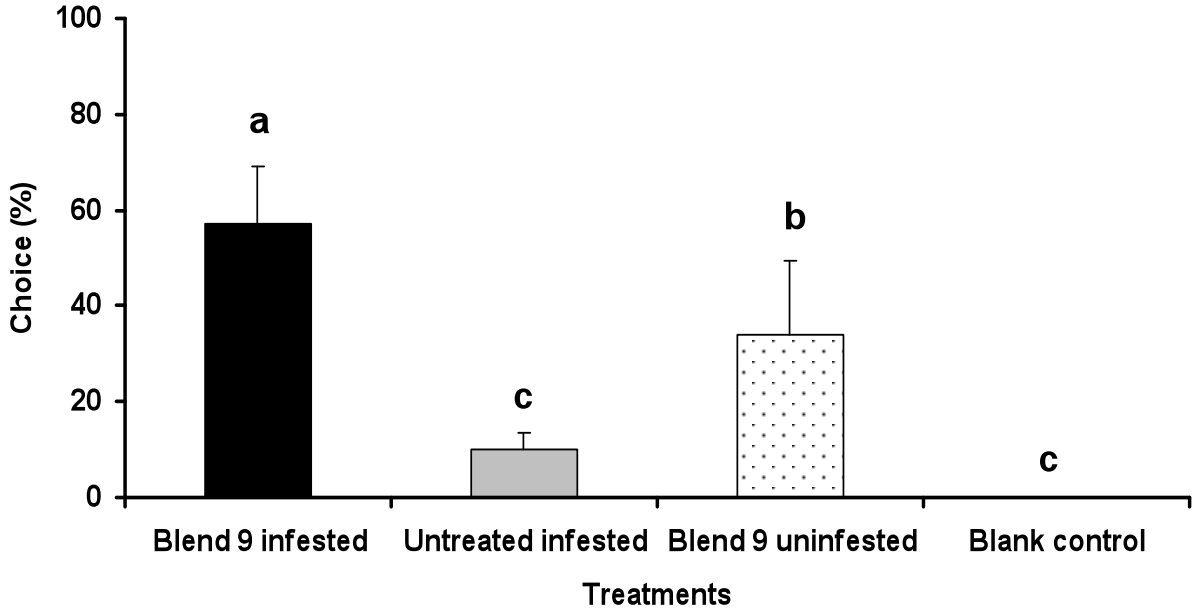

100
80
60
40
20
0
Choice (%)
a
a
b
c
Blend 9 infested
Untreated infested
Blend 9 uninfested
Blank control
Treatments

Figure 8


100
80
60
40
20
0
Choice (%)
a
c
b
c
Blend 9 infested
Untreated infested
Blend 9 uninfested
Blank control
Treatments

CHAPTER 5

SPECIES AND SEXUAL DIFFERENCES IN BEHAVIORAL RESPONSES OF A SPECIALIST AND GENERALIST PARASITOID SPECIES TO HOST-RELATED VOLATILES

5.1. Introduction

Parasitoids use various types of host-related plant volatiles for foraging and host location (Dicke and Sabelis 1988, Turlings et al. 1990, 1991, De Moraes et al. 1998). Host-related plant volatiles can be sub-divided into two major groups: constitutive compounds, and inducible or herbivore-induced plant volatiles. Constitutive compounds are present constantly in plants and released immediately in response to mechanical damage or at the beginning of herbivore feeding. These include green leaf volatiles (GLVs) such as (Z)-3-hexenal, hexanal, and (Z)-3-hexen-1-ol (Turlings et al. 1990, Dicke et al. 1993, Loughrin et al. 1994, McCall et al. 1994, Cortesero et al. 1997, Smid et al. 2002, Gouinguené et al. 2005). Herbivore-induced plant volatiles (HIPVs) are emitted as a delayed response to herbivore feeding damage. HIPVs in cotton (*Gossypium hirsutum* L) and similar plants include (Z)-3-hexenyl butyrate, (*E,E*)-α-farnesene, (*E*)-β-farnesene, (*E*)-β-ocimene, and linalool (Dicke 1994, Loughrin et al. 1994, McCall et al. 1994, Cortesero et al. 1997, Röse et al. 1998, Ngumbi et al. 2009).

The relationship between the degree of specialization of parasitoids and their responses to different suites of host-related volatiles is an important and current evolutionary question (Vet et al. 1993, Geervliet et al. 1996, Cortesero et al. 1997, Bernays 2001, Chen and Fadamiro 2007, Ngumbi et al. 2009, 2010). Specialist parasitoids which attack fewer host species are predicted to

utilize as host location cues host specific volatile signals (e.g., certain HIPVs) (Cortesero et al. 1997). In contrast, since information on host identity is relatively unimportant to natural enemies which attack a wide variety of host species (Vet and Dicke 1992), generalist parasitoids may have evolved to use general host-related volatiles (such as GLVs and common HIPVs) as host location cues.

Recent and ongoing studies by my group have employed a comparative approach to test the above predictions by investigating the electrophysiological responses of two parasitoid species (Hymenoptera: Braconidae) with different degrees of host specificity*, Microplitis croceipes* (Cresson) and *Cotesia marginiventris* (Cresson), to different suites of host-related plant volatiles. *Microplitis croceipes* is a relatively specialist parasitoid specific to *Heliothis* and *Helicoverpa* larvae (Eller 1990), whereas *C. marginiventris* is a generalist parasitoid of caterpillars of a wide range of lepidopteran species, including *Spodoptera exigua* (Hübner), *Helicoverpa zea* (Boddie), and *Heliothis virescens* (Fab) (Lepidoptera: Noctuidae) (Jalali et al. 1987, Turlings et al. 1990, Röse et al. 1998). For the most part, the results of our studies which utilized electroantennogram (EAG) and coupled gas chromatography electroantennogram detection (GC-EAD) techniques (Chen and Fadamiro 2007, Ngumbi et al. 2009, 2010) support the prediction that specialist parasitoids are relatively more responsive to some HIPVs, whereas generalist parasitoids are more responsive to GLVs. However, electrophysiological results may not always correlate with behavior, making it important to conduct comparative behavioral tests with our parasitoid models.

Female parasitoids have remained the focus of most studies on olfactory response of parasitoids to host-related compounds (Cortesero et al. 1997) with only few studies paying attention to male response (Whitman and Eller 1992, Park et al. 2001). This is expected since

females are the primary sex involved in host location and thus are predicted to be more responsive to host-related volatiles (Jyothi et al. 2002, Whitman and Eller 1990, Chen and Fadamiro 2007). Furthermore, since host-related volatiles may play different roles in the ecology of female (host location) and male (mate location) parasitoids, it is possible that each sex may show differential responses to different types of host-related volatiles (Li et al. 1992, Park et al. 2001).

In this study, I compared the behavioral responses of both sexes of the specialist (*M. croceipes*) and generalist (*C. marginiventris*) parasitoid models to host-related plant volatiles. Y-tube olfactometer bioassays were conducted to test for innate differences in the behavioral responses of naïve females and males of both parasitoid species to select synthetic compounds representing two categories of host-related volatiles: (i) GLVs (hexanal and (Z)-3-hexen-1-ol); and (ii) HIPVs ((Z)-3-hexenyl acetate, linalool, (Z)-3-hexenyl butyrate, and (*E,E*)-α-farnesene). Based on the results of foundational electrophysiological studies summarized above (Chen and Fadamiro 2007, Ngumbi et al. 2009, 2010), I hypothesized that (i) *M. croceipes* (specialist) would show greater behavioral responses to HIPVs, whereas *C. marginiventris* (generalist) would show greater behavioral responses to GLVs, and (ii) that females of both parasitoid species would show greater behavioral responses than conspecific males to host-related volatiles.

5.2 Materials and Methods

5.2.1 Insects. The parent cultures of *M. croceipes* and *C. marginiventris* were provided by the USDA-ARS, Insect Biology and Population Management Research Laboratory (Tifton, Georgia) and the University of Georgia (Tifton campus, contact: John Ruberson) respectively. *Microplitis croceipes* was reared on caterpillars of *H. virescens,* its preferred host (Stadelbacher

et al. 1984, King et al. 1985), whereas *C. marginiventris* was reared on caterpillars of its main host *S. exigua* (Jalali et al. 1987). The rearing procedures for both parasitoids were similar to those of Lewis and Burton (1970). Eggs purchased from Benzone Research (Carlisle, PA, USA) were used to start laboratory colonies of the two lepidopteran host species, *H. virescens* and *S. exigua*. Caterpillars of both species were reared on a laboratory-prepared pinto bean diet (Shorey and Hale 1965) at 25 ± 1°C, 75 ± 5% RH and under a L14:D10 photoperiod. For each parasitoid species, newly emerged adults were collected prior to mating, sexed, and placed in pairs of individuals of opposite sex (mated individuals) in a 6-cm diameter plastic Petri dish supplied with water and sugar sources. Water was provided by filling a 0.5 ml microcentrifuge tube with distilled water and threading a cotton string through a hole in the cap of the tube. About 5 drops (2 µl per drop) of 10% sugar solution were smeared on the inside of the Petri dish cover with a cotton-tipped applicator. Mated parasitoids (aged 3-5 days) were used for the bioassays.

5.2.2 Test Compounds. Six compounds were tested in this study: hexanal, (Z)-3-hexen-1-ol, (Z)-3-hexenyl acetate, linalool, (Z)-3-hexenyl butyrate, and (*E,E*)-α-farnesene. Compounds were purchased from Sigma® Chemical Co. (St. Louis, MO, USA) with purity >97%. Solutions of synthetic volatile compounds were formulated in hexane. Each compound was tested at two doses (1 and 100 µg/µl).

5.2.3 Behavioral Bioassays. A Y-tube olfactometer (Analytical Research Systems, Inc, Gainesville, FL) was used to test the attraction of 3-5 days old naïve female and male *M. croceipes* and *C. marginiventris* to the six selected synthetic plant volatiles. The system consists of a central tube (13.5 cm long, 24 mm diameter) and two lateral arms (5.75 cm long, 24 mm

diameter). A sieve inlay in the lateral arms and extending glass tube 5.25 cm away from the connection prevents escape of insects and serves as an end point of each lateral arm. Humidified and purified air was passed into the extending glass tube through a Teflon® connection at 150 mL/min. The Y-tube olfactometer was inverted following preliminary experiments which showed that the parasitoids preferred to walk vertically up the glass tube and not horizontally (unpublished data). Illumination was provided by vertically hanging an office lamp (20 W, 250 Lux) above (~ 50 cm high) from the olfactometer tube. Parasitoids were introduced individually into the central arm of the Y-tube. The initial choice of a parasitoid that responded by walking into one of the two arms and remaining there at least 15 s was recorded. If a parasitoid did not make a choice within 5 min of being released, it was removed and discarded. Parasitoids that did not walk into any of the arms were not counted. After four individual parasitoids had been tested, the olfactometer arms were flipped around (180^{o}) to minimize any positional effect. After eight individuals had been bioassayed, the olfactometer set-up was rinsed with soapy water, then with acetone, and then air-dried. Each compound was delivered as a 10-µL sample placed on filter paper strips (7 x 40 mm, Whatman® No. 1). After allowing for solvent evaporation (~15 s), a filter paper strip was inserted into a designated arm of the olfactometer. A similar filter paper strip with solvent (hexane) was inserted into the second arm and served as a control. We compared for 1) effect of parasitoid species (same sex) on behavioral response and ii) effect of sex on behavioral response. For each species, 30 naïve individuals per sex were bioassayed to each test compound/dose. The two species, sexes and doses were tested daily in separate experiments using a random order. Olfactometer data (30 replicates per sex) were analyzed by the use of a chi-square (χ^2) test ($P < 0.05$; JMP® 7.0.1, SAS Institute 2007).

5.3 Results

5.3.1 ***Microplitis croceipes*.** Female *M. croceipes* showed significant attraction in a Y-tube olfactometer to most of the tested HIPVs (i.e. (Z)-3-hexenyl acetate, linalool and (Z)-3-hexenyl butyrate) at the two doses but not to (*E,E*)-α-farnesene or the two GLVs (hexanal and (Z)-3-hexen-1-ol) (Table 1, Fig. 1a). Males also showed significant attraction to two HIPVs (i.e. (Z)-3-hexenyl acetate and (*E,E*)-α-farnesene) and to hexanal (a GLV) at the high dose (Table 1, Fig. 1b).

Comparing the two sexes, sex exerted a significant effect on behavioral response of *M. croceipes* but this was dose-dependent in many cases. Females showed significantly greater responses than males to hexanal at the low dose (1 µg: $\chi^2 = 4.4$, df = 1, $P = 0.0359$), (Z)-3-hexenyl acetate at the high dose (100 µg: $\chi^2 = 4.4$, df = 1, $P = 0.0359$), linalool at both doses (1 µg: $\chi^2 = 4.3$, df = 1, $P = 0.0372$; 100 µg: $\chi^2 = 5.5$, df = 1, $P = 0.0191$), and (Z)-3-hexenyl butyrate at both doses (1 µg: $\chi^2 = 5.6$, df = 1, $P = 0.0175$; 100 µg: $\chi^2 = 6.4$, df = 1, $P = 0.0112$) (Fig. 2). In contrast, males showed significantly higher response than females to hexanal at the high dose (100 µg: $\chi^2 = 4.3$, df = 1, $P = 0.0372$) and (*E,E*)-α-farnesene at the low dose (1 µg: $\chi^2 = 4.3$, df = 1, $P = 0.0372$) (Fig. 2).

5.3.2 ***Cotesia marginiventris*.** Female *C. marginiventris* showed significant attraction to both doses of the two tested GLVs (hexanal and (Z)-3-hexen-1-ol) and to linalool at the low dose. However, no significant attraction was recorded to the remaining three HIPVs (i.e. (Z)-3-hexenyl acetate, (Z)-3-hexenyl butyrate and (*E,E*)-α-farnesene) (Table 1, Fig. 3a). Males, on the other hand, showed significant attraction to both doses of (Z)-3-hexen-1-ol, linalool and (*E,E*)-α-farnesene) (Table 1, Fig. 3b).

Comparing both sexes of *C. marginiventris*, females showed significantly greater attraction than males only to hexanal at both doses (1 µg: $\chi^2 = 5.2$, df = 1, $P = 0.0224$; 100 µg: $\chi^2 = 4.6$, df = 1, $P = 0.0306$). In contrast, males showed significantly greater attraction than females to linalool at the low dose (1 µg: $\chi^2 = 4.3$, df = 1, $P = 0.0372$) and (*E,E*)-α-farnesene at the low dose (1 µg: $\chi^2 = 4.6$, df = 1, $P = 0.0306$) (Fig. 4).

5.3.3 Comparing Both Parasitoid Species. Significant differences were recorded in the responses of both parasitoid species to the tested compounds. Female *M. croceipes* (specialist) showed significantly greater responses than female *C. marginiventris* (generalist) to the HIPVs, (Z)-3-hexenyl acetate (1 µg: $\chi^2 = 8.5$, df = 1, $P = 0.0035$), linalool (1 µg: $\chi^2 = 4.3$, df = 1, $P = 0.0372$), and (Z)-3-hexenyl butyrate (1 µg: $\chi^2 = 9.8$, df = 1, $P = 0.0018$; 100 µg: $\chi^2 = 12.5$, df = 1, $P = 0.0004$) (Fig. 5a). In contrast, female *C. marginivetris* were relatively more attracted to the GLVs, hexanal (1 µg: $\chi^2 = 5.2$, df = 1, $P = 0.0224$; 100 µg: $\chi^2 = 8.5$, df = 1, $P = 0.0035$) and (Z)-3-hexen-1-ol (1 µg: $\chi^2 = 4.9$, df = 1, $P = 0.0268$) (Fig. 5a).

Similar results were obtained when males of both species were compared. Male *M. croceipes* showed significantly greater responses than male *C. marginiventris* to the HIPVs, (Z)-3-hexenyl acetate (1 µg: $\chi^2 = 6.8$, df = 1, $P = 0.0090$) and (Z)-3-hexenyl butyrate (1 µg: $\chi^2 = 5.5$, df = 1, $P = 0.0185$), whereas male *C. marginiventris* showed relatively greater attraction to hexanal (1 µg: $\chi^2 = 4.4$, df = 1, $P = 0.0359$), (Z)-3-hexen-1-ol (1 µg: $\chi^2 = 4.3$, df = 1, $P = 0.0372$) and linalool (1 µg: $\chi^2 = 4.3$, df = 1, $P = 0.0372$; 100 µg: $\chi^2 = 11.8$, df = 1, $P = 0.0006$) (Fig. 5b). These results suggest that the specialist parasitoid is in general more responsive to the HIPVs, whereas the generalist parasitoid is more responsive to the GLVs.

5.4 Discussion

The results revealed key sexual and species differences in behavioral responses of my parasitoid models to host-related volatiles, and may have important ecological ramifications. As predicted, the specialist parasitoid, *M. croceipes* was more responsive to most of the herbivore-induced plant volatiles (HIPVs), whereas the generalist (*C. marginiventris*) showed relatively greater behavioral responses to the green leaf volatiles (GLVs). Females of both species also showed greater responses than conspecific males to most of the tested volatiles. These findings are in agreement with the results of previous studies by my group which showed differential electrophysiological responses of both parasitoid species to host-related volatiles (Chen and Fadamiro 2007, Ngumbi et al. 2009, 2010). In the above studies, which utilized EAG and GC-EAD techniques, *M. croceipes* consistently showed greater electrophysiological responses to the HIPVs such as (Z)-3-hexenyl acetate, and (Z)-3-hexenyl butyrate, whereas *C. marginiventris* showed greater responses to the GLVs such as (Z)-3-hexenal, *trans*-2-hexenal, and (Z)-3-hexenol.

Few studies have systematically compared behavioral responses of specialist and generalist parasitoids to host-related volatiles (Elzen et al. 1987, Vet et al. 1993, Geervliet et al. 1996, Cortesero et al. 1997, Röse et al. 1998). In general, a specialist parasitoid typically showed greater response than a generalist to host-related odor (Elzen et al. 1987, Vet et al. 1993), However, I am not aware of any studies which reported differential responses of specialist and generalist parasitoids to GLVs and HIPVs, as recorded in the present study. Thus, my results showing that the specialist parasitoid is more responsive to the HIPVs while the generalist is more responsive to GLVs are intriguing and may have ecological significance. Specialist parasitoids like *M. croceipes* are likely to have evolved the ability to respond more to the HIPVs,

which are specifically linked to their hosts (Cortesero et al. 1997). (Z)-3-hexenyl acetate and (Z)-3-hexenyl butyrate are major HIPVs emitted by cotton plants damaged by caterpillars (Loughrin et al. 1994, McCall et al. 1994, Ngumbi et al. 2009), and have been reported to elicit behavioral responses in *M. croceipes* (Whitman and Eller 1992). Recently, I showed that both compounds are emitted in greater quantities by plants damaged by *H. virescens*, a key host of *M. croceipes,* compared to plants damaged by *S. exigua*, a non-host (Ngumbi et al. 2009), suggesting that these compounds could play an important role in host location behavior of *M. croceipes* in natural settings.

Similarly, the results which showed that the generalist (*C. marginiventris*) was more attracted to the GLVs appear to be in correlation with the behavioral ecology and foraging behavior of this species. GLVs are ubiquitous volatiles commonly emitted by various plants (Cortesero et al. 1997, Hoballah et al. 2002, D'Alesandro and Turlings 2005, Hoballah and Turlings 2005). Thus, it would seem adaptive for generalist parasitoids, which attack numerous hosts on numerous plants, to be more responsive to GLVs. My results suggest that GLVs are important host location cues for *C. marginiventris*, and possibly similar generalist parasitoids.

The important sexual differences recorded in this study are consistent with the results of a previous study in which females of *M. croceipes* and *Netelia heroica* Townes (Hymenoptera: Ichneumonidae) showed greater behavioral responses to host-related volatiles than males (Whitman and Eller 1990), and are in agreement with our current knowledge of parasitoid host location behavior. The female is the primary sex involved in host location. It is logical to expect females to show greater responses than males to host-related volatiles (in particular GLVs), especially at low doses, since evolution would favor females that were able to arrive immediately at the site of host plant attack (Chen and Fadamiro 2007). On the other hand, male parasitoids are

probably exploiting host-related volatiles for mating and may have evolved greater sensitivity to HIPVs, in particular at high doses, since selection pressure would favor males that were best able to locate sites where females are likely to be found, as signaled by the production of HIPVs (Chen and Fadamiro 2007). This may explain the results in which males of both parasitoid species showed greater behavioral responses than females to linalool and (*E,E*)-α-farnesene.

The compounds tested in this study are constituents of blends of volatiles emitted by caterpillar damaged cotton plants (Loughrin et al. 1994, Cortesero et al. 1997, Ngumbi et al. 2009). In nature, parasitoids typically exploit the whole blend of volatiles for host location. However, attraction of many parasitoid species to certain individual components of the blend, including some of the compounds tested in the present study, has also been documented (Du et al. 1998, Powell et al. 1998, de Boer and Dicke 2004). Based on recent EAG and GC-EAG studies (Chen and Fadamiro 2007, Ngumbi et al. 2009), I selected a subset of compounds that are key components of the blend of volatiles produced by caterpillar damaged plant in order to carry out extensive and detailed behavioral responses of both parasitoid species to these compounds. My results, therefore, form a foundation for future studies that would be designed to investigate the behavioral responses of both parasitoid species to complex odor blends mimicking the natural blends emitted by cotton plants damaged by different caterpillar species.

In summary, my results showed that specialist and generalist parasitoids appear to employ different types of host-related volatiles as host location cues. The data support the prediction that specialist parasitoids that utilize fewer numbers of host species are likely to possess olfactory detection systems which are more highly sensitive and narrowly tuned (selective) to host-related volatiles than generalist parasitoids (Vet and Dicke 1992, Cortesero et al. 1997, Smid et al. 2002, Chen and Fadamiro 2007, Ngumbi et al. 2009, 2010). The results of

this behavioral study are in correlation with previously published reports on the electrophysiological responses of both parasitoid species (Chen and Fadamiro 2007, Ngumbi et al. 2009, 2010). Increased knowledge of parasitoid host specificity and host location strategies and identification of attractive volatile compounds should enhance the performance of parasitoids as biological control agents.

5.5 Acknowledgements

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Table 1. Chi-square analysis of behavioral responses of *M. croceipes* and *C. marginiventris* to six host-related volatiles

Compound	Dose (µg)	*Microplitis croceipes*			*Cotesia marginiventris*		
		df	χ^2	*P*	df	χ^2	*P*
Female							
Hexanal	1	1	1.1	0.3010	1	29.1	<0.0001*
	100	1	2.4	0.1201	1	17.9	<0.0001*
(Z)-3-hexen-1-ol	1	1	0.3	0.6054	1	23.1	<0.0001*
	100	1	2.4	0.1201	1	13.6	0.0002*
(Z)-3-hexenyl acetate	1	1	17.9	<0.0001*	1	2.4	0.1201
	100	1	9.9	0.0017*	1	2.4	0.1201
Linalool	1	1	6.7	0.0091*	1	2.4	0.1201
	100	1	4.3	0.0377*	1	6.8	0.0091*
(Z)-3-hexenyl butyrate	1	1	36.1	<0.0001*	1	0.0	1.0000
	100	1	29.1	<0.0001*	1	2.4	0.1201
(E,E)-α-farnesene	1	1	2.4	0.1201	1	0.0	1.0000
	100	1	0.0	1.0000	1	1.1	0.3010
Male							
Hexanal	1	1	9.9	0.0017	1	1.1	0.3010
	100	1	6.8	0.0091*	1	0.0	1.0000
(Z)-3-hexen-1-ol	1	1	2.4	0.1201	1	6.8	0.0091*
	100	1	2.4	0.1201	1	9.9	0.0017*
(Z)-3-hexenyl acetate	1	1	9.9	0.0017*	1	4.3	0.0377
	100	1	1.1	0.3010	1	4.3	0.0377
Linalool	1	1	2.4	0.1201	1	6.8	0.0091*
	100	1	6.8	0.0091	1	17.9	<0.0001*
(Z)-3-hexenyl butyrate	1	1	2.4	0.1201	1	9.9	0.0017
	100	1	0.3	0.6054	1	2.4	0.1201
(E,E)-α-farnesene	1	1	6.8	0.0091*	1	17.9	<0.0001*
	100	1	9.9	0.0017*	1	9.9	0.0017*

Asterisk (*) indicates significant difference between test compound and hexane (control) (χ^2 test, $P < 0.05$).

Figure Legend

Figure 1. Response of *Microplitis croceipes* females (**a**) and males (**b**) in a Y-tube olfactometer when given a choice between hexane (control) and host-related plant volatiles. In this and other figures, volatile compounds were tested at two doses (1 and 100 μg). Asterisk (*) indicates significant differences between stimulus and control (χ^2 tests, $P < 0.05$).

Figure 2. Sexual differences in the response of *Microplitis croceipes* to host-related plant volatiles in a Y-tube olfactometer. Asterisk (*) indicates significant differences between the sexes (χ^2 tests, $P < 0.05$).

Figure 3. Response of *Cotesia marginiventris* females (**a**) males (**b**) in a Y-tube olfactometer when given a choice between hexane (control) and host-related plant volatiles. Asterisk (*) indicates significant differences between stimulus and control (χ^2 tests, $P < 0.05$).

Figure 4. Sexual differences in the response of *Cotesia marginiventris* to host-related plant volatiles in a Y-tube olfactometer. Asterisk (*) indicates significant differences between the sexes (χ^2 tests, $P < 0.05$).

Figure 5. Comparing behavioral responses of *Microplitis croceipes* versus *Cotesia marginiventris* females (**a**) and males (**b**) to host-related volatiles in a Y-tube olfactometer. Asterisk (*) indicates significant differences between the species (χ^2 tests, $P < 0.05$).

Figure 1

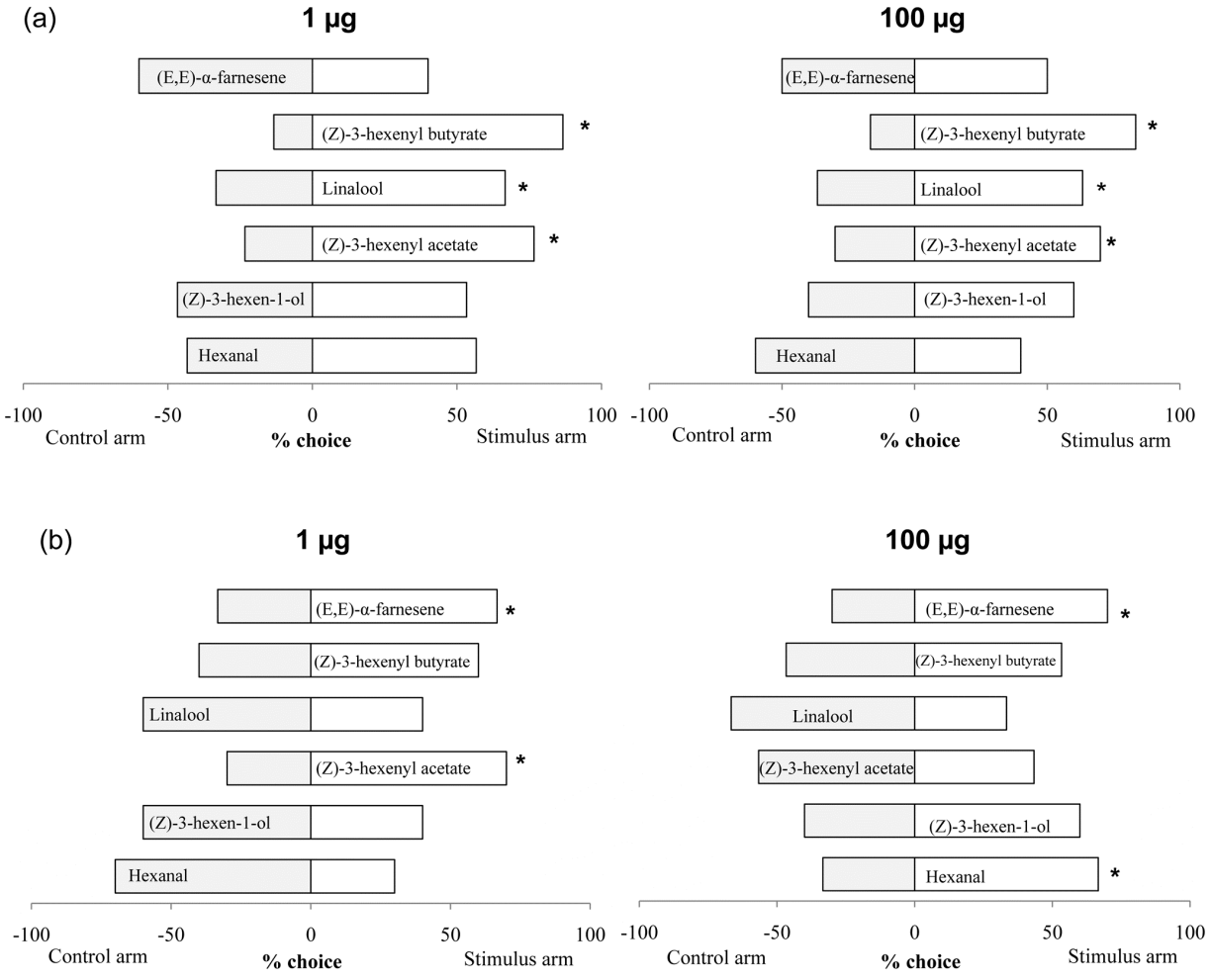

(a)
1 µg
100 µg
(E,E)-α-farnesene
(Z)-3-hexenyl butyrate
Linalool
(Z)-3-hexenyl acetate
(Z)-3-hexen-1-ol
Hexanal
*
-100
-50
0
50
100
Control arm
% choice
Stimulus arm
(b)
1 µg
100 µg
(E,E)-α-farnesene
(Z)-3-hexenyl butyrate
Linalool
(Z)-3-hexenyl acetate
(Z)-3-hexen-1-ol
Hexanal
*
-100
-50
0
50
100
Control arm
% choice
Stimulus arm

Figure 2

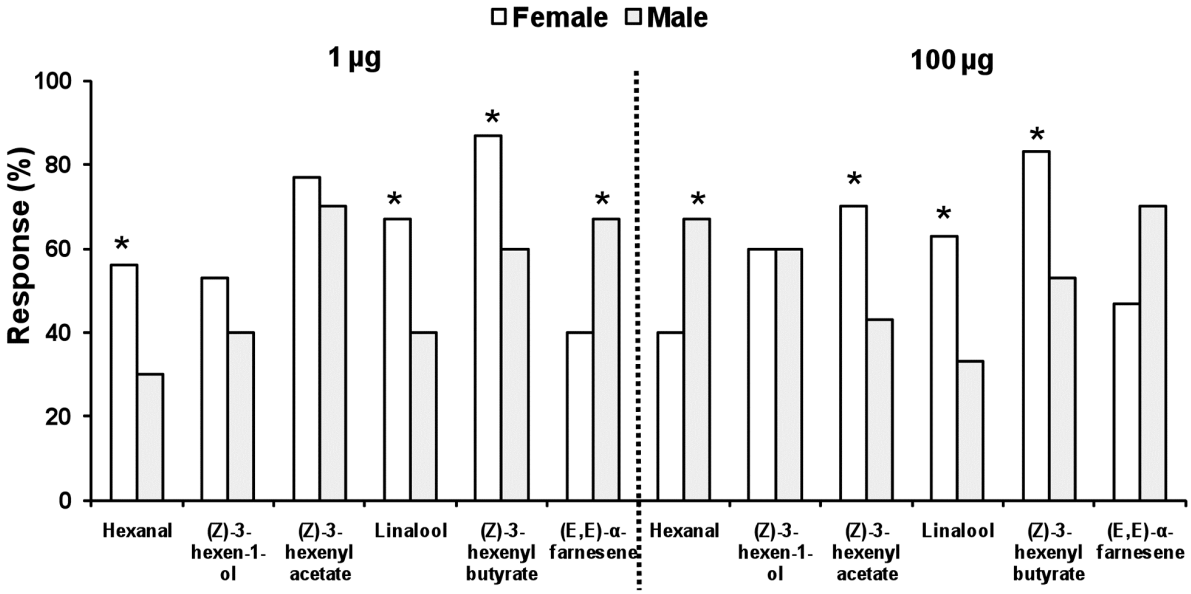

Female
Male
1 µg
100 µg
Response (%)
100
80
60
40
20
0
*
Hexanal
(Z)-3-hexen-1-ol
(Z)-3-hexenyl acetate
Linalool
(Z)-3-hexenyl butyrate
(E,E)-α-farnesene

Figure 3

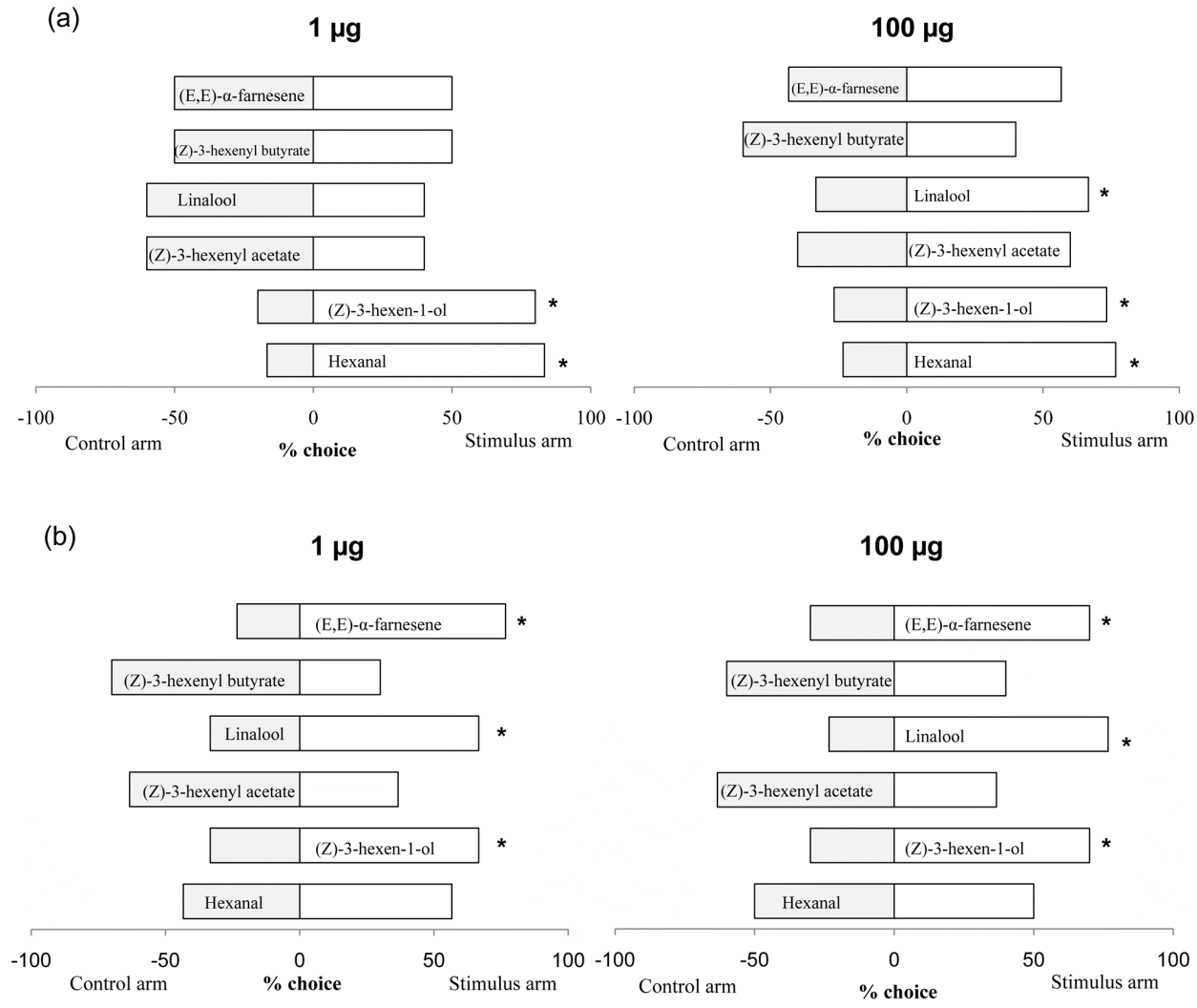

(a)
1 µg
(E,E)-α-farnesene
(Z)-3-hexenyl butyrate
Linalool
(Z)-3-hexenyl acetate
(Z)-3-hexen-1-ol
Hexanal
*
*
-100
-50
0
50
100
Control arm
% choice
Stimulus arm
100 µg
(E,E)-α-farnesene
(Z)-3-hexenyl butyrate
Linalool
(Z)-3-hexenyl acetate
(Z)-3-hexen-1-ol
Hexanal
*
*
-100
-50
0
50
100
Control arm
% choice
Stimulus arm
(b)
1 µg
(E,E)-α-farnesene
(Z)-3-hexenyl butyrate
Linalool
(Z)-3-hexenyl acetate
(Z)-3-hexen-1-ol
Hexanal
*
*
-100
-50
0
50
100
Control arm
% choice
Stimulus arm
100 µg
(E,E)-α-farnesene
(Z)-3-hexenyl butyrate
Linalool
(Z)-3-hexenyl acetate
(Z)-3-hexen-1-ol
Hexanal
*
*
-100
-50
0
50
100
Control arm
% choice
Stimulus arm

Figure 4

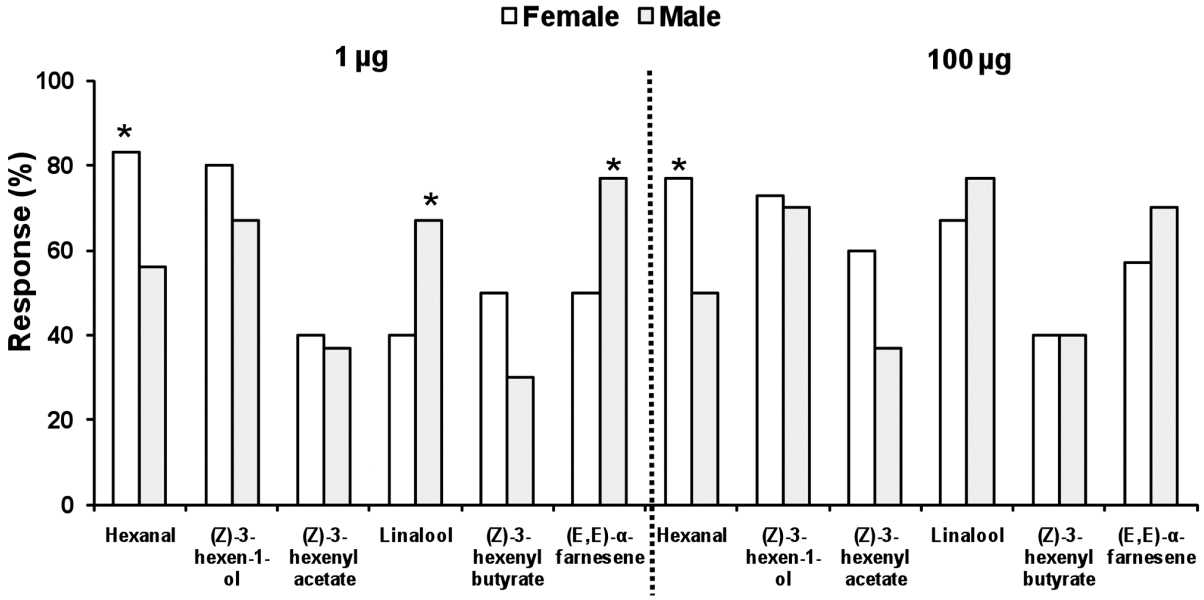
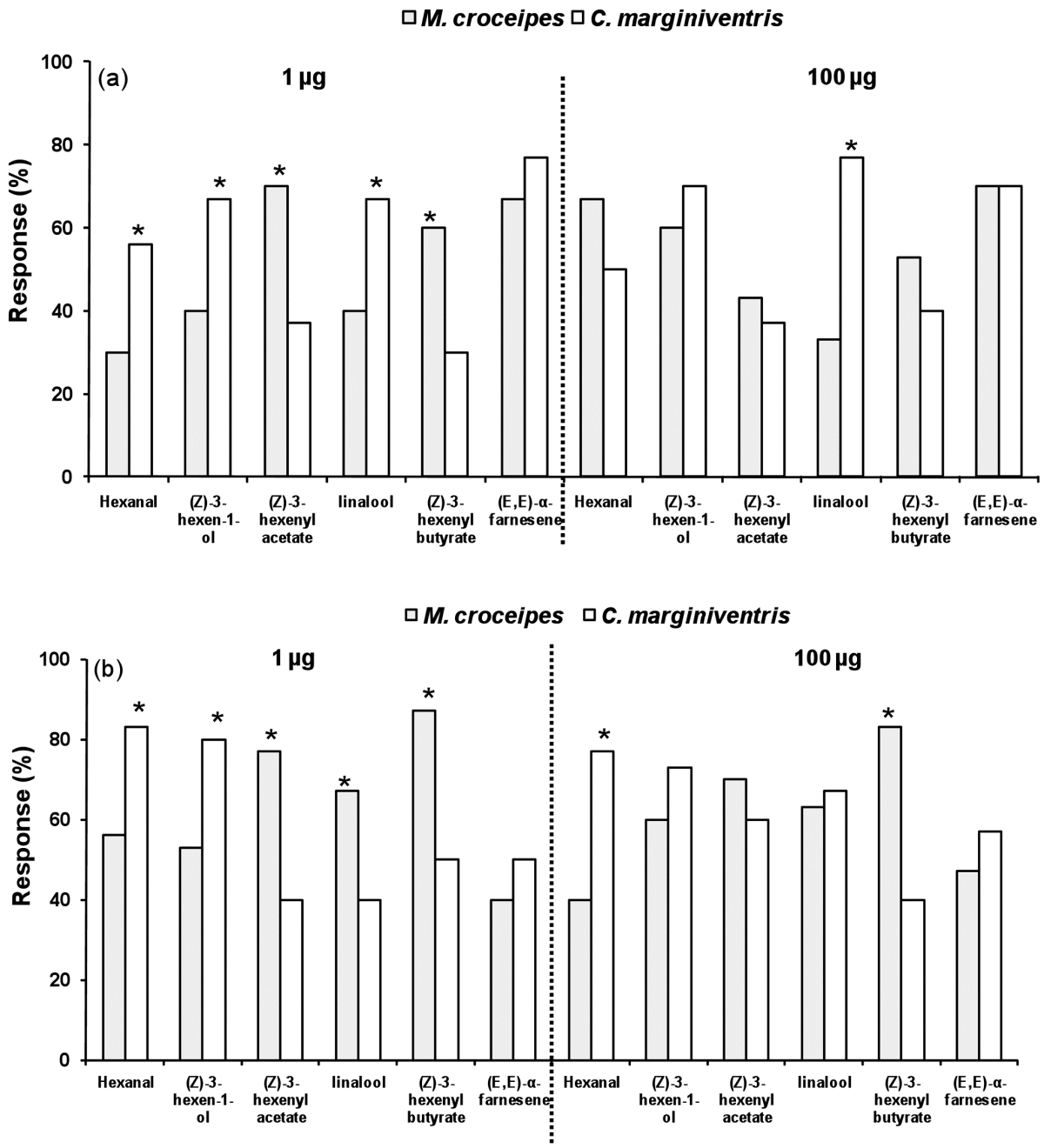

Female
Male
1 µg
100 µg
Response (%)
100
80
60
40
20
0
*
*
*
*
Hexanal
(Z)-3-hexen-1-ol
(Z)-3-hexenyl acetate
Linalool
(Z)-3-hexenyl butyrate
(E,E)-α-farnesene
Hexanal
(Z)-3-hexen-1-ol
(Z)-3-hexenyl acetate
Linalool
(Z)-3-hexenyl butyrate
(E,E)-α-farnesene

Figure 5


M. croceipes
C. marginiventris
(a)
1 µg
100 µg
Response (%)
100
80
60
40
20
0
Hexanal
(Z)-3-hexen-1-ol
(Z)-3-hexenyl acetate
linalool
(Z)-3-hexenyl butyrate
(E,E)-α-farnesene
(b)