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

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Short Title. GC-EAD responses of two parasitoids to host-related volatiles

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Abstract - Plants emit volatile blends that may be quantitatively and/or qualitatively different in response to attack by different herbivore species. These differences may convey herbivore-specific information to parasitoids and are predicted to play a role in mediating host specificity in specialist parasitoids. Here, we tested the above prediction by using as models two parasitoids (Hymenoptera: Braconidae) of cotton caterpillars with different degree of host specificity: *Microplitis croceipes*, a specialist parasitoid of *Heliothis* spp., and *Cotesia marginiventris*, a generalist parasitoid of caterpillars of several genera including *Heliothis* spp. and *Spodoptera* spp. We compared 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) versus *S. exigua* (a host species for *C. marginiventris*). Based on a recent study in which we reported intriguing differences in the EAG responses of both parasitoid species to different types of host related volatiles, we hypothesized that *M. croceipes* (specialist) will show relatively greater GC-EAD responses to the herbivore-induced plant volatile (HIPV) components of cotton headspace, whereas *C. marginiventris* (generalist) will show greater response to the green leaf volatile (GLV) components. Thirty volatile components were emitted by cotton plants in response to feeding by either of the two caterpillar species, however 18 components were significantly elevated in the headspace of *H. virescens* damaged plants. Sixteen volatile components consistently elicited GC-EAD responses in both parasitoid species. As predicted, *C. marginiventris* showed significantly greater GC-EAD responses than *M. croceipes* to most GLV components, whereas several HIPV components elicited comparatively greater responses in *M. croceipes*. These 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.

Key Words - *Microplitis croceipes*, *Cotesia marginiventris*, *Heliothis virescens*, *Spodoptera exigua*, GC-EAD, herbivore-induced plant volatiles.

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 plants 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, it has been shown that the blends of volatile compounds released from herbivore damaged plants differ qualitatively and quantitatively depending on the plant species and variety (Dicke et al., 1990; Loughrin et al., 1994; Hoballah et al., 2002), the herbivore

species (De Moraes et al., 1998; Loughrin et al., 1994; McCall et al., 1994), and the stage of the herbivore (Takabayashi et al., 1991; Du et al., 1996). For instance, it was shown that corn (*Zea mays* L.) plants infested by beet armyworm *Spodoptera exigua* (Hübner) caterpillars emitted linalool, (3*E*)-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, (3*E*)-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).

92 The question of whether specialist and generalist parasitoids show differential response to
93 different suites of host-related volatiles has been a major focus of evolutionary ecology in recent
94 years (Vet et al., 1993; Geervliet et al., 1996; Bernays, 2001; Chen and Fadamiro, 2007; Stilmant
95 et al., 2008). It is predicted that specialist parasitoids utilizing fewer number of hosts are likely to
96 possess a relatively more highly sensitive (high olfactory sensitivity to host-related chemical
97 cues) and narrowly-tuned (selective) host detection olfactory system than generalist parasitoids
98 (Vet and Dicke, 1992; Cortesero et al., 1997; Smid et al., 2002; Chen and Fadamiro, 2007).
99 However, only a few studies have compared olfactory response and sensitivity to host-related
100 volatiles in specialist and generalist parasitoids to date, and have produced contrasting results
101 (Elzen et al., 1987; Vet et al., 1993; Geerveliet et al., 1996; Chen and Fadamiro, 2007). On the
102 one hand, some studies reported relatively greater response for specialists compared to
103 generalists (Elzen et al., 1987; Vet et al., 1993). In contrast, Geervliet et al. (1996) recorded no
104 differences in the behavioral responses of the specialist, *Cotesia rubecula* Marshall and the
105 generalist, *Cotesia marginiventris* (Cresson) to host-related volatiles, and both species were
106 unable to distinguish between plant volatiles induced by their hosts versus plant volatiles induced
107 by nonhost species. Similarly, Smid et al. (2002) reported no differences in the receptive range of
108 the specialist, *C. rubecula* and the generalist, *Cotesia glomerata* L. to a wide range of host-
109 related odor compounds. Such discrepancies in the above studies suggest that diverse species of
110 specialist and generalist parasitoids may respond differently to different types of host-related
111 volatiles. Furthermore, even within a broad category of specialist or generalist parasitoids,
112 differences may still exist among species based on the degree of specialization (De Moraes et al.,
113 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*. *Microplitis 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*, *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 in which we recorded differences in the electroantennogram (EAG) responses of both parasitoid species to various synthetic host-related volatile compounds (Chen and Fadamiro, 2007), we 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.

METHODS AND MATERIALS

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/vermiculate/peat moss mixture. Plants used for headspace volatile collections were 4-6 weeks old.

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.

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). The 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 groups of 2 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

159 distilled water and threading a cotton string through a hole in the cap of the tube. About 4-6
160 drops (2 µl per drop) of 10% sugar solution were smeared on the inside of the Petri dish cover
161 with a cotton-tipped applicator. Female parasitoids (aged 3-5 days old) of both species were used
162 for the experiments.

163 *Collection and GC Analysis of Headspace Volatiles.* The methodology and protocols used for
164 volatile collection were similar to those reported by Gouinguené et al. (2005), but with some
165 modifications. Headspace volatiles were collected both from caterpillar damaged and undamaged
166 cotton plants. To induce the production of HIPVs from cotton plants, 30 second instar
167 caterpillars of *H. virescens* or *S. exigua* were allowed to feed on a potted cotton plant for 12 hr
168 prior to volatile collection. The pot with the potting soil was wrapped with aluminum foil to
169 minimize evaporation of water and volatiles from the soil. The plant (with the feeding
170 caterpillars) was then placed in a volatile collection chamber (Analytical Research Systems, Inc.,
171 Gainesville, FL.) consisting of a 5 l glass jar. A purified (using activated charcoal) air stream of
172 500 ml/min was passed through the jar at room temperature for 24 hr. The results of a pilot test
173 which compared headspace volatile collection for 24 hr versus 12 hr showed no noticeable
174 differences in the number or relative proportion of the peaks, however the 24 hr duration was
175 selected because it produced consistent profiles in which all the key peaks were detected in
176 relatively higher amounts. Headspace volatiles were trapped using a trap containing 50 mg of
177 Super-Q (Alltech Associates, Deerfield, IL) and eluted with 200 µl of methylene chloride. The
178 resulting extracts (200 µl) were stored in a freezer (at -20 °C) until use. Another container with
179 potting soil without plant was used to check for miscellaneous impurities and background noise.
180 The collection system was checked and controlled for breakthrough of the trap during sampling.
181 One µl of each headspace volatile extract was injected into a Shimadzu GC-17A equipped with a

flame ionization detector (FID). The dimension of 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 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.

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, the 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 same as previously described (Chen and Fadamiro, 2007). Glass capillaries (1.1 mm I.D.) filled with Ringer solution were used as electrodes. 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 analog signal was detected through a probe (INR-II, Syntech®), captured and processed with a data acquisition controller (IDAC-4, Syntech®), and later analyzed with software (GcEad 32, Syntech®) 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 significant and consistent qualitative and quantitative differences among the treatments.

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 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.*

227 *virescens* damaged versus *S. exigua* damaged cotton plants were established by using the
228 Student's t-test ($P < 0.05$, SAS Institute, 1998).
229
230 *GC-EAD Analyses with Synthetic Blend.* In order to confirm the observed differences in the GC-
231 EAD responses of both parasitoids to the headspace extracts, a synthetic blend mimicking the
232 headspace of caterpillar-infested cotton plants was prepared. This blend was formulated to mimic
233 closely the active components of the headspace of cotton plants infested with *H. virescens*,
234 although the same compounds were detected also in the headspace of cotton plants infested with
235 *S. exigua.* It consisted of 13 synthetic volatile compounds which were identified as key
236 biologically active components in the headspace volatiles of cotton plants infested with *H.*
237 *virescens*, and blended at an approximate ratio in which they were detected in the headspace. The
238 compounds were purchased from the above source with purity > 97% and included *cis*-3-
239 hexenal, *trans*-2-hexenal, *cis*-3-hexen-1-ol, *cis*-3-hexenyl acetate, *trans* -2-hexenyl acetate,
240 linalool, *cis*-3-hexenyl butyrate, *trans*-2-hexenyl butyrate, indole, *cis*-jasmone, α-farnesene, α-
241 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,
242 7.2, 0.4, 4.6, 4.3, and 10.2, respectively. Each compound was diluted in hexane and blended at
243 the above ratio to obtain a 100 µg/µl solution.. A 3-µl aliquot of the blend (100 µg/µl) was
244 injected for a GC-EAD run. Five successful GC-EAD recordings were obtained for each
245 parasitoid species as described above.
246
247 *Quantification of GC-EAD Responses.* GC-EAD responses of both parasitoid species to different
248 volatile components were quantified by using a measurement marker tool available with the GC-
249 EAD software (GcEad 32). This tool enabled the quantification of EAD peaks in microvolts

(μV). Significant differences in GC-EAD responses of both parasitoid wasps to each volatile component were then established by using the Student's t-test ($P < 0.05$: SAS Institute, 1998).

RESULTS

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* (Figure 1A, B). The same identical 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 (Figure 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.

GC-EAD Responses. 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 (Figures 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 significantly 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) (Table 2, Figures 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 significantly greater responses in *M. croceipes*, compared to *C. marginiventris*. In addition, α-humulene also elicited greater response in *M. croceipes* than in *C. marginiventris*, but this was significant only for *H. virescens*-infested headspace extract. Also, *M. croceipes* showed relatively greater GC-EAD responses than *C. marginiventris* to indole and *cis*-jasmone, but these differences were significant only for *S. exigua*-infested extract. 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 (Table 2, Fig.

294 4). A confirmatory test in which the synthetic blend was tested at a reduced amount (i.e., 1 µl of
295 a 0.1 µg/µl solution of the blend was injected for a GC-EAD run) produced similar results as
296 those shown in Figure 4, suggesting that the amounts tested in the initial experiment with
297 synthetic blend were not too high or physiologically irrelevant.

298

299 DISCUSSION

300 The results of this study showed that *M. croceipes* and *C. marginiventris* females were
301 capable of responding antennally to many but not all of the caterpillar-induced cotton volatiles,
302 with both parasitoid species showing differential electrophysiological responses to the different
303 components of the volatile blends. Compared to undamaged plants, cotton plants emitted
304 detectable amounts of a wide range of volatiles, specifically 30 volatile compounds, in response
305 to damage by *H. virescens* or *S. exigua*. In general, our results are in agreement with those
306 previously reported by other authors on the induction of cotton volatiles by caterpillar species
307 (Loughrin et al., 1994; McCall et al., 1994), but with some important differences. Loughrin et al.
308 (1994) and McCall et al. (1994) reported 23 and 22 compounds, respectively from the headspace
309 of caterpillar-infested cotton plants, most of which were identified also in the present study.
310 These compounds included GLVs such as *cis*-3-hexenal, *trans*-2-hexenal, and *cis*-3-hexen-1-ol,
311 and HIPVs such as *cis*-3-hexenyl acetate, linalool, (*E*,*E*)-4,8-dimethyl-1,3,7-nonatriene, *cis*-3-
312 hexenyl butyrate, *trans*-2-hexenyl butyrate, *trans*-2-hexenyl-2-methyl butyrate, indole, *cis*-
313 jasmone, (*E*,*E*)-α-farnesene, α-humulene, and *trans*-nerolidol. However, we also detected
314 additional volatile compounds which were not reported by Loughrin et al. (1994) and McCall et
315 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 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 major 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 change in proportions or ratios of volatile compounds 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. It is important to note that the use of plant volatiles by both parasitoids to locate host-infested plants may suggest that both are generalists in terms of host habitat 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; Park et al., 2001; Smid et al., 2002; Gouinguené et al., 2005). The reason why parasitoids do not perceive all the components of the headspace volatile of caterpillar-damaged plants is an interesting evolutionary question which deserves to be addressed. It is note worthy 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, Gouinguene 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).

362 The major difference recorded in our study was in the intensity of GC-EAD response of
363 both parasitoids to several compounds. For the first time, we utilized a measurement tool in the
364 GC-EAD software to quantify and then establish significant differences in GC-EAD responses of
365 the two parasitoid species to the various volatile components. The generalist, *C. marginiventris*
366 showed significantly greater GC-EAD responses than the specialist, *M. croceipes* to most GLV
367 components, whereas several HIPV components elicited comparatively greater responses in *M.*
368 *croceipes*. Similar differences in the intensity of response of parasitoids to host-related
369 compounds were also reported by Gouinguené et al. (2005). The authors reported that the
370 generalist parasitoids, *C. marginiventris* and *C. sonorensis* showed a greater sensitivity to cotton
371 GLVs *cis*-3-hexanal, *trans*-2-hexanal and *cis*-3-hexen-1-ol) than the more restricted *M.*
372 *rufiventris.* Our results in which females of the generalist *C. marginiventris* showed
373 comparatively greater GC-EAD responses to GLVs (*cis*-3-hexanal, *trans*-2-hexanal and *cis*-3-
374 hexen-1-ol), which are continuously present in the plant and released in freshly damaged plants
375 support our hypothesis, and are somewhat in agreement with previous electrophysiological
376 (Gouinguené et al., 2005; Chen and Fadamiro, 2007) and behavioral studies (Cortesero et al.,
377 1997; Hoballah et al., 2002; D'Alessandro and Turlings, 2005; Hoballah and Turlings, 2005).
378 Similar to our results, Gouinguené et al. (2005) also reported that *C. marginiventris* showed little
379 or no antennal response to several HIPVs including β-myrcene, β-caryophyllene, bergamotene,
380 and β-farnesene. In contrast, the specialist *M. croceipes* showed greater GC-EAD responses to
381 the HIPVs, which are more specifically linked to its host. These findings were verified by the
382 results of the GC-EAD tests with the synthetic blend, which also showed the same differences in
383 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*). Our GC data showed that the essential difference between the volatile blends of cotton plants induced by *H. virescens* versus *S. exigua* is 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. The 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 our 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 without doubt a challenging task for specialist parasitoids, such as *M. croceipes*. Thus, it is likely that other unknown minor compounds as well as host-specific volatiles may play also a role in differentiation of host versus non-host by *M. croceipes*.

In contrast, no obvious differences were observed in the response of *C. marginiventris* to volatile blends induced by both caterpillar species. Our 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. However, *C. glomerata* was able to discriminate between plant volatiles induced by its hosts versus volatiles induced by non-host species after learning (Geervliet et al., 1998). This suggests that associative learning may improve the overall ability of *C. marginiventris* to respond to the HIPV components of the volatile blends, as has been reported for some other generalist parasitoids (Turlings et al., 1989, 1993; Vet and Groenewold, 1990; Vet, 1999; Steidle and van Loon, 2003; Tamo et al., 2006). Indeed, there is evidence that associative learning may improve response of *C. marginiventris* to induced volatiles (D'Alessandro and Turlings, 2005). Furthermore, the results of an ongoing study in our laboratory suggest that associative learning may enhance the behavioral response of *C. marginiventris* to host-related volatiles (unpublished data).

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 has 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* in our laboratory, we recorded relatively greater numbers of olfactory sensilla placodea on *M. croceipes* than on *C. marginiventris* antennae (unpublished data). 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 our 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*. Additionally, unknown minor compounds as well as host-specific volatiles may play also a role in the differentiation of different host-plant complexes. Further discrimination 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[a]	*H. virescens*-infested		*S. exigua*-infested		Uninfested	
		Amount (ng ± SE)[b]	Relative %	Amount (ng ± SE)[b]	Relative %	Amount (ng ± SE)[b]	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

[a] *In order of elution during gas chromatography*

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

Means across the same row for the same headspace extract 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).

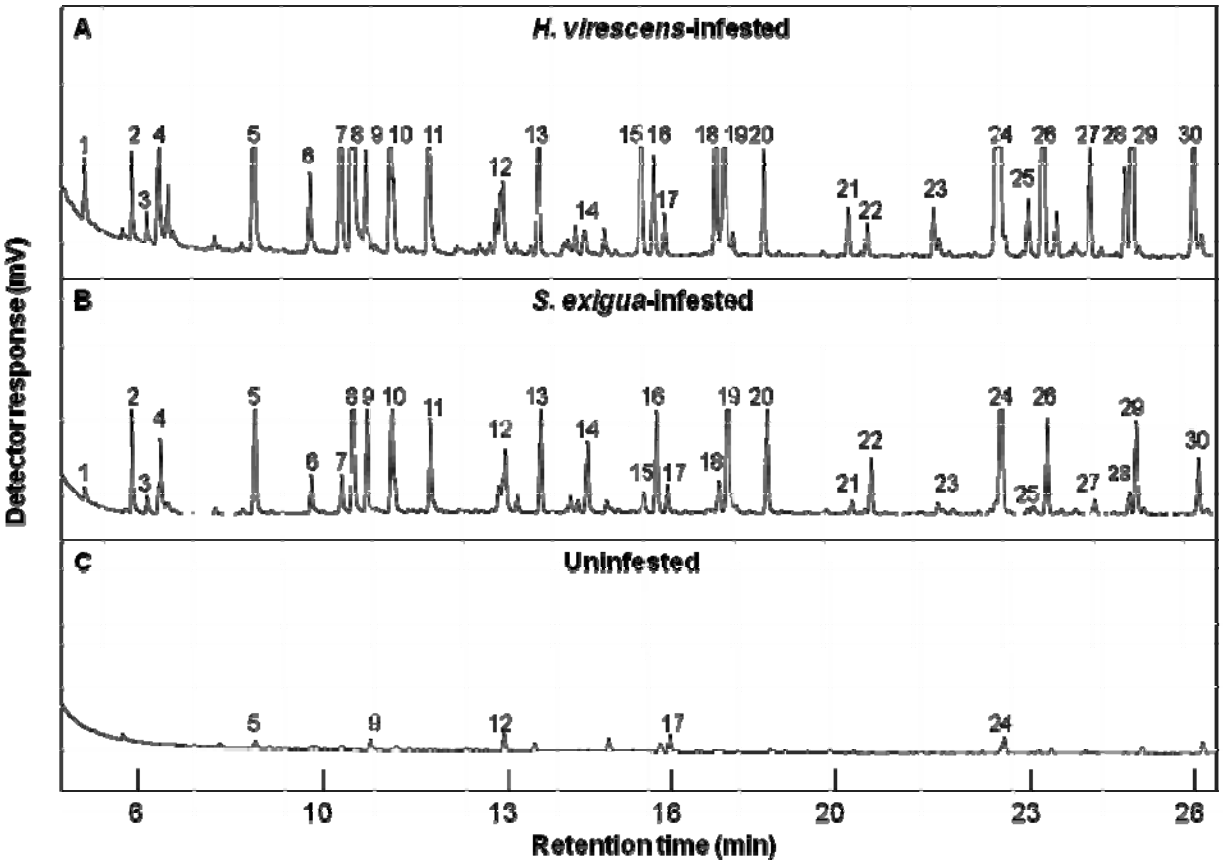
687 FIGURE LEGENDS

688 **FIG. 1.** Chromatographic profiles of headspace volatiles collected from cotton plants infested
689 with *H. virescens* (A) or *S. exigua* (B) caterpillars, versus undamaged control plants (C).
690 Identified compounds: (1) *cis*-3-hexenal; (2) *trans*-2-hexenal; (3) *cis*-3-hexen-1-ol; (4) trans-2-
691 hexen-1-ol; (5) α-pinene; (6) β-pinene; (7) myrcene; (8) *cis*-3-hexenyl acetate; (9) *trans*-2-
692 hexenyl acetate; (10) limonene; (11) β-ocimene; (12) linalool; (13) unknown; (14) (*E*)-4,8-
693 dimethyl-1,3,7-nonatriene; (15) *cis*-3-hexenyl butyrate; (16) *trans*-2-hexenyl butyrate ; (17) *n*-
694 decanal (18) *cis*-3-hexenyl-2-methyl butyrate; (19) *trans*-2-hexenyl-2-methyl butyrate; (20)
695 indole; (21) isobutyl tiglate; (22) (*E*)-2-hexenyl tiglate; (23) *cis*-jasmone; (24) caryophyllene;
696 (25) α-*trans* bergamotene; (26) α-farnesene; (27) α-humulene; (28) β-farnesene; (29) β-
697 hemachalene; (30) *trans*-nerolidol.
698
699 **FIG. 2.** GC-EAD responses of *M. croceipes* (A) and *C. marginiventris* (B) to headspace volatiles
700 from *H. virescens* damaged cotton plants. GC-EAD active compounds: (1) *cis*-3-hexenal; (2)
701 *trans*-2-hexenal; (3) *cis*-3-hexen-1-ol; (4) *cis*-3-hexenyl acetate; (5) *trans* -2-hexenyl acetate; (6)
702 linalool; (7) (*E*)-4,8-dimethyl-1,3,7-nonatriene; (8) unknown; (9) *cis*-3-hexenyl butyrate; (10)
703 *trans*-2-hexenyl butyrate ; (11) *trans*-2-hexenyl-2-methylbutyrate; (13) *cis*-jasmone; (14) α-
704 farnesene, (15) α-humulene; (16) *trans*-nerolidol. Note that responses of *C. marginiventris* to
705 some of the HIPV components were almost too low to be detectable in this and the next two
706 figures. GC-EAD responses of both species to the various compounds are quantified in Table 2.
707
708 **FIG. 3.** GC-EAD responses of *M. croceipes* (A) and *C. marginiventris* (B) to headspace volatiles
709 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. GC-EAD responses of both species to the various compounds are quantified in Table 2.

FIG. 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 an 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. GC-EAD responses of both species to the various compounds are quantified in Table 2.

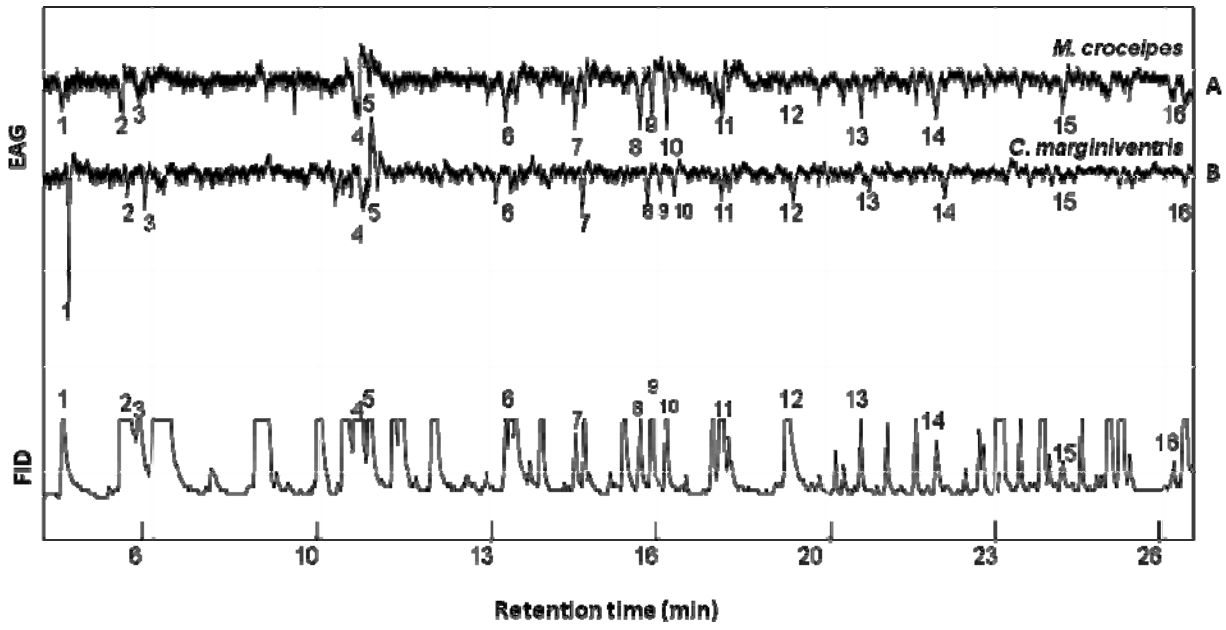
FIG. 1


A
H. virescens-infested
B
S. exigua-infested
C
Uninfested
Detector response (mV)
Retention time (min)
6
10
13
16
20
23
26

736

737 FIG. 2

738



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744 FIG. 3

745

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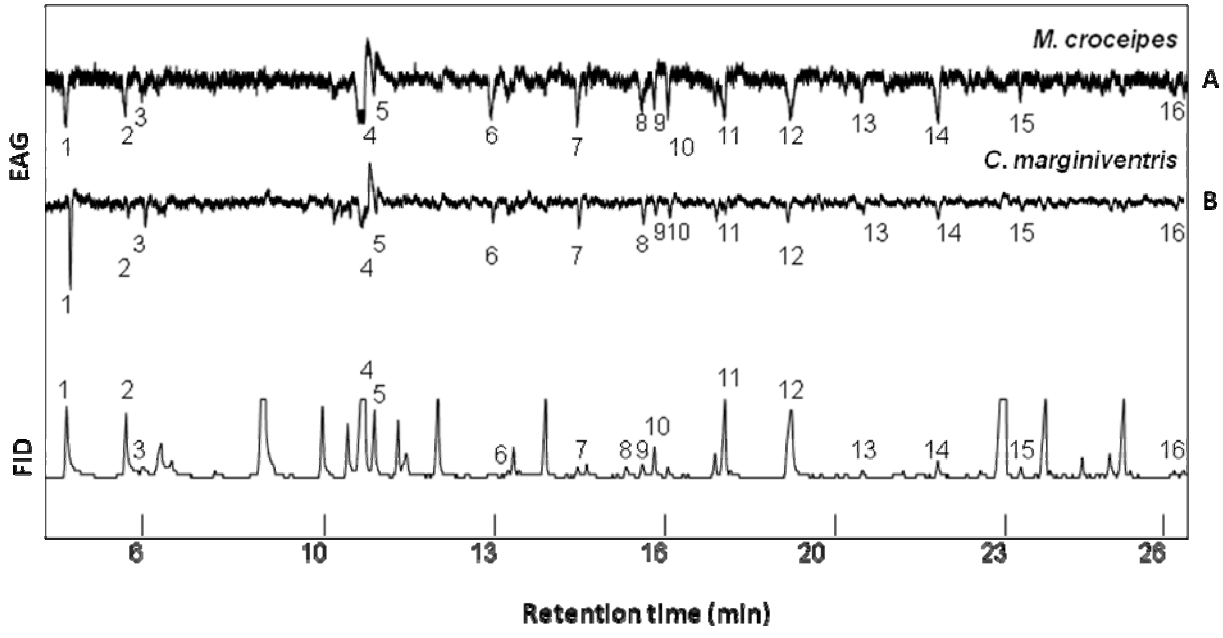

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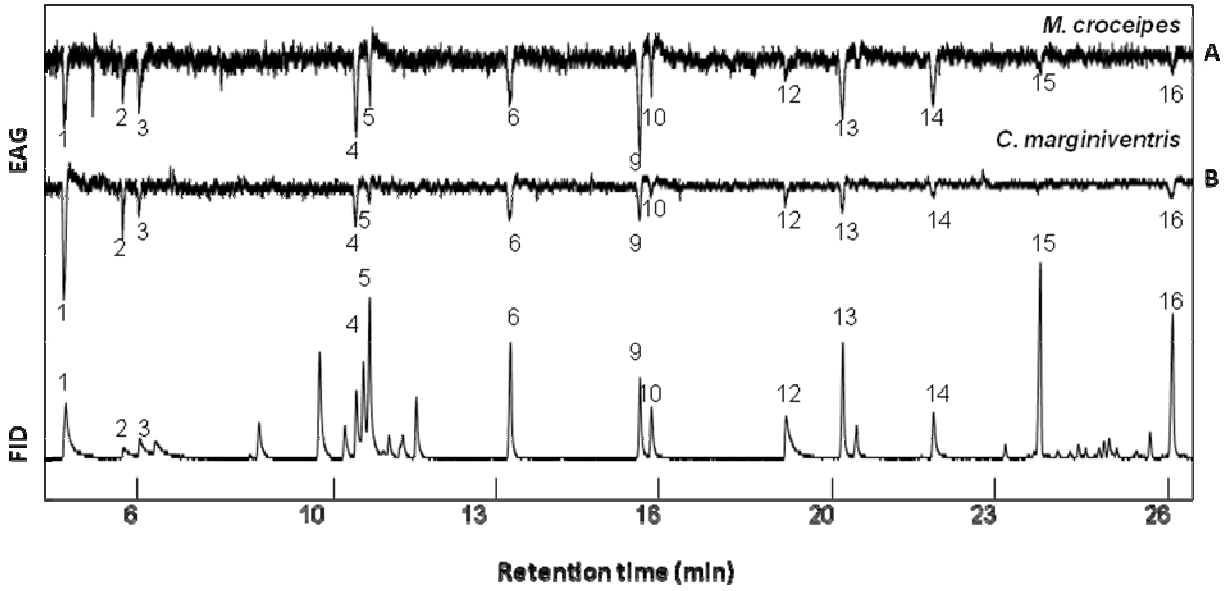
748

749 FIG. 4

750

751



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