Pest Management Science SCI

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Journal:	*Pest Management Science*
Manuscript ID	PM-20-0662
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	19-Jun-2020
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Key Words:	Aphidius colemani, Bacillus, electroantennogram, multitrophic interactions, natural enemy, VOCs, chemical ecology, pest management

Identification and application of bacterial volatiles to attract a generalist aphid parasitoid: from laboratory to greenhouse assays

Running title: Parasitoid attraction by bacterial volatiles

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Abstract

BACKGROUND: Recent studies have shown that microorganisms emit volatile compounds that affect insect behaviour. However, it remains largely unclear whether microbes can be exploited as a source of attractants to improve biological control of insect pests. In this study, we used a combination of coupled gas chromatography-electroantennography (GC-EAG) and Y-tube olfactometer bioassays to identify attractive compounds in the volatile extracts of three bacterial strains that are associated with the habitat of the generalist aphid parasitoid *Aphidius colemani*, and to create mixtures of synthetic compounds to find attractive blends for *A. colemani*. Subsequently, the most promising blend was evaluated in two-choice cage experiments under greenhouse conditions.

RESULTS: GC-EAG analysis revealed 20 compounds that were linked to behaviourally attractive bacterial strains. A mixture of two EAG-active compounds, styrene and benzaldehyde applied at a respective dose of 1 µg and 10 ng, was more attractive than the single compounds or the culture medium of the bacteria in Y-tube olfactometer bioassays. Application of this synthetic mixture under greenhouse conditions resulted in significant attraction of the parasitoids, and outperformed application of the bacterial culture medium.

CONCLUSION: Compounds isolated from bacterial blends were capable of attracting parasitoids both in laboratory and greenhouse assays, indicating that microbial cultures are an effective source of insect attractants. This opens new opportunities to attract and retain natural enemies of pest species and to enhance biological pest control.

Keywords: *Aphidius colemani*; *Bacillus*; electroantennogram; multitrophic interactions; natural enemy; VOCs

1 INTRODUCTION

Biological control using natural enemies such as arthropod predators and parasitoids has become an important alternative method of pest management,[1] but the efficacy of biological pest control can be seriously hampered when naturally occurring enemies are not sufficiently abundant or effective. To increase the efficacy of biological control, naturally occurring parasitoids and predators are often complemented with the release of commercially reared natural enemies.[1,2] While this temporarily increases the local density of natural enemies, a major challenge in biological pest control remains to attract and retain beneficial insects within the crop so that they maintain high population densities in the longer term and sufficiently reduce the local abundance of pests.[2,3]

Insect- and plant-derived semiochemicals can be manufactured and deployed to manipulate the behaviour of natural enemies. Examples include volatiles produced when plants are attacked by herbivores (herbivore-induced plant volatiles, HIPVs), and alarm, sex or aggregation pheromones of pests or natural enemies.[4,5] These chemicals can be sprayed onto crops or deployed in dispensers at regular intervals in the crop.[4] While most research in this field has focused on cues derived from plants and insects,[3,6] there is mounting evidence that microorganisms emit volatile compounds (mVOCs, microbial volatile organic compounds) that also play a role in insect behaviour.[7,8] In some cases, mVOCs strongly attract insects by signalling the presence of appropriate resources such as food sources and oviposition sites,[9-11] whereas others have been found to deter insects.[12]

Despite an increased understanding of the role of microbial volatiles as insect semiochemicals,[7,8,13] little is still known whether they can be exploited as a source of

attractants of pest natural enemies. In most cases, insects respond to complex mixtures of volatile compounds in specific ratios.[14,15] However, other studies have shown that insects may also respond to single compounds.[16,17] Additionally, there are examples indicating that simplified blends of synthetic volatiles, representing only a limited set of the volatiles from a natural blend, can be as attractive as the natural blends.[18,19] This suggests that, despite the rich plethora of volatiles that is generally available from natural resources, only a select number of compounds evoke a behavioural response in the insects. So far, identification of active microbial compounds affecting parasitoid foraging behaviour, or mixtures thereof, and study of their performance under field conditions remain largely unexplored. Such studies would allow to fully grasp the potential of microbial volatiles to develop new semiochemical-based strategies to improve biological pest control efficacy.

In previous research using laboratory assays with *Aphidius colemani* Viereck (Hymenoptera: Braconidae) we showed that parasitic wasps are attracted to volatile blends emitted by bacteria isolated from the parasitoids' habitat.[20] Preliminary analyses of the volatile blends showed that bacteria that significantly attracted the parasitoids produced blends that contained significantly lower amounts of esters, organic acids, aromatics and cycloalkanes than repellent strains.[20] In this study, we tested the behavioural and electrophysiological responses of *A. colemani* females to the volatile blends of three bacterial strains producing attractive mVOCs. Subsequently, five EAG-active compounds were selected and tested individually, as well as in blends, for their effects on parasitoid olfactory responses under laboratory conditions. Finally, two-choice cage experiments with plants treated with the behaviourally most active synthetic blend

versus control plants were performed to assess their attractive potential under greenhouse conditions. As a comparison, the cell-free cultivation medium of one of the bacterial strains was included.

2 MATERIALS AND METHODS

2.1 Study organisms

Three bacterial isolates that produce volatile blends that are attractive to *A. colemani*[20,21] were used in this study (Table S1, Supporting Information). Strains were isolated from different sources from the parasitoid's habitat. They included an isolate from the aphid *Macrosiphum euphorbiae* (ST18.16/150), an isolate from an *Aphidius* wasp (ST18.16/133), and an isolate from *Dendrocerus aphidum*, which is an hyperparasitoid of *Aphidius* (ST18.16/043). Based on sequencing of the *rpoB* gene, isolates were assigned to *Bacillus circulans* (ST18.16/150), *Bacillus pumilus* (ST18.16/133) and *Bacillus* sp. (ST18.16/043) (Table S1, Supporting Information). Strains were stored at −80°C in tryptic soy broth (TSB, Oxoid, Hampshire, UK) containing 25% (v/v) glycerol. Insect responses were investigated using adult females of *A. colemani*. Parasitoids were obtained in the form of parasitized aphid mummies from Biobest (Westerlo, Belgium) (Aphidius-system®). Mummies were placed inside a nylon insect cage (20×20×20 cm, BugDorm, MegaView Science Co., Ltd., Taichung, Taiwan) and kept under controlled conditions (22°C, 70% relative humidity and a 16:8-h light:dark photoperiod) until parasitoid emergence. All experiments were performed with food- and water-inexperienced females that were <24 hours old.

2.2 Production of mVOCs

For production of mVOCs, the procedure by Goelen et al.[20] was used. Briefly, bacterial strains were plated on tryptic soy agar (TSA, Oxoid, Hampshire, UK) and incubated at 25°C for 24h. Next, bacterial cells were inoculated in 10 mL TSB and incubated overnight at 25°C with agitation at 120 rpm. Bacterial cells were then washed and diluted to a cell suspension with an optical density (OD 600 nm) of 1. Next, 1.5 mL of the obtained suspension was inoculated in a 250 mL Erlenmeyer flask containing 150 mL GYP25 medium[20]. Erlenmeyer flasks were sealed with silicone plugs and incubated at 25°C at 120 rpm. Each strain was cultivated in triplicate, and non-inoculated, blank medium was included as a control. After 48h of incubation, the media were centrifuged at 10,000 g for 15 min and filter-sterilized to obtain cell-free supernatants. The samples were then stored in small aliquots in sterile, amber glass vials at -20°C until further use.

2.3 Identification of physiologically active mVOCs

In order to determine which mVOCs elicited an electrophysiological response in *A. colemani*, first microbial volatiles were collected by dynamic headspace collection (air entrainment).[22] Specifically, volatiles were collected for 1h from 150 µL cell-free cultivation medium inside a 4 mL glass screw top GC vial (Thermo Scientific, Waltham, USA). In- and outlet ports were created by fitting Swagelock ports onto 19Gx2" syringe needles (AganiTM, Terumo®, Leuven, Belgium) which were pierced through the 12 mm polytetrafluorethylene (PTFE)/silicone septum (Supelco, Bellefonte, USA) of the GC vial. Activated charcoal filtered air was supplied through the inlet port at a rate of 400 mL/min. Air subsequently passed over the medium in the GC vial and headspace volatiles

were adsorbed on Porapak Q filters (0.05 g, 50/80 mesh; Supelco, Bellefonte, USA) that were fitted on the outlet port through which air was drawn at a rate of 300 mL/min. Prior to entrainment, Porapak Q filters were washed with diethyl ether and conditioned by heating to 132°C in an activated charcoal-filtered nitrogen stream for 2h. Air entrainment of 150 µL of blank GYP25 medium was included as a control. All connections in the air entrainment setup were made using PTFE tubing. Entrained volatiles were eluted in 750 µL diethyl ether and were stored in 1.1 mL glass microvials at -20°C until further use. GC-FID analysis yielded highly similar mVOC profiles across the biological replicates for each treatment. Therefore, all remaining experiments were performed with only one of the three replicates.

After air entrainment, coupled gas chromatography-electroantennography (GC-EAG) was performed using antennal preparations of female parasitoids. Before analysis, air entrainment samples were concentrated to 50 µL under an activated charcoal-filtered nitrogen stream. GC-EAG analyses were performed three times, and for each replicate a new antennal preparation was used. The GC-EAG system was equipped with a 6890N GC machine (Agilent Technologies, Santa Clara, USA) fitted with a cold on-column injection system and a non-polar HP-1 capillary column (50 m; 0.32 mm internal diameter; 0.52 µm film thickness), and used a flame ionization detector (FID).[23] The carrier gas was helium. The oven temperature was initiated at 30°C and was maintained there for 2 min before being raised to 250°C at a rate of 5°C/min. The GC column effluent was split equally between the FID and the heated transfer line which delivered the separated compounds into an activated charcoal filtered, humidified air stream that flew towards the antennal preparation. Antennal preparations were made by chilling the

parasitoid in ice for 1 min, excising the head, removing one entire antenna, and then removing the tip of the last antennal segment to ensure good contact with the recording electrode. The antenna was then brought into contact with the Ag-AgCl ground electrode by inserting the antennal base into a glass capillary housing the electrode and filled with saline solution (composition as in Maddrell[24], but without the glucose). The distal end was brought into contact with the recording electrode in a similar way. Detected signals were amplified by a high impedance amplifier (UN-06; Ockenfels Syntech GmbH, Kirchzarten, Germany) and analysed using customized Syntech software. Outputs from the FID and the EAG amplifier were analysed simultaneously with custom software. Only volatiles with a consistent electrophysiological response peak in all three replicates were considered as EAG-active.

Next, EAG-active mVOCs were tentatively identified by coupled GC-MS using 4 µL of the concentrated air entrainment samples on a Waters Autospec Ultima mass spectrometer (Manchester, UK) coupled to an Agilent 6890 GC (Agilent Technologies, Santa Clara, USA; cold on-column injector, 50 m × 0.32 mm internal diam, 0.52 µm film thickness HP-1- column). Ionization was performed by electron impact at 70 eV and 220°C. The GC oven temperature was initiated at 30°C and maintained for 5 min and then raised to 250°C at 5°C/min. Helium was the carrier gas. Peak identities were tentatively determined by manually comparing mass spectra with those from mass spectral databases using NIST MS Search v2.0 software with the NIST 2011 library, and by comparison of GC retention indices (Kováts index = KI).

2.4 Y-tube behavioural assays

In order to test the attractiveness of the microbial volatile blends and EAG-active volatiles or blends thereof, a Y-tube olfactometer bioassay was performed as described by Goelen et al.[20] For each bacterial strain, 150 µL of the cell-free cultivation medium was loaded on a filter paper (37 mm; Macherey-Nagel, Düren, Germany) and subsequently put in one of the olfactometer odour chambers. The second chamber received another filter on which 150 µL blank medium was loaded as a control. For assessing parasitoid response to EAG-active compounds, benzaldehyde (≥99.5%), butyl acetate (99.7%), 1,3-diacetyl benzene (97.0%), styrene (≥99.0%) (all purchased from Sigma-Aldrich, Saint Louis, USA) and 1,2-dimethyl benzene (o-xylene) (≥99.0% Fluka, Bucharest, Romania) were used. Compounds were dissolved in diethyl ether prior to loading 10 µL of the mixture on a filter paper. After 30 seconds (which allowed the diethyl ether to evaporate), the filter paper was placed in one of the odour chambers of the olfactometer setup, while in the other chamber another filter paper was placed on which 10 µL diethyl ether had been added as a control. In a first experiment, the different test compounds were diluted in diethyl ether in different concentrations, resulting in seven different doses, i.e. 1, 10, 50 and 100 ng, and 1, 10 and 50 µg, which were then each tested in the Y-tube olfactometer. In a second experiment, two synthetic volatile blends were tested, which are further referred to as "Blend 1" and "Blend 2". Blend 1 consisted of two compounds to which *A. colemani* showed significant preference in the first experiment, i.e. benzaldehyde and styrene. The blend was produced by combining both compounds in their most attractive dose as determined in the first experiment (i.e. 10 ng for benzaldehyde and 1 µg for styrene). In addition, four other doses of the blend were tested with the same ratio of both

compounds (Table S2, Supporting Information). Blend 2 consisted of five EAG-active compounds and was created by adding the different compounds at relative amounts resembling the ratios in the mVOC blend of one of the bacterial strains (ST18.16/133), and was tested at five different doses (Table S3, Supporting Information).

All experiments were conducted with 60 female individuals, which were released in twelve cohorts of five individuals, and olfactory response was evaluated 10 min after their release. Parasitoids that did not make a choice within 10 min after release were considered as non-responding individuals and were eliminated from statistical analysis. Parasitoid olfactory response was analysed using a Generalized Linear Mixed Model (GLMM) based on a binomial distribution with a logit link function (logistic regression) using bacterial isolate, compound or blend as fixed factor (performed in R with the 'glmer' function from the lme4 package). Each release of one cohort of five individuals served as a replicate. To adjust for overdispersion and to prevent pseudoreplication, the release of each cohort (n = 12) was included in the model as a random factor. The number of parasitoids choosing for the control or treatment side in each cohort was entered as response variable. To examine the preference of the investigated parasitoids, we tested the null hypothesis (H0) that the parasitoids showed no preference for any olfactometer arm (i.e. 50:50 response) by testing H_0: logit = 0, which equals a 50:50 distribution. Results were presented by calculating the Preference Index (PI), which is the difference between the number of parasitoids choosing for the volatile compounds and the parasitoids choosing for the control divided by the total number of responding insects.

2.5 Cage experiments

Following the laboratory bioassays, the most promising blend was tested in a two-choice cage experiment in a greenhouse compartment (average temperature 22 ± 4°C, day light). As a comparison, the cell-free cultivation medium of one of the bacterial strains was included. Experiments were performed in a 2×3×2 m cage that was closed at all sides with a fine mesh. Nine-week-old sweet pepper plants (*Capsicum annuum* cv. IDS) were placed onto elevated platforms (height: 40 cm) in each corner of the cage (Fig. S1, Supporting Information). Using a vaporizer, plants were treated by spraying them with either the synthetic blend of 1 ng/µL benzaldehyde and 100 ng/µL styrene (Blend 1) or the cell-free cultivation medium of strain ST18.16/133 ("Treatment"; two plants), or a control solution (diethyl ether or non-inoculated GYP25 medium) ("Control"; two plants). Specifically, the leaves of the plants were sprayed with 20 puffs by which on average 2.5 mL was deposited onto the leaves of each plant. Treatment and control plants were always placed diagonally relative to each other. To evaluate the ability of the volatile mixtures to affect the behavioural response of *A. colemani*, 60 females were released from an elevated platform (height: 40 cm) in the centre of the cage 30 min after the plants had been sprayed (Fig. S1, Supporting Information). To record the parasitoids' responses, a transparent, non-odorous glue plate (40×25 cm; Biobest, Westerlo, Belgium) was placed directly behind each plant to trap the parasitoids that visited this part of the cage (Fig. S1, Supporting Information). Forty-eight hours after parasitoid release, traps were removed and trapped parasitoids were counted. The experiment was replicated eight times on four different experimental days. For each replicate, plants were renewed, and the positions of treatment and control plants were switched. Parasitoid behavioural response was analysed

as mentioned earlier using a GLMM based on a binomial distribution with a logit link function (logistic regression), but using blend (synthetic blend vs. bacterial culture medium) as fixed factor. Each release of 60 individuals served as a replicate. The total number of parasitoids choosing for the control or treatment plants in each replicate was entered as response variable.

3 RESULTS

3.1 Electrophysiological responses of *A. colemani* to mVOCs

In total, 20 EAG-active compounds were found in the mVOCs released by the bacteria (Fig. S2, Supporting Information), nine of which were tentatively identified by GC-MS and KI comparison (Table 1). While most of the EAG-responses were elicited by compounds unique to a certain strain, five EAG-active compounds originated from the mVOCs of more than one strain (Table 1). Specifically, the EAG-active compounds styrene and *o*-xylene were found in the volatile extracts of three strains, while benzaldehyde, 1,3-diacetylbenzene and a so far unknown compound were found in the volatile blends produced by two strains (Table 1).

3.2 Olfactory responses to EAG-active compounds and blends thereof

Behavioural assays with five selected EAG-active compounds revealed that parasitoids showed a significant behavioural response to two compounds: styrene and benzaldehyde (Fig. 1). Compound dose significantly affected parasitoid response (styrene: $\chi^2 = 23.33$, $df = 6$, $P = 0.003$; benzaldehyde: $\chi^2 = 18.73$, $df = 6$, $P = 0.016$). Parasitoids had a significant preference for styrene at 1 µg dose (PI = 0.38, $P = 0.005$), and for

benzaldehyde at 50 ng (PI = 0.29, $P = 0.035$) and 10 ng (PI = 0.31, $P = 0.011$) doses (Fig. 1). Olfactory response to 10 or 50 ng benzaldehyde was comparable with the response to the bacterial cultivation medium (PI = 0.30 - 0.33), while the response to 1 µg styrene was more pronounced (Fig. 1). Results for benzaldehyde also suggest that doses equal or higher than 1 µg elicit a negative response in *A. colemani*. Furthermore, results revealed that 10 ng of butyl acetate was significantly repellent to *A. colemani* (PI = -0.36; $P = 0.011$) (Fig. 1).

Of the two synthetic blends tested, parasitoids were significantly attracted to Blend 1 ($\chi^2 = 21.15$, $df = 4$, $P < 0.001$), while the effect of Blend 2 was not significant in any of the doses tested ($\chi^2 = 5.90$, $df = 4$, $P = 0.207$) (Fig. 2). Parasitoid females had a significant preference for the 0.75× (PI = 0.32; $P = 0.043$), 1× (PI = 0.50, $P < 0.001$) and 1.5× dose (PI = 0.28, $P = 0.022$) of Blend 1, while they were significantly deterred by the 2× dose (PI = -0.28, $P = 0.046$) (Fig. 2). A combination of 1 µg styrene and 10 ng benzaldehyde elicited a considerably stronger response (PI = 0.50) in comparison to the responses to the individual compounds ($PI_{styrene} = 0.38$, $PI_{benzaldehyde} = 0.31$) and the mVOCs of the bacterial cell-free media (PI = 0.30 - 0.33).

3.3 Parasitoid behavioural response under greenhouse conditions

Parasitoid behavioural response in the two-choice cage experiment varied significantly between synthetic Blend 1 and the cell-free cultivation medium of strain ST18.16/133 ($\chi^2 = 5.75$, df = 4, $P = 0.016$). Plants treated with Blend 1 were visited by significantly more parasitoids than the control plants (PI = 0.35, $P < 0.001$). Specifically, 50 to 80% of the total number of trapped individuals were caught near the treatment plants across the

different replicates (Fig. 3). Plants treated with the cultivation medium of ST18.16/133 elicited no significant response relative to the control plants (PI = 0.03, $P = 0.677$). In the experiment with Blend 1, on average 20 out of the 60 released insects (33.0%) were caught on the sticky plates, whereas this was considerably lower in the experiment with the cultivation medium of ST18.16/133, where on average 11.5 insects (19.2%) were trapped. It has to be noted that our method used to evaluate insect response may have underestimated the number of responding parasitoids as only individuals trapped on the glue plates behind the plants were taken into account.

4 DISCUSSION

Our results showed that *A. colemani* females were able to detect several, but not all, mVOCs produced by the bacteria. This suggests that only certain mVOCs play a role in parasitoid olfactory behaviour, which is in agreement with previous research on plant- or host-associated volatiles.[14] Although GC-EAG analyses allow the determination of electrophysiologically active compounds, an EAG response does not necessarily indicate behavioural activity.[25] In our study, only two of five tested EAG-active compounds (benzaldehyde and styrene) evoked an innate behavioural response in the Y-tube bioassays. Further, the olfactory response varied in a dose-dependent manner, ranging from no or negative responses to positive responses. This has previously been observed for HIPVs in braconid parasitoids.[18,26] Interestingly, styrene at a dose of 1 µg and benzaldehyde at 10 ng or 50 ng doses elicited a similar or even stronger preference in *A. colemani* than the cell-free cultivation medium of the bacteria. Similar findings have been reported for *Psyttalia* parasitoids, which were more or equally attracted to individual

synthetic *Ceratitis capitata*-induced fruit volatiles than to the odour of infested fruits themselves.[27] The higher sensitivity to benzaldehyde compared to styrene suggests it is a more ecologically relevant compound. Benzaldehyde is widely emitted by plants, and flowers in particular,[28] which may explain the high preference observed for this compound. Bacterial VOC blends are generally composed of typical fermentation products like methylated, low molecular weight alcohols and corresponding aldehydes and organic acids.[13,29] However, some compounds emitted by microbes are also commonly reported as plant volatiles or insect pheromones.[20] It is therefore possible that the parasitoids were attracted to benzaldehyde in the context of it being a floral volatile rather than coincidental production by bacteria as side-products of their primary and secondary metabolism.[30] However, recent findings have shown that many mVOCs are not simply side-products, but display certain biological activities, e.g. to aid microbial dispersal by insect vectors.[31] Further research is needed to unravel the ecological role of volatiles produced by bacteria.

Although no behavioural responses were observed for a number of EAG-active compounds, or specific concentrations of EAG-active compounds, it has to be noted that these compounds or concentrations may still exert an effect within a blend of volatiles. Previous research has demonstrated that insects that are attracted to a specific blend can be unaffected by or even repelled by the individual compounds of that blend.[15,32] In addition, it has to be considered that the parasitoids used in this study had not been previously exposed to the mVOCs tested. It is possible that compounds that did not elicit an innate response in our studies, may elicit a conditioned response as a result of

associative learning, when parasitoids experience these volatiles in association with feeding or oviposition events.[33]

Parasitoids were not only attracted by individual compounds, but also by mixtures of synthetic mVOCs. Specifically, a strong positive response was observed for a synthetic mixture of styrene and benzaldehyde when combined at a ratio of 100/1. Moreover, at a dose of 1 µg styrene and 1 ng benzaldehyde, parasitoid preference for the blend was considerably higher than for the individual compounds. At these amounts, the blend attracted 75% of the responding individuals (PI = 0.50), which is comparable to levels of positive response obtained with synthetic plant volatiles and volatiles from aphid-infested plants in *Aphidius* species.[26,34] Additionally, *A. colemani* response to our two-component blend was also stronger than to a bacterial cultivation medium, which suggests that the latter may contain compounds that have a masking or inhibitory effect on the key compounds responsible for the attractiveness of the blend.[35,36] These findings could also be interpreted as an indication that parasitoids had an innate response to simple blends with typical floral volatiles like benzaldehyde.[28] Several examples exist where the response to a blend containing a select number of synthetic compounds exceeded the response to the natural blend.[35,37]

By contrast, the synthetic mixture of EAG-active compounds mimicking the behaviourally active cultivation medium of bacterial strain ST18.16/133 did not induce a positive behavioural response in *A. colemani*, despite the presence of styrene and benzaldehyde in the mixture. However, the amounts and proportions of styrene and benzaldehyde in this blend were different compared to the active two-compound blend. It is also possible that one or more key compounds that were present in the bacterial cultivation medium were absent in the synthetic mixture of five compounds. Previous

research has shown that removing key compounds from an attractive volatile blend can disrupt attraction to that blend.[38] It is therefore possible that one or more of the unidentified EAG-active compounds in the bacterial volatile emissions have been essential in eliciting the attractive response in *A. colemani*. Additional research is required to identify these EAG-active compounds and assess their effects on parasitoid olfactory response, both individually and in mixtures.

In contrast to the laboratory assays, the cell-free cultivation medium of strain ST18.16/133 did not show significant attraction of *A. colemani* in the cage experiments. This confirms previous research showing that results from laboratory experiments cannot always be extrapolated to more realistic environments and over longer distances.[37,39] Under natural conditions, there are more complex background odours originating from diverse sources which can compete or interact with attractants, thereby reducing the signal-to-noise ratio and disturbing the insect's response.[37,40] In contrast, application of the two-component mixture of styrene and benzaldehyde resulted in significant attraction of the parasitoids to the treated plants. Parasitoid responsiveness to the synthetic blend was also significantly higher compared to the cell-free cultivation medium, further demonstrating the attractiveness of the two-compound blend. However, additional research is needed to establish whether the observed effects were directly caused by the applied blend of synthetic volatiles, or whether they were the result of an interaction between the applied compounds and the plants, inducing the production of attractive volatiles.[40]

5 CONCLUSIONS

In conclusion, this study demonstrated that mVOCs emitted by bacteria elicited behavioural and electrophysiological responses in *A. colemani* parasitoids. The olfactory response of *A. colemani* to synthetic blends based on bacterial volatile emissions was largely dependent on the dose and ratio of the different compounds. Moreover, synthetic volatile blends were able to attract *A. colemani* parasitoids under greenhouse conditions, while this was not the case for the more complex bacterial cell-free cultivation medium. This opens opportunities to construct simple synthetic blends to attract or retain natural enemies of pest species at the greenhouse or field scale. Future research is needed to assess whether attracting natural enemies with such compounds will also enhance biological control efficacy.

ACKNOWLEDGEMENTS

We would like to thank the Flemish Fund for Scientific Research (FWO; 1S15116316N) and Flanders Innovation & Entrepreneurship (VLAIO; HBC.2018/2022) for financial support. Further, we are grateful to Dries Belmans and Frank van Neerbos for help with the experiments.

CONFLICT OF INTEREST DECLARATION

All authors declare that no competing interests exist.

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Table 1. Compounds[a] identified by coupled GC-EAG, using female *Aphidius colemani* antennae, in volatile extracts collected from the cell-free cultivation medium of three bacterial strains that are attractive to *A. colemani* and the blank medium

				Neutral	Attractive		
EAG response[b]	**RT (min)[c]**	**RI[d]**	**Compound**	**Blank medium**	**ST18.16/133**	**ST18.16/043**	**ST18.16/150**
A1, D1	4.28	705	heptane	33.1			53.6
C1	4.57	727	unknown 1			15.4	
B1	4.72	738	unknown 2		1.7		
A2	4.77	741	2,4-dimethyl hexane	1.4			
A3	5.35	780	unknown 3	0.8			
B2	**5.66**	**798**	**butyl acetate**		**4.2**		
D2	6.17	837	ethyl cyclohexane				1.1
D3	6.58	868	cyclohexanone				18.9
B3, C2, D4	**6.92**	**890**	**styrene**		**1.2**	**0.8**	**1.3**
B4, C3, D5	**7.02**	**896**	**o-xylene**		**2.2**	**3.2**	**6.0**
C4	7.09	901	unknown 4			0.9	
B5	7.42	929	unknown 5		1.0		
B6, D6	**7.50**	**935**	**benzaldehyde**		**1.3**		**1.6**
B7, D7	7.75	956	unknown 6		4.6		11.3
D8	8.02	976	unknown 7				0.7
A4	10.30	1175	unknown 8	2.7			
B8	11.21	1263	unknown 9		1.1		
C5	11.91	1335	unknown 10			0.9	
B9, C6	**12.53**	**1399**	**1,3-diacetylbenzene**		**1.2**	**3.4**	
A5	12.95	1447	unknown 11	1.2			

[a]Peak areas of each compound that elicited a EAG-response are shown for each strain as determined by an HP-1 equipped GC. Compounds indicated in bold were selected for further experiments.
[b]Letter and number combinations refer to the different panels and marked EAG-active peaks in Fig. S2 (Supporting Information).
[c]Retention times of associated compounds as identified in the GC-EAG analyses.

[d]Retention indices (Kováts index) relative to retention times of C7-C22 n-alkanes on an HP-1 GC column.

FIGURE LEGENDS

Figure 1. Olfactory responses of adult *Aphidius colemani* females when given the choice between one of seven different doses ranging from 1 ng to 50 µg of five synthetic volatile compounds (i.e. butyl acetate, styrene, *o*-xylene, benzaldehyde and 1,3-diacetylbenzene) and a diethyl ether blank in a Y-tube olfactometer bioassay. Parasitoid response is expressed as Preference Index (PI), calculated by dividing the difference between the number of parasitoids choosing the synthetic volatiles and the parasitoids choosing the control by the total number of responding insects. In total, 60 individuals were tested (12 releases of 5 females; $n = 12$) for each dose. Non-responders were excluded from the statistical analysis. Olfactory response of *A. colemani* to the mVOCs of the bacterial strains ST18.16/133, ST18.16/043 and ST18.16/150 were included as a reference. Grey bars indicate non-significant olfactory responses ($P > 0.05$), green bars indicate significant attractive responses ($P \leq 0.05$) and red bars indicate significant repellent responses ($P \leq 0.05$) when compared to a theoretical 50:50 distribution within a choice test (Generalized Linear Mixed Model). ** $0.001 \leq P < 0.01$; * $0.01 \leq P \leq 0.05$; ns, non-significant. Overall parasitoid responsiveness was higher than 67%.

Figure 2. Olfactory responses of adult *Aphidius colemani* females when given the choice between one of five different doses of a synthetic volatile blend and a diethyl ether blank in a Y-tube olfactometer bioassay. Synthetic blends tested included (A) Blend 1, consisting of two compounds (benzaldehyde and styrene) and (B) Blend 2, consisting of five compounds (butyl acetate, *o*-xylene, benzaldehyde, styrene, and 1,3-diacetylbenzene). For Blend 1, dose 1× was composed of 1 µg styrene and 10 ng

benzaldehyde; for Blend 2, dose 1× consisted of 3.40 µg butyl acetate, 1.81 µg *o*-xylene, 1.07 µg benzaldehyde, 1.00 µg styrene, and 0.98 µg 1,3-diacetylbenzene. Blends were tested at a volume of 10 µl. The volatile composition of the synthetic blends tested is illustrated by the pie charts. Parasitoid response is expressed as Preference Index (PI), calculated by dividing the difference between the number of parasitoids choosing the volatile blend and the parasitoids choosing the control by the total number of responding insects. In total, 60 individuals were tested (12 releases of 5 females; $n = 12$) for each dose. Non-responders were excluded from the statistical analysis. Olfactory response of *A. colemani* to the mVOCs of the bacterial strains ST18.16/133, ST18.16/043 and ST18.16/150 is included as a reference. Grey bars indicate non-significant olfactory responses ($P > 0.05$), green bars indicate significant attractive responses ($P \leq 0.05$) and red bars indicate significant repellent responses ($P \leq 0.05$) when compared to a theoretical 50:50 distribution within a choice test (Generalized Linear Mixed Model). *** $P < 0.001$; * $0.01 \leq P \leq 0.05$; ns, non-significant. Overall parasitoid responsiveness was higher than 80%.

Figure 3. Responses of adult *Aphidius colemani* females under greenhouse conditions when given the choice between two sweet pepper plants treated with a volatile blend and two control plants. Experiments included application of (A) Blend 1 and diethyl ether as a control, and application of (B) the cell-free cultivation medium of ST18.16/133 and blank GYP25 medium as a control. The volatile composition of the blends tested is illustrated by the pie charts. Blend 1 was composed of 100 ng/µL styrene and 1 ng/µL benzaldehyde. Plants were sprayed with a vaporizer (20 puffs) by which on average 2.5

mL was deposited onto the leaves of each plant. Parasitoid response is expressed as the mean Preference Index (PI) (±SE) of eight replicates (n = 8). For each replicate, 60 individuals were released, and parasitoid response was evaluated 48h after insect release by counting the number of trapped wasps on transparent, odourless glue plates behind the plants. The green bar indicates an average significant attractive response ($P \leq 0.05$), while the grey bar indicates an average non-significant olfactory response ($P > 0.05$) when compared to a theoretical 50:50 distribution within a choice test (Generalized Linear Mixed Model). *** $P < 0.001$; ns, non-significant. Average responsiveness for Blend 1 was 33.0%, for the ST18.16/133 culture medium it was 19.2%.

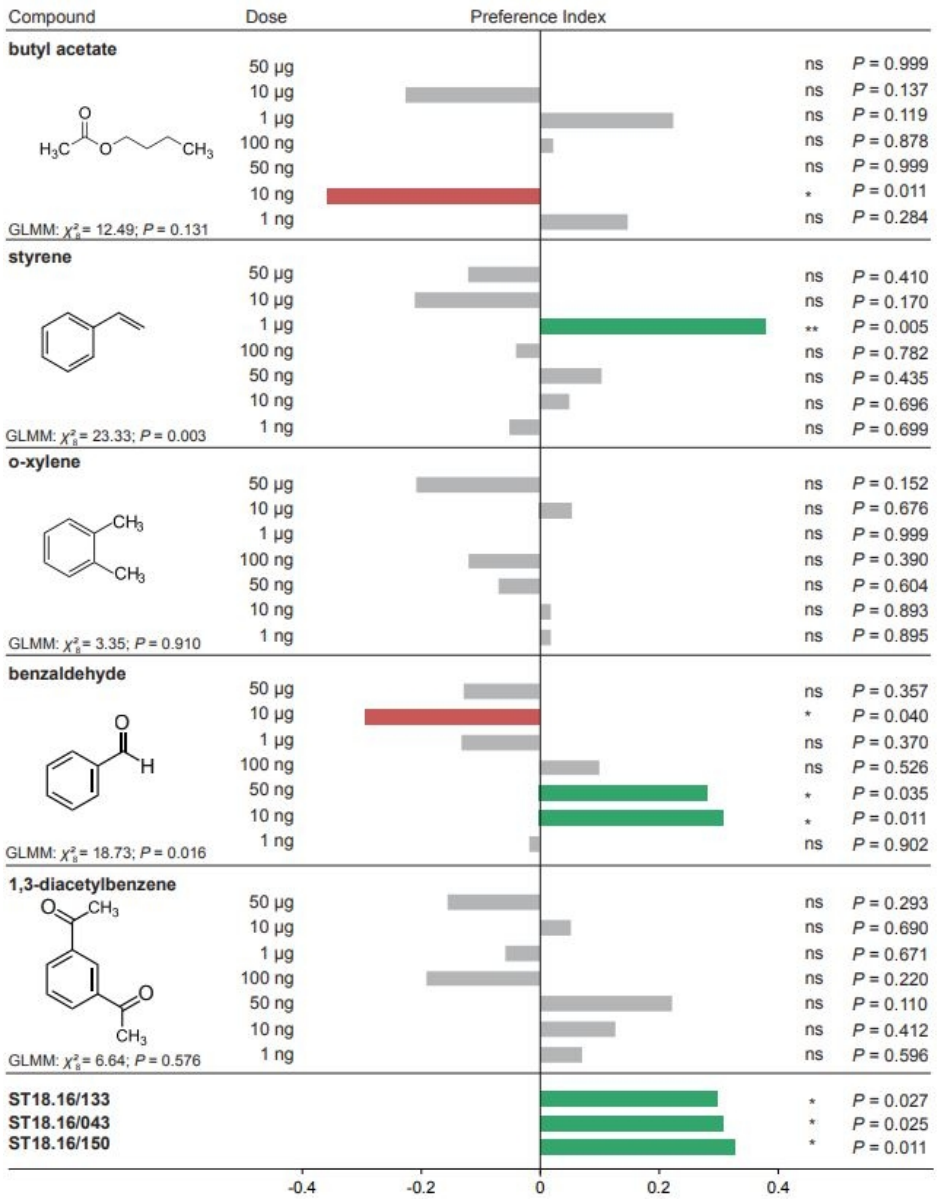

Figure 1. Olfactory responses of adult Aphidius colemani females when given the choice between one of seven different doses ranging from 1 ng to 50 µg of five synthetic volatile compounds (i.e. butyl acetate, styrene, o-xylene, benzaldehyde and 1,3-diacetylbenzene) and a diethyl ether blank in a Y-tube olfactometer bioassay. Parasitoid response is expressed as Preference Index (PI), calculated by dividing the difference between the number of parasitoids choosing the synthetic volatiles and the parasitoids choosing the control by the total number of responding insects. In total, 60 individuals were tested (12 releases of 5 females; n = 12) for each dose. Non-responders were excluded from the statistical analysis. Olfactory response of A. colemani to the mVOCs of the bacterial strains ST18.16/133, ST18.16/043 and ST18.16/150 were included as a reference. Grey bars indicate non-significant olfactory responses (P > 0.05), green bars indicate significant attractive responses (P ≤ 0.05) and red bars indicate significant repellent responses (P ≤ 0.05) when compared to a theoretical 50:50 distribution within a choice test (Generalized Linear Mixed Model). ** 0.001 ≤ P < 0.01; * 0.01 ≤ P ≤ 0.05; ns, non-significant. Overall parasitoid responsiveness was higher than 67%.

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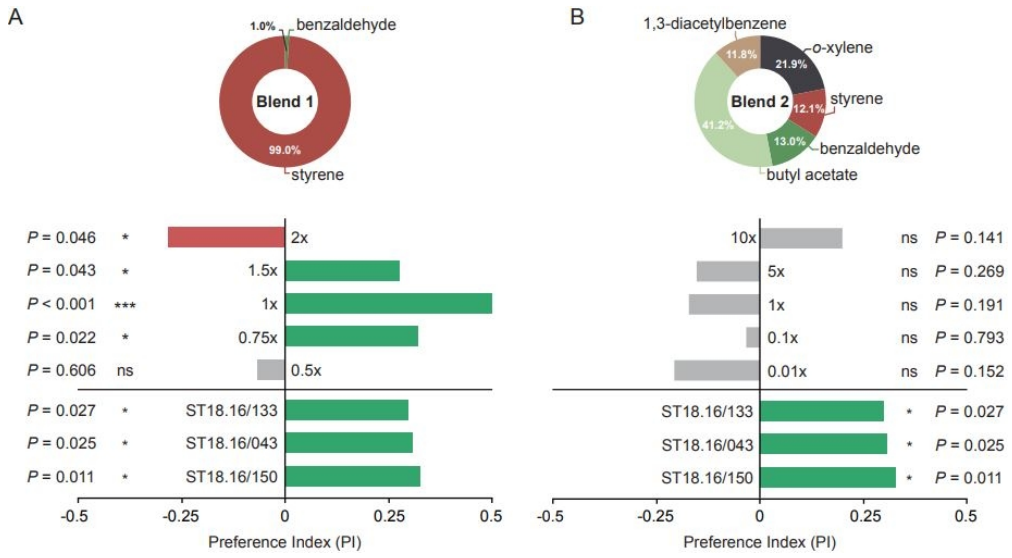

Figure 2. Olfactory responses of adult Aphidius colemani females when given the choice between one of five different doses of a synthetic volatile blend and a diethyl ether blank in a Y-tube olfactometer bioassay. Synthetic blends tested included (A) Blend 1, consisting of two compounds (benzaldehyde and styrene) and (B) Blend 2, consisting of five compounds (butyl acetate, o-xylene, benzaldehyde, styrene, and 1,3-diacetylbenzene). For Blend 1, dose 1× was composed of 1 µg styrene and 10 ng benzaldehyde; for Blend 2, dose 1× consisted of 3.40 µg butyl acetate, 1.81 µg o-xylene, 1.07 µg benzaldehyde, 1.00 µg styrene, and 0.98 µg 1,3-diacetylbenzene. Blends were tested at a volume of 10 µl. The volatile composition of the synthetic blends tested is illustrated by the pie charts. Parasitoid response is expressed as Preference Index (PI), calculated by dividing the difference between the number of parasitoids choosing the volatile blend and the parasitoids choosing the control by the total number of responding insects. In total, 60 individuals were tested (12 releases of 5 females; n = 12) for each dose. Non-responders were excluded from the statistical analysis. Olfactory response of A. colemani to the mVOCs of the bacterial strains ST18.16/133, ST18.16/043 and ST18.16/150 is included as a reference. Grey bars indicate non-significant olfactory responses ($P > 0.05$), green bars indicate significant attractive responses ($P \leq 0.05$) and red bars indicate significant repellent responses ($P \leq 0.05$) when compared to a theoretical 50:50 distribution within a choice test (Generalized Linear Mixed Model). *** $P < 0.001$; * $0.01 \leq P \leq 0.05$; ns, non-significant. Overall parasitoid responsiveness was higher than 80%.

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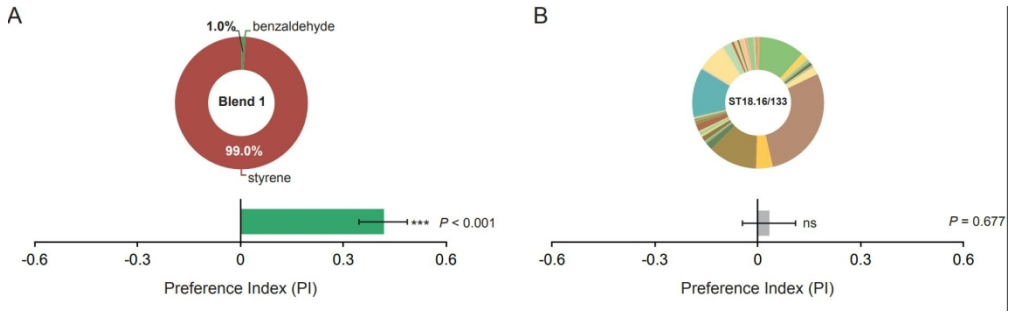

Figure 3. Responses of adult Aphidius colemani females under greenhouse conditions when given the choice between two sweet pepper plants treated with a volatile blend and two control plants. Experiments included application of (A) Blend 1 and diethyl ether as a control, and application of (B) the cell-free cultivation medium of ST18.16/133 and blank GYP25 medium as a control. The volatile composition of the blends tested is illustrated by the pie charts. Blend 1 was composed of 100 ng/µL styrene and 1 ng/µL benzaldehyde. Plants were sprayed with a vaporizer (20 puffs) by which on average 2.5 mL was deposited onto the leaves of each plant. Parasitoid response is expressed as the mean Preference Index (PI) (±SE) of eight replicates (n = 8). For each replicate, 60 individuals were released, and parasitoid response was evaluated 48h after insect release by counting the number of trapped wasps on transparent, odourless glue plates behind the plants. The green bar indicates an average significant attractive response ($P \leq 0.05$), while the grey bar indicates an average non-significant olfactory response ($P > 0.05$) when compared to a theoretical 50:50 distribution within a choice test (Generalized Linear Mixed Model). *** $P < 0.001$; ns, non-significant. Average responsiveness for Blend 1 was 33.0%, for the ST18.16/133 culture medium it was 19.2%.

502x153mm (96 x 96 DPI)

Identification and application of bacterial volatiles to attract a generalist aphid parasitoid: from laboratory to greenhouse assays

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Table S1. Bacterial isolates used in this study

Isolate identifier (GenBank Accession N°[a])	Olfactory response of *A. colemani*[b]	Phylogenetic affiliation based on *rpoB* sequence similarity[c]				Source of isolation
		Phylum	Family	Closest match in GenBank	Identity (%)[d]	
ST18.16/133 (MN232849)	Attractive	Firmicutes	Bacillaceae	*Bacillus pumilus*	100.00	*Aphidius ervi*
ST18.16/043 (MN232830)	Attractive	Firmicutes	Bacillaceae	*Bacillus* sp.	80.24	*Dendrocerus aphidum*
ST18.16/150 (MN232831)	Attractive	Firmicutes	Bacillaceae	*Bacillus circulans*	92.20	*Macrosiphum euphorbiae*

[a]Accession number of *rpoB* sequences deposited in GenBank.
[b]Olfactory response of *A. colemani* parasitoids to the mVOCs produced by the strains in a Y-tube olfactometer bioassay.
[c]Based on a Blast search against GenBank (July 2019).
[d]Fragment length was 1102 bp.

Table S2. Composition (µg) of the five different doses of Blend 1[a] tested in the Y-tube olfactometer bioassay

Tested dose	Composition		
	Styrene	Benzaldehyde	Total
2x	2.000	0.020	2.020
1.5x	1.500	0.015	1.515
1x[b]	1.000	0.010	1.010
0.75x	0.750	0.008	0.758
0.5x	0.500	0.005	0.505
Relative amount	99.0%	1.0%	100%

[a]Blend 1 consisted of two volatile compounds which elicited a significant EAG-response in the GC-EAG analysis.
[b]The 1x dose contains the sum of doses of styrene and benzaldehyde at which they were most attractive individually in the Y-tube olfactometer bioassay. For all doses tested relative composition of the compounds was kept constant, i.e. 1.0% benzaldehyde and 99.0% styrene.

Table S3. Composition (µg) of the five different doses of Blend 2[a] tested in the Y-tube olfactometer bioassay

Tested dose	Composition[b]					
	***o*-Xylene**	**Styrene**	**Benzaldehyde**	**Butyl acetate**	**1,3-Diacetylbenzene**	**Total**
10x	18.10	10.00	10.74	34.05	9.75	82.64
5x	9.05	5.00	5.37	17.02	4.88	41.32
1x	1.81	1.00	1.07	3.40	0.98	8.26
0.1x	0.18	0.10	0.11	0.34	0.10	0.83
0.01x	0.018	0.010	0.011	0.034	0.010	0.083
Relative amount	21.9%	12.1%	13.0%	41.2%	11.8%	100%

[a]Blend 2 consisted of five volatile compounds which elicited a significant EAG-response in the GC-EAG analysis.

[b]The relative amount of each of the five compounds of Blend 2 resembled the headspace composition of these compounds in the ST18.16/133 cell-free medium as detected by the GC.

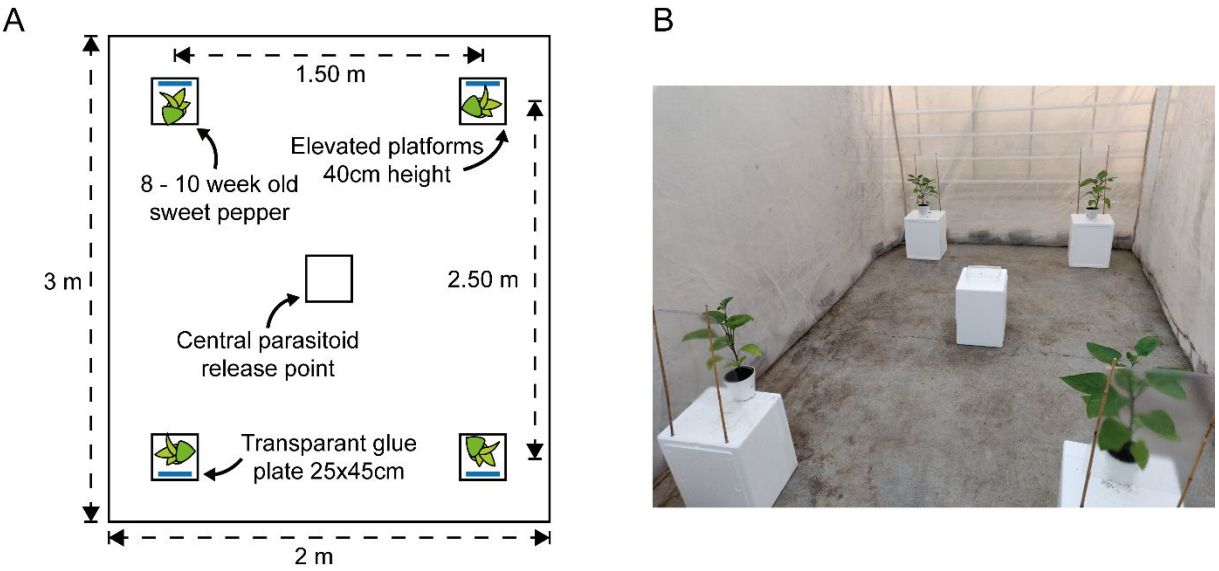

Figure S1. Experimental setup of the cage experiments under greenhouse conditions. (A) Schematic diagram of the experimental setup used in the cage experiments, which were performed in a 2x3x2 m cage fitted with a fine mesh. Nine-week-old pepper plants were placed on elevated platforms in each corner of the cage. Plants were treated by spraying them with a synthetic mVOC solution or bacterial culture medium (Treatment; 2 plants), and a control solution (Control; 2 plants). Control and treatment plants were placed diagonally relative to each other inside the cage. Directly behind each plant a transparent glue plate was placed to catch visiting parasitoids. In each replicate of each experiment, 60 *Aphidius colemani* females were released from a central release point on an elevated platform. (B) Photograph of the experimental setup.

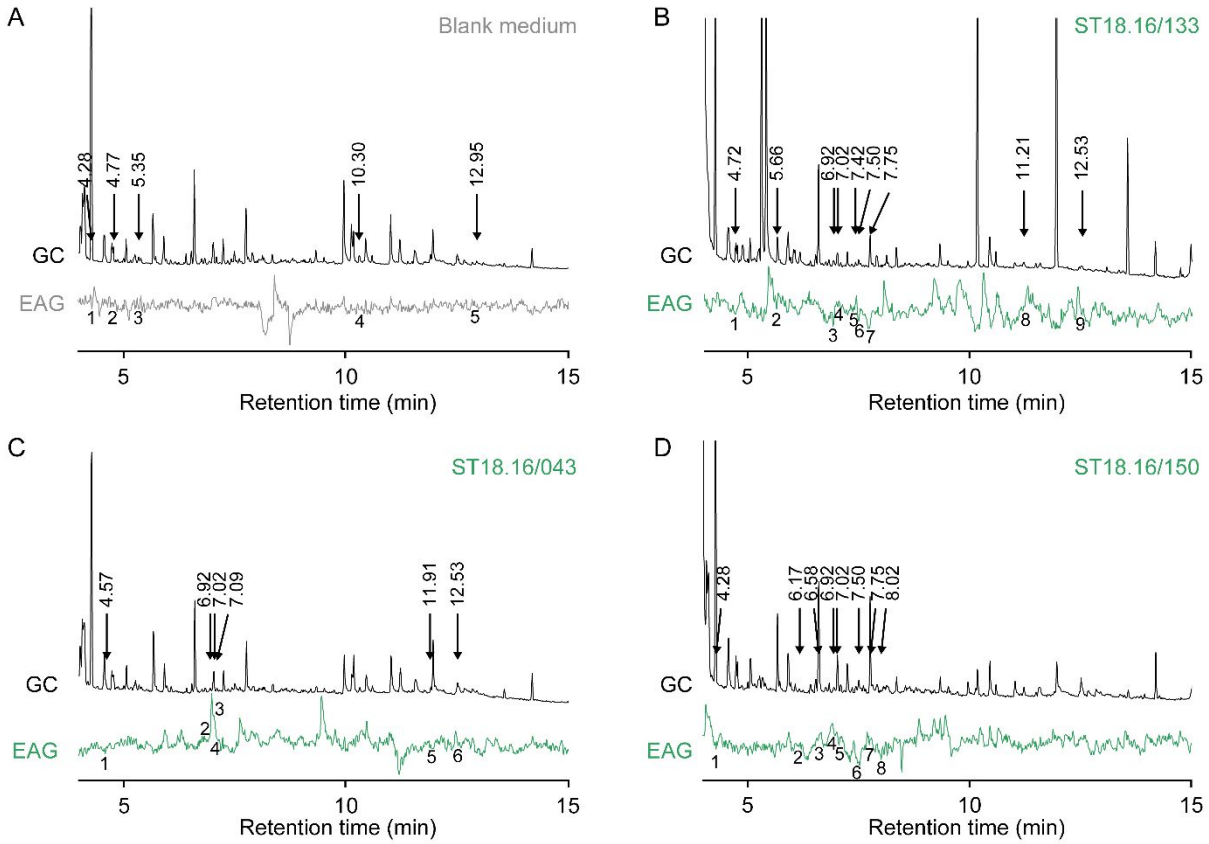

Figure S2. Coupled GC-EAG with female *Aphidius colemani* antennae on volatiles entrained from the cell-free cultivation medium of the three tested bacterial strains and the blank medium. Upper trace: result of the GC analysis; lower trace: EAG-response. Only EAG-active peaks that are found in all three replicates tested are marked with a number and associated retention times (min) on the GC chromatogram (for more details, see Table 1). The colour of the electroantennogram indicates the effect of the volatile blend of the tested strain on the olfactory response of *Aphidius colemani*, i.e. grey = neutral and green = attractive in a Y-tube olfactometer bioassay.