Journal of Chemical Ecology
https://doi.org/10.1007/s10886-020-01147-9

Bacteria Affect Plant-Mite Interactions Via Altered Scent Emissions

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Received: 13 August 2019 / Revised: 18 December 2019 / Accepted: 8 January 2020

Abstract
Epiphytic bacteria have been shown to affect the composition of volatiles released by plants and as a consequence the behavior of other organisms towards the plant, such as herbivores and/or pathogens. In this study, we explored the effects of inoculation with three bacterial strains, namely *Pseudomonas syringae*, *Pantoea ananatis,* and *Pseudomonas putida*, on the composition of leaf volatile organic compounds (VOCs) emitted by bean plants (*Phaseolus vulgaris* L.). In addition, we examined responses of the two-spotted spider mite (*Tetranychus urticae*) to VOCs by measuring leaf damage and oviposition of female adults after bacterial inoculation. Colonized bean plants emitted different VOCs depending on the bacterial inoculum. The quantities of volatiles 1-undecanol and (*Z*)-3-hexen-1-ol significantly increased after *P. syringae* inoculation, while methyl salicylate and anisole increased in response to *P. ananatis*. *T. urticae* females preferred control plants over plants inoculated with *P. syringae* or *P. putida* in olfactometer assays, while no particular preference was recorded in the presence *of P. ananatis*. Furthermore, leaf damage caused by spider mites was 3-fold lower in plants inoculated with *P. syringae* than in control plants and plants inoculated with *P. ananatis*. Subsequently, the number of eggs laid on leaves inoculated with *P. syringae* was significantly lower than on those inoculated with *P. ananatis* or on the control ones. Moreover, a significantly higher number of spider mites selected methyl salicylate odor source over 1-undecanol, in a two-choice bioassay. The results demonstrate the bacterial involvement in plant-arthropod interactions and suggest further investigation on the potential use of bacteria as biocontrol agents in agriculture.

Keywords Spider mites · Plant -bacteria- mite interactions · Epiphytic bacteria · Bacteria induced leaf volatiles · *Pseudomonas* · *Pantoea*

Introduction

Plants interact with their biotic environment in versatile ways by attracting, deterring or even killing interaction partners, i.e. bacteria, fungi, and arthropods such as mites. Many of these interactions between plants and microorganisms, and also between plants and herbivores occur synchronously or sequentially and may affect each other via altered scent emissions. Thus, the plants' physiology and phenotype may be a result of previous interactions with significant effects for plant diseases and/or tissue damage by herbivores (Humphrey et al. 2014).

In this interplay, plant emitted volatile organic compounds (VOCs) are of major importance as plant communication tools (Junker et al. 2017). VOCs are compounds with low molecular weight and high vapor pressure that serve a multitude of biological functions (Dudareva et al. 2013; Raza et al. 2016). Apart from their important and well-known function in attracting pollinators and other beneficial insects, they can serve as means for "plant–plant" and "plant–insect" communication (Hijad et al. 2013). There is strong evidence showing that these VOC emissions also play a relevant role in structuring plant–microbe interactions on aboveground plant surfaces (Farré-Armengol et al. 2016; Junker and Tholl 2013). Furthermore, bacteria and fungi inhabiting plant surfaces contribute to plant VOCs in different ways, presumably by inducing the synthesis of "new" compounds or catabolizing others emitted by the plants' metabolism, by producing volatiles as part of their own metabolism, or/and by utilizing plant volatiles as carbon sources (Helletsgruber et al. 2017; Peñuelas et al. 2014).

Metabolites from different compound classes, such as terpenoids, phenylpropanoids, and products of lipoxygenase metabolism have been identified in VOCs emitted by plants

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Published online: 20 January 2020

during plant-microbe interactions and disease establishment. Release of volatiles that have a strong antibacterial activity, such as (*E*)-2-hexanal and (*Z*)-3-hexenol derived from the lipoxygenase pathway, have been found in bean emissions in response to pathogen attack, suggesting their importance in modulating plant defense mechanisms (Croft et al. 1993; Ongena et al. 2004). These studies and many others exploring plant volatile release in response to microorganisms are based on experiments quantifying pathogen colonization of the aboveground plant parts and disease progression (Hammerbacher and Coutinho 2019 and references herein). Much less is known about the contribution of common phyllospheric bacterial epiphytes on leaf VOCs (Junker et al. 2011a) and subsequently on other organisms reaching the phyllosphere, such as herbivores.

To address this knowledge gap, in this study we explored the possible impact of leaf associated bacteria on leaf volatile emissions, and also the host selection behavior of a common, polyphagous herbivore, the spider mite, *Tetranychus urticae*. In particular, we examined whether bacterial strains with different life histories could affect both leaf volatile emissions and spider mite feeding and oviposition preferences. For this purpose, three bacterial strains were chosen, i.e. (i) a common epiphytic *Pseudomonas syringae* pv *syringae* strain (representatives of this species are potentially phytopathogenic), (ii) a nonpathogenic *Pantoea ananatis* (former *Erwinia herbicola*) strain; and (iii) a saprophytic *Pseudomonas putida* strain.

The selection of the particular bacterial strains was based on two criteria, life strategies and spatial distribution. Strains of all three bacteria are widely distributed on different ecosystems, and usually found on aerial plant tissues. Among them, *P. syringae* and *P. ananatis* are mainly plant epiphytes with different characteristics and *P. putida* is a saprophytic soil bacterium that can also be found in the phyllosphere. More specifically, *P. syringae* is a common leaf associated bacterium that has been isolated from over 180 host plants (Hirano and Upper 1990; Ravindran et al. 2015). More than 50 pathovars of this bacterium, and nine distinct genomospecies have been elucidated (Gardan et al. 1999). *P. syringae* pv *syringae* belongs to genomospecies 1, in which common epiphytes that maintain resident populations in the absence of plant disease are included (Hirano and Upper 1990; Ravindran et al. 2015). Their ability to tolerate UV radiation and grow high epiphytic populations has been associated with UV-inducible plasmid-borne *rulAB* genes, which confer mutagenic DNA repair (Lindow and Brandl 2003). Moreover *P. syringae* strains have been found to elicit emission of leaf volatiles in different plants such as tobacco (Huang et al. 2003) and *Arabidopsis thaliana* and trigger plant defenses (Attaran et al. 2008). Strains of the second selected bacterium, *Pantoea sp.*, show an incredible ability of adaptation to a broad range of hosts, including leaf surfaces of various plants and different environmental conditions (Dutkiewicz et al. 2016).

Of major importance for epiphytic survival is their ability to produce carotenoids which allow them to persist UV radiation and allows successful epiphytic growth (Walterson and Stavrinides 2015). Strains of *P. agglomerans* and *P. ananatis* have been shown to promote plant growth and defense mostly against phytopathogenic microorganisms (Dutkiewicz et al. 2016; Walterson and Stavrinides 2015) Thus, antimicrobial activity of specific strains has been resulted in developing commercial biocontrol agents to help control fireblight of apple and pear trees (Walterson and Stavrinides 2015). Species from the genus *Pantoea* also commonly occur as symbionts of many species of insects such as thrips (de Vries et al. 2001). On the other side, *Pseudomonas putida* is a common saprophyte bacterium in well-aerated soil and water systems and can reach leaf surfaces through splashing or aerosols (Karamanoli et al. 2012). Apart from its ability to recycle different organic compounds including those with aromatic rings, strains of this bacterium are able to produce IAA (Patten and Glick 2002) and are considered as plant growth promoting rhizobacteria (PGPR). We include this bacterium in our study, because it shows a totally different life style from the other two, and we were interested to compare its impact in our system with the two selected bacteria.

We chose to explore the effects of bacterial colonizers with different life histories on plant interactions with spider mites because these herbivores feed on over 1100 plant species and cause serious economic losses in agriculture around the globe. Apart from their wide host range, rapid life cycle and easy maintenance under laboratory conditions, together with recently developed tools for their genomic elucidation makes spider mites suitable candidates for plant-herbivore interactions studies (Rioja et al. 2017). Spider mite host selection has been shown to be mediated by plant volatiles. In particular, terpenoids accumulating in trichomes of wild tomato that are constitutively synthesized in the glandular trichomes of all green plant parts, contribute to reduce mite infestation to these plants (Bleeker et al. 2012). From the other side, mite attacks may alter leaf volatile release, as a plant defense response. For instance, cultivated tomato volatile emissions after spider mite attacks consist of increased quantities of monoterpenes, sesquiterpenes, aromatics, aldehydes, ketones, alcohols, and esters (Kant et al. 2004 and references herein); and also phenolic methyl salicylate and the homoterpene 4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT, Dicke et al. 1998). This induced release of leaf VOCs may enhance direct plant defenses and/or attract mite's natural enemies (Kant et al. 2004; Shimoda et al. 2005). Earlier studies on spider mite interactions with beans (*Phaseolus lunatus*) showed that herbivore-induced volatiles activated multifunctional signaling cascades involving ethylene and jasmonic acid signaling (Arimura et al. 2002). Although, there is evidence that microbes may decisively influence these interactions, their role in modulation of plant defenses and VOC release in response to spider mite attack has been largely untested (Schausberger

2018). Our study contributes to the understanding of how bacteria influence plant VOC release and subsequently spider mite behavior and provides a basis for future studies on bacterial involvement in plant-arthropod interactions and the potential application of bacteria as biocontrol agents.

Methods and Materials

Microorganisms, Spider Mites, and Plants

Bacterial Strains and Media Three different strains were used: *Pseudomonas syringae* pv *syringae* strain B728a isolated from bean leaves and grown on King's media B (KB) (Loper and Lindow 1987), *Pantoea ananatis* strain 26SR6, isolated from corn and grown on nutrient agar with 2.5% glycerol (NAG) (Lindow 1982), and one strain isolated from oregano leaves in the laboratory of Agricultural Chemistry of Aristotle University of Thessaloniki and characterized as *Pseudomonas putida* with accession number EU 275365 (Karamanoli et al. 2012). All strains were kept in glycerol stocks under deep freeze (-80 °C) and recultured in agar LB medium (Sigma-Aldrich) plates to obtain viable cultures just prior to experimentation. Fresh overnight bacterial cultures of the tested strains were prepared in phosphate-buffered saline (PBS) medium (18 h of incubation at 22–24 °C) to be used in the experiments. Among the three bacterial strains, *P. syringae* and *P. ananatis* were used in all bioassays in which inoculated plants were employed, while *P. putida* was used only in the initial bioassays in which leaf scent compounds from colonized leaves were analyzed, and subsequently mite attractiveness was recorded towards the same colonized bean leaves.

Spider Mites The colony of *Tetranychus urticae* Koch (Tetranychidae) was established with individuals collected from bean plants in the area of Thessaloniki, northern Greece and has been maintained in the laboratory of Applied Zoology and Parasitology, Aristotle University of Thessaloniki, under control conditions (26 ± 1 °C and 16 day cycle) on detached bean leaves maintained on wet cotton-wool in plastic cups with water, as described earlier (Koveos et al. 1995). Adult females used in each bioassay were 10–15 days old, and they were starved for 24 h before experimentation.

Plant Material Common bean *Phaseolus vulgaris* (Fabaceae) was used as a model plant. Individual bean plants, were grown in plastic pots (10 cm in diameter) containing a sand–peat mixture in a greenhouse under standard conditions (22–25 °C during the 16 h - light and at 15–18 °C the 8 h - dark regime). When the plants reached the stage of second fully developed leaf, five plants per treatment were randomly assigned and used for bacterial inoculation, volatile collection and/or spider mites establishment.

Plant Inoculation We inoculated bean plants by spraying leaves to run off (approximately 20 mL), with bacterial suspensions prepared in PBS medium using a hand sprayer with 200 mL of capacity. Bacterial suspensions in PBS of about 10^6 CFU ml^{-1} were prepared as described above. Control plants were sprayed to run off with 20 mL of sterile PBS solution. All plants were then placed under 100% RH for 24 h and subsequently they were used for VOC collection and mite behavioral assays. Bacterial establishment on leaf surfaces was evaluated by serial dilution plating also 24 h after inoculation (Karamanoli et al. 2012).

Volatile Collection Leaf scent samples from inoculated and control bean plants were sampled with a static headspace method using PDMS tubes (Rotilabo-Silikonschlauch inner diameter 1,0 mm outer diameter 1,8 mm Carl Roth GmbH+ Co. KG, Product-Nr. 9555.1). One fully expanded bean leaf, composed from three leaflets, was enclosed within a 10 cm × 15 cm polyester oven bag (Toppits) without detaching it from the plant. We minimized plant handling during leaf enclosure into the bag for VOC sampling to avoid causing any stress on the plants. A PDMS tube was placed with forceps inside the bag, near the leaves but not touching them. The tube remained inside the bag for 3 h to collect the volatiles emitted. After the samplings, PDMS tubes containing the VOC samples were immediately stored in vials at −5 °C. VOC samplings were conducted 24 and 48 h after inoculation. PDMS tubes were stored for periods shorter than one month before they were desorbed and analyzed by gas chromatography-mass spectrometry (GC-MS).

GC-MS Analyses and VOC Quantification Scent samples were analyzed using an automatic thermal desorption system (TD-20; Shimadzu, Tokyo, Japan) coupled with a GC–MS (modelQP2010 Ultra EI; Shimadzu). The GC-MS was equipped with a ZB-5 fused silica column (5% phenyl polysiloxane; 60 m long, inner diameter 0.25 mm, film thickness, 0.25 μm, Phenomenex, Aschaffenburg, Germany) and the column flow (carrier gas: helium) was set to 1.5 ml/min. The GC oven temperature started at 40 °C (split ratio 1:1), then increased by 6 °C per minute to 225 °C and held constant for 1 min. The MS interface worked at 225 °C. Mass spectra were taken at 70 eV (in EI mode) from m/z 30 to 350. The GC/MS data were processed using the GCMSolution package (Version 2.72, Shimadzu Corporation, Kyoto, Japan). For identification of the compounds, masss spectral libraries (ADAMS, ESSENTIALOILS-23P, FFNSC 2, W9 N11) were used and comparison of their mass spectra and retention times with authentic standards and published literature. Kovats indices were generated using n-alkane mixture (Larue et al.

2015). The compounds found in the leaf samples were compared to those found in the blanks (empty oven bags) to determine which compounds were emitted by leaves. For quantitative analysis of VOCs, calibration curves were done by injecting known amounts of the available authentic standards into the GC/MS system, and calculating the mean ratios between the injected amounts and the resulting peak areas for each compound. The dry weight (DW) of leaves whose emissions were sampled were measured and emissions were then standardized for leaf DW (ng g^{-1}DW of leaf)

Behavioral Bioassays

Mite Response towards Colonized Plant Volatiles To investigate whether spider-mite behavior was affected by leaves colonized by different bacteria, we tested mite preference towards colonized and control leaves. In particular, after collecting volatile samples, the inoculated plants were used for evaluating spider mites' responses to leaf volatiles in a mobile olfactometer equipped with a four field arena that allowed behavioral assays as described earlier (Junker et al. 2011b). Briefly, the mites were allowed to choose between the scents released from colonized leaves connected to two opposed fields and from non-colonized (control) leaves in the remaining two fields of the arena. High air pressure (100 ml min^{-1}) was filtered with charcoal and distilled water and then directed over a Teflon tube into an odorless PET oven bag in which leaves from colonized or control plants were enclosed. Afterwards, the airstream was transferred via Teflon tube into one of the four arms, and an adult female was gently placed in the central part of the arena. After 60, 90, 120, 150, 180, 210 and 240 s, the presence of mites for either of the fields was scored, and the final preference was recorded. In total, 60 adult females per treatment were tested.

Mite Response Towards Individual Volatile Compounds To investigate whether mite behavior was affected by volatile constituents of bean leaf scent identified by GC-MS analysis, we tested mite preference towards individual volatile compounds. Solutions of 1 mM of all tested volatiles, which included: anisole, ocimene, 3-hexen-1-ol, 3-hexenyl acetate, 1-undecanol, methyl salicylate, 1-octen-3-ol (all purchased from Sigma-Aldrich) were prepared by diluted in ethanol (99%) and further diluted in tap water. Ethanol was always less than 1% in the final solution. We also prepared mixture of these volatiles in order to test for possible synergistic or antagonistic effects. In particular we prepared 5 mixtures of volatiles in different concentrations: mixture 1 = methyl salicylate, 1-undecanol, 3- hexanyl acetate (1:1:1); mixture 2 = methyl salicylate, 1-undecanol, 3-hexanyl acetate (1:1:1); mixture 3 = anisole, 1-undecanol, 3-hexanyl acetate (1:2:1); mixture 4 = ocimene, methyl salicylate, 1- octen-3-ol (1:2:1); and mixture 5 = ocimene, 1-undecanol, 1- octen-3-ol (1:1:1). Aqueous solutions of 1% ethanol were used as control odor source. Spider mites' responses to individual volatiles and their mixtures were examined in a Y-tube olfactometer as described by Koveos et al. (1995). The olfactometer consisted of a Y-shaped, 3 cm diameter, glass tube, with a Y-shaped steel wire positioned to lead the spider mites from the trunk towards either of the two arms where the odor source or neutral air was connected. Ambient air was filtered with charcoal and distilled water and then directed to the tube via the odour source to the Y-tube arms. Air flow was maintained at 0.5 m s^{-1} at the end of each tube, which corresponded to 1.16 m s^{-1} at the end of the trunk. Spider mite adult females were individually introduced at the starting point (the trunk of the Y-tube) on the steel wire and their choice for one of the two arms was recorded for 240 s. Individuals that did not select either arm within this period were excluded of the statistical analysis. We cleaned the arms with ethanol after each trial and switched the arm containing the sample odor with that containing the control odor in the olfactometer every three trials. In total, 80 mites were used. Further, based on the results with Y tube olfactometer, we selected two volatile substances which evoked the most promising effects i.e. 1-undecanol and methyl salicylate and tested the responses of mites to them using a trapezoid-shaped bridge test as described by Wei et al. (2014). In brief, a trapezoid – shape bridge cut from a transparent acetate sheet and folded to a trapezoid shape (length long side: 3 cm, pillar: 1 cm, width: 0.5 cm, thickness: 1 mm; Wei et al. 2014) was positioned such that it connected with two bean leaf sections treated with either volatile compound laying on a wet cotton-wool disk in an open Petri dish. A female adult was placed at the middle of the bridge, and allowed to walk to either side, in order to make a choice by mounting either leaf disk (2 cm in diameter) cut from an intact bean plant just before the experiment. Once it entered one of the leaf sections, its choice was recorded as "first choice" the Petri dish was covered and placed in the growth chamber. After 24 h of incubation the number of eggs deposited on each leaf disk were recorded. In total, 60 mites were used in each odor-source (Wei et al. 2014).

Evaluation of Leaf Damage Caused by Mite Infestation Bean plants, colonized by either *P. syringae* or *P. ananatis* were artificially infested by spider mites by *transferring* 10 adult females from the stock colony on three apical leaves per plant. Subsequently, the infested plants were maintained in an incubator at 20 ± 4 °C, 55–65% RH, 16:8 LD) for twelve days. The percentage of damaged leaf area by the mites, was estimated using Progress Gryphax computer program (JENOPTIK Optical Systems GmbH, Germany) under stereoscope, at the end of experimental period.

Egg Production of Adult Females Leaf disks (2 cm diameter) from inoculated (either with *P. syringae* or *P. ananatis*) and

control leaves were gently removed, and placed on wet cotton wool inside small falcon tubes. On each leaf disk a newly emerged adult female from the stock colony was transferred. Every two days, the number of eggs laid on each leaf disk was scored and removed. The tubes were kept in an incubator at 20 ± 2 °C, 55–65% RH and a photoperiod of LD 16:8, till the mites were dead (approximately eighteen days). For each treatment 40 replicates (leaf disks with an adult female) were used.

Statistical Analyses To test for differences in scent emission of bean leaves inoculated with bacterial strains or control leaves, we performed a Bray-Curtis-distance-based redundancy analysis (R package *vegan*) with proportional emission rates of VOCs as dependent variables and treatment (control or inoculated with one of the three bacterial strains) and time after inoculation as explanatory variables. To analyze mite preference behavior towards different volatile compounds in the behavioral bioassays χ^2 tests were used. The percentages of leaf damage after mite infestation of inoculated leaves by bacteria and control bean plants were compared by *analysis* of variance (ANOVA) following Tukey's (HSD) test. Differences in the mean number of eggs laid in different treatment (*P. syringae or P. ananatis* bacterial suspensions) and control leaf disks at each time point, were assesses by a paired two-tailed *t*-test. Prior to ANOVA, data was checked for normality and homogeneity of variances. No violations from the assumptions were detected.

Results

Leaf Volatile Compounds VOC emissions from bean leaves were affected after inoculation with bacteria. The composition of VOCs differed between the control leaves and leaves inoculated with a bacterial strain (distance-based redundancy analysis: treatment: $F_{3,25} = 2.17$, $P = 0.014$, Fig. 1). The emissions of six compounds i.e. (*Z*)-3-hexene-1-ol, anisole, 1-octen-3-ol, 1-undecanol, 4,8-dimethyl-1,3,7 nonatriene, methyl salicylate, significantly differed between inoculated by bacteria and control leaves (ANOVA: $F_{3,51} > 3.23$, $P < 0.029$; Tab. 1). Sampling time of volatiles, i.e. 24 or 48 h after inoculation affected only two compounds (4,8-dimethyl-1,3,7 nonatriene, methyl salicylate; $F_{1,51} > 4.95$, $P < 0.031$; Tab. 1). Additionally, the interaction between treatment and time affected two compounds (anisole, methyl salicylate; $F_{3,51} > 3.27$, $P < 0.029$; Tab. 1). Moreover, 1-undecanol and (*Z*)-3-hexen-1-ol increased only in response to *P. syringae* inoculation (both 24 and 48 h after inoculation), while only (*Z*)-3- hexen-1-ol increased in response to *P. ananatis* (only 48 h after inoculation). Overall, 1-undecanol was detected only in leaves inoculated by *P. syringae*, while methyl salicylate increased significantly only in response to *P. ananatis* inoculation.

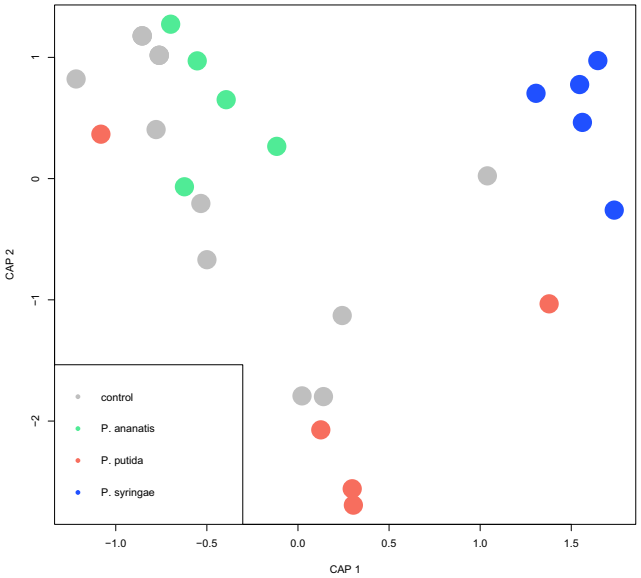

Fig. 1 Differences in volatile compound composition of control bean leaves (grey bullets), and leaves inoculated with *P. syringae* (blue bullets), *P. ananatis* (green bullets), and *P. putida*(red bullets). Ordination plots show similarity of leaf scent samples after constrained analysis of principal coordinates, axes represent the coordinates of each sample after non-metric multidimensional scaling. Samples located closer to each other are also more similar in leaf scent composition

Behavioral Bioassays In a four field arena olfactometer, in which spider mites were allowed to choose between leaves inoculated with bacteria and control leaves, significantly more female mites were attracted by control leaves, at least when leaves were inoculated by *P. syringae* or *P. putida* (Fig.2 χ^2 - test, $P = 0.013$ and $P = 0.004$, respectively). Additionally, when leaves were colonized by *P. ananatis* mites did not exhibit a particular preference between colonized and non - colonized leaves (Fig. 2, χ^2 - test, $P = 0.052$).

Responses to individual compounds were more variable and most compounds did not evoke a preference or repellence to female mites in the Y-tubed olfactometer bioassay (Fig. 3). However, mites showed a preference for methyl salicylate over the control (Fig. 3, χ^2 - test, $P = 0.018$), while they were repelled by 1–undecanol (Fig. 3, χ^2-test, $P = 0.017$). Moreover, when five different mixtures of volatiles were tested, mites exhibited preference only to mixture 4 (ocimene: methyl salicylate:1- octen-3-ol, 1:2:1), (Fig. 4, χ^2 - test, $P = 0.046$), indicating that increased concentration of methyl salicylate in the mixture attracted the mites stronger than the other mixtures and the control plants .

In order to elucidate whether 1-undecanol and methyl salicylate affect both mite behavior and reproduction, their impact on spider mite preference and oviposition were comparatively evaluated in a trapezoid bridge test. Although female adults preferred methyl salicylate over 1-undecanol in their first choice (Fig. 5a, χ^2 - test, $P = 0.035$), average number of eggs in the presence of the two volatiles did not differ within the first 24 h after establishment (Fig. 5b, pairwise *t* -test, $P = 0.725$).

Table 1 Volatile compounds (ng g^{-1} DW) detected from the bean leaf headspace by GC-MS analysis, 24 and 48 h after bacterial inoculation (mean ± se, $n = 5$)

Compound	RI	*P. syringae*		*P. ananatis*		*P. putida*		Control	
		24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
(z)-3-Hexen-1-ol	854	152.1 ± 68.4	60.0 ± 21.5	12.0 ± 5.1	62.7 ± 22.6	1.1 ± 0.7	20.7 ± 11.0	8.17 ± 1.0	31.6 ± 8.2
Anisole	921	0.6 ± 0.3	1.1 ± 0.2	2.9 ± 0.7	1.1 ± 0.2	0.04 ± 0.02	1.5 ± 0.4	0	1.1 ± 0.3
1-Octen-3-ol	978	6.0 ± 2.4	4.8 ± 2.2	1.0 ± 0.3	3.7 ± 1.0	0.6 ± 0.2	1.5 ± 0.6	1.1 ± 0.6	1.7 ± 0.3
(z)-3-Hexenyl acetate	1005	140.3 ± 66.7	28.2 ± 7.2	22.2 ± 3.3	35.2 ± 14.1	17.7 ± 7.7	145.3 ± 91.5	31.7 ± 12.5	28.5 ± 5.7
E-beta-Ocimene	1050	32.6 ± 19.5	27.6 ± 15.3	4.7 ± 1.1	6.5 ± 2.5	4.0 ± 1.4	4.7 ± 3.1	16.8 ± 1.7	2.2 ± 0.3
4,8-Dimethyl-1,3,7 nonatriene	1118	27.7 ± 8.6	44.6 ± 22.1	130.1 ± 53.7	67.4 ± 19.4	18.4 ± 10.0	10.1 ± 5.2	60.1 ± 10.2	10.9 ± 1.6
Methyl salicylate	1202	0.4 ± 0.2	0	36.5 ± 11.4	7.0 ± 2.7	5.1 ± 3.6	0.4 ± 0.2	5.3 ± 0.2	0
1-Undecanol	1373	14.3 ± 3.8	7.4 ± 2.5	0	0	0.6 ± 0.2	0.1 ± 0.06	0	0

* The identification was based on mass spectrum matching with four spectral databases and published data

Leaf Damage Caused by Mite Infestation The damage caused by spider mites to leaves of bean plants was found to vary significantly depending on the different bacterial strains (ANOVA: $F_{2,87} = 21.436$; $P < 0.001$). Leaves inoculated with *P. syringae* suffered a significantly lower damage compared to the respective ones inoculated with *P. ananatis* and the control leaves (Fig. 6).

Egg Production of Adult Females Bacterial inoculation of bean leaves by *P. syringae* reduced the egg production of females fed on them, compared to the control non - inoculated leaves (ANOVA $F_{1,\ 16} = 6.71$, $P < 0.05$). The mean number of eggs laid during the entire adult life on the control non-inoculated leaves was significantly higher than on those fed on leaves inoculated with *P. syringae* (Fig. 7, pair-wise *t*-test, $P < 0.05$). In each observation day, the number of females not laying any eggs was higher on leaves inoculated with *P. syringae* than on the control leaves albeit the difference being significant only in the two last sampling times according to pairwise t-test (Fig. 7, pairwise *t*-test, $P < 0.05$). By contrast, inoculation of leaves with *P. ananatis* did not have any negative effect on egg production of *T. urticae* females (Fig. 8, pairwise *t*-test, $P < 0.05$).

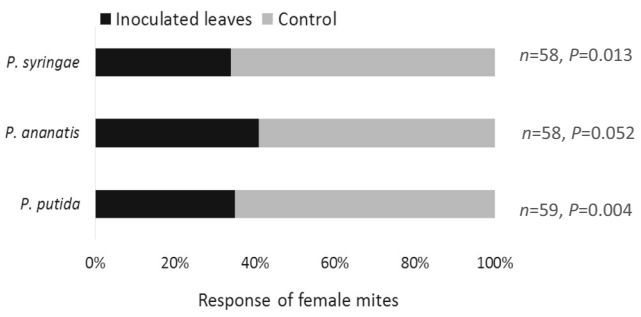

Fig. 2 Olfactory responses of female mites for control and inoculated leaves with *P. syringae, P ananatis* or *P. putida* stains. *P*-values show significant mite preferences between inoculated and control leaves χ^2–test; n = sample size for each comparison

Discussion

Our results demonstrate that certain strains of epiphytic bacteria differently affect the identified bean leaf VOCs and the responses of *T. urticae* to them. We tested three widely distributed bacterial strains with different life strategies, i.e. a nonpathogenic *P. ananatis* strain, a epiphytic, potentially pathogenic strain of *P. syringae* and a saprophyitic soil bacteria *P. putida* strain. Bacterial inoculation induced strain-specific changes the scent bouquet released by bean leaves. Plants inoculated with *P. ananatis* and control plants did not strongly differ in scent composition, whereas inoculation with *P. syringae* resulted in a distinct scent bouquet. Next to compositional changes, inoculation with bacterial strains resulted in increases or decreases in the emission of individual compounds.

The inoculation of bean leaves with the potentially phytopathogenic *P. syringae* increased the emissions of GLVs such as (*Z*)-3-hexenol and (*Z*)-3-hexanyl acetate within the first 24 h after inoculation. Lipoxygenase metabolism induction was also reported for both lima beans and tobacco plants, which released high amounts of (*Z*)-3-hexenol 18–20 h after inoculation by *P. syringae* pv. *syringae* (Croft et al. 1993; Heiden et al. 2003). Interestingly, the above-mentioned compounds have been identified as antibacterial agents in the past (Croft et al. 1993). Furthermore, 1-undecanol was emitted in higher quantities after inoculation with *P. syringae*. Although, undecanol has been detected among leaf VOCs of undamaged plants (Sarkar et al. 2016) and plants after virus infection (Cheung et al. 2015), this compound has also been identified as a member of the VOC mixtures released by two *Pseudomonas* species in response to fungal pathogens, i.e. *P. fluorescence* (Raza et al. 2016) and *P. chlororaphis* (Spence et al. 2014). To the best of our knowledge, this is the first time that this compound is detected in leaf VOCs in response to *P. syringae*, so the elucidation of its origin (plant's

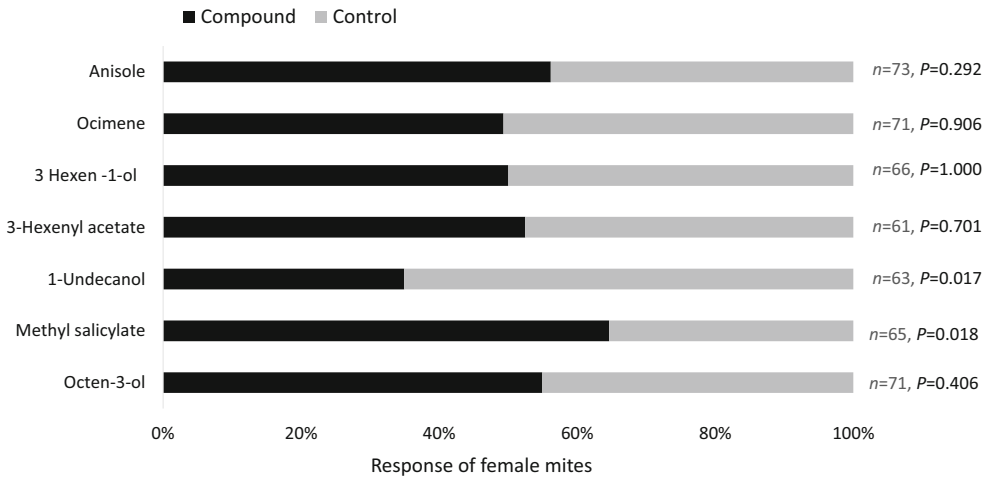

Fig. 3 Olfactory responses of female mites to individual volatile compounds (selected according to GC-MS analysis of bean leaf head space) and neutral air. *P*-values show significant mite preferences between individual compounds and neutral air χ^2 – test; *n* = sample size for each comparison

or bacterial) and also its function deserve further investigation. Against our expectations, methyl salicylate was not detected among the emitted volatiles after *P. syringae* inoculation, although, this compound is a common volatile signal in regulation of plant defense mechanisms after plant infection by phytopathogenic microorganisms (Huang et al. 2003; Liu et al. 2010). However, quantitative effects on the emissions of VOCs due to the inoculation with *P. syringae* may be highly strain- and timing-specific, which is indicated by studies on different strains of this bacterial species (Huang et al. 2003).

Apart from release of methyl salicylate from *P. ananatis* 24 h after inoculation, leaves inoculated with *P. ananatis* and *P. putida*, did not release significant quantities of the above mentioned VOCs compared to the control plants. The only compound that was found in high amounts in all treatments, including controls, is (*Z*)-3-hexenyl acetate. This compound has been evaluated as priming agent in plants after fungal attack (Ameye et al. 2015), and also as a signal of leaf mechanical damage caused either from biotic or abiotic stress (Ebel et al. 1995). Its presence in our system cannot be evaluated since it was detected in both treated plants and controls. Further, the inoculation of bean plants by *P. putida* did not induce any particular volatile emission within 24 h after inoculation, indicating that the presence of a saprophytic bacterium, even at high cell numbers do not affect leaf VOC emission.

Our behavioral assays demonstrate that changes in scent emission of bean leaves in response to bacterial inoculation translate into changes of the interactions between bean leaves and spider mites. Spider mites avoided the odors of leaves inoculated with *P. syringae* and *P. putida* in the olfactometer trial, while they did not avoid *P. ananatis* colonized leaves. Likewise, leaves inoculated with *P. syringae* suffered a lower feeding damage than the control leaves and those inoculated with *P. ananatis.* Similarly, the number of eggs laid on leaves inoculated with *P. syringae* was lower than on the control leaves and those inoculated with *P. ananatis*. These results suggest that bacteria may mediate the plant response to

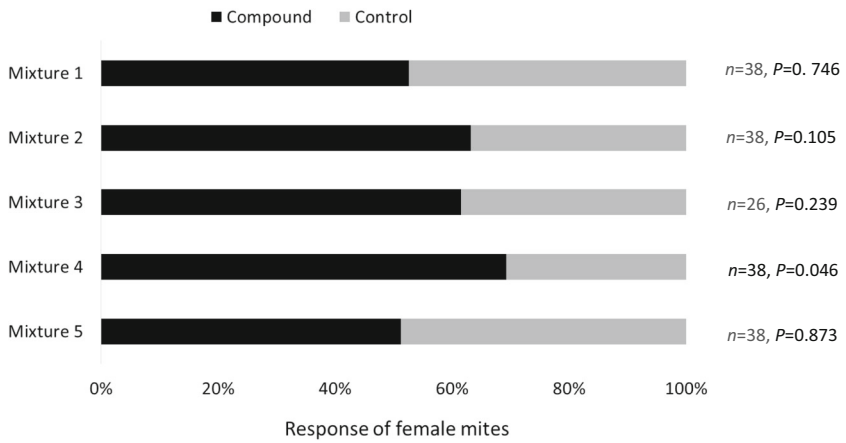

Fig. 4 Olfactory responses of female mites to five mixtures of the volatile compounds (selected according to GC-MS analysis of bean leaf head space) and neutral air; Mixture 1 = methyl salicylate:1-undecanol:3-hexanyl acetate, 1:1:1; Mixture 2 = methyl salicylate:1-undecanol:3-hexanyl acetate, 1:1:1; Mixture 3 = anisole:1-undecanol:3-hexanyl acetate, 1:2:1; Mixture 4 = ocimene: methyl salicylate:1- octen-3-ol, 1:2:1; and Mixture 5 = ocimene:1-undecanol: octen-3-ol, 1:1:1. *P*-values show significant mite preferences between mixtures and neutral air χ^2 – test; *n* = sample size for each comparison

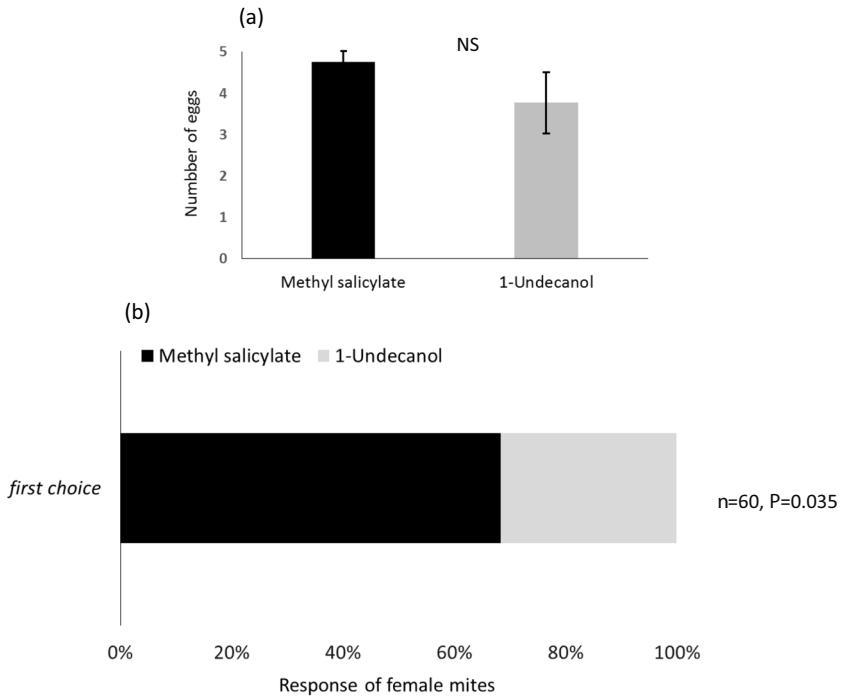

Fig. 5 **(a)** First choice of female spider mites in a two-choice behavioral assay towards bean leaf discs treated either with 1-undecanol or methyl salicylate; sample size and χ^2-test significance level is given to the right, and **(b)** the mean number of eggs laid on each leaf disk within 24 h (NS = non-significant)

herbivore attack and consequently affect the severity of leaf damage by herbivores (Sarmento et al. 2011; Schausberger 2018 and references herein). Alternatively, bacteria may directly affect the behavior of herbivores as suggested by Peters et al. (2017). We showed that bean leaves colonized by *P. ananatis* emitted methyl salicylate, which turned out to be an attractant for spider mites potentially leading to higher oviposition rates and leaf damage. Similarly, Agut et al. (2015) highlighted that Cleopatra mandarin VOCs with high levels of methyl salicylate increased attractiveness to spider mites. They suggested that this behavior is probably attributed to the negative cross-talk between the jasmonic acid and salicylic acid pathways, and also that high levels of methyl salicylate could be used by spider mites (here we used *T. urticae*) as indicators of low JA pathway activation (Agut et al. 2015). Although, no data about spider mite preference to volatiles derived from lipoxygenase metabolism exist to the best of our knowledge, our results demonstrate that mites do not show any particular preference to these compounds, at least in the concentrations used in the olfactometer bioassay. On the contrary, when these compounds are emitted from infested plants can serve as indirect defenses attracting mite's natural enemies (Kant et al. 2004; Shimoda et al. 2005).

Further, we found that spider mites showed preference for methyl salicylate, but were repelled by 1-undecanol, either when they had to choose over the control or when the two compounds were tested against each other to the trapezoid behavioral assay. Interestingly, 1-undecanol was detected only

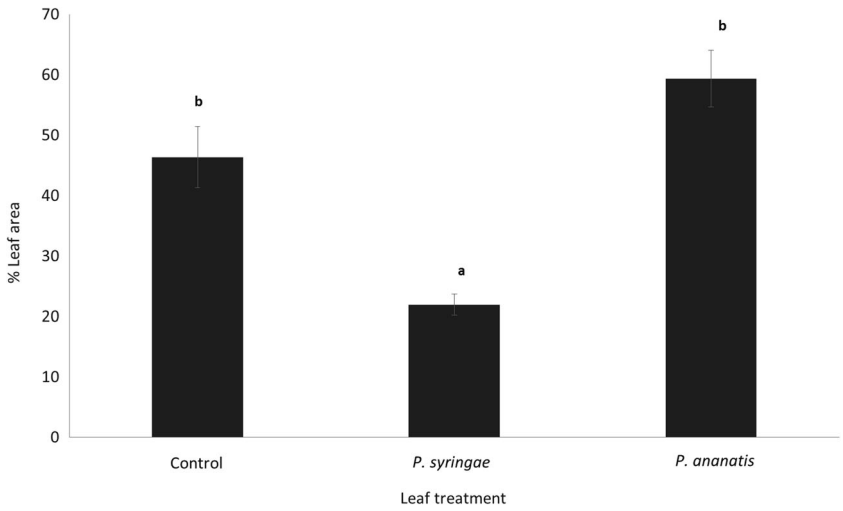

Fig. 6 Leaf damage (% mean ± se) caused by female mites after inoculation of bean plants with *P. syringae* or *P. ananatis* bacterial strains. Means followed by the same letter did not differ significantly according to *Tuckey's HSD* after ANOVA (ANOVA $F_{2,87} = 21.436$; $P < 0.001$)

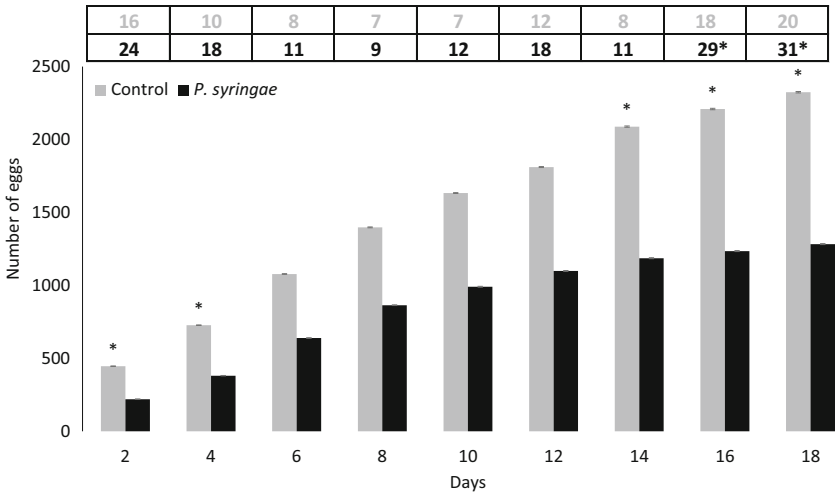

Fig. 7 Egg production (mean cumulative number of eggs laid per 2 days (± se) of adult females fed on bean leaf disks inoculated or not with *P. syringae*. The number of females not laying any eggs in each observation day is shown in the boxes. Asterisks indicate significant differences ($P < 0.05$) according to pairwise *t* -test ($n = 40$)

when bean plants were inoculated with *P. syringae* and not with the other strains. This compound has been already evaluated as antibacterial agent (Mukherjee et al. 2013) and also it was detected in VOCs bouquet after 48 h feeding by three different insects (Sarkar et al. 2016). Further, this compound does not seem to influence egg production of female mites, thus the above findings indicate that it functions rather as a repelling cue for spider mites and not as an acaricidal agent. As mentioned also above, it's origin (plant or bacterial), and also its exact impact on spider mites is worth further elucidation.

According to our results, there is a clear indication that there are differences in leaf volatile composition caused by different bacterial colonizers altering the behavior and reproduction of female mites. It was shown that, when *P. ananatis* is used as bacterial inoculum, spider mites increase their feeding activity causing a high damage to bean plants. This finding indicates that one extensively accepted symbiotic bacterium, some of whose strains produce antibiotics that are active against serious plant diseases (Walterson and Stavrinides 2015), may provoke attraction to a serious plant herbivore that causes great losses in agriculture. On the other hand, *P. syringae* for which some strains have been reported to be serious plant pathogens, initiates the release of one volatile with repellent activity to spider mite and probably reduces the mites' performance on bean leaves. This result is of major importance since one common leaf bacterial inhabitant, which potentially can also cause disease, seems to play a role in plant defense under the presence of an herbivore. Taking into consideration that this bacterium may be a central player in the microbiome of many plants, being member of their core or satellite microbiome (Compant et al. 2019), its influence on plant defense mechanisms and on herbivore performance should be further elucidated. Verification of 1-udecanal origin (plant or bacterial), and experimentation on plant responses during plant - herbivore interactions, including metabolomic alterations and defense related gene expression will help us to clarify the ecological relevance of bacterial presence in this

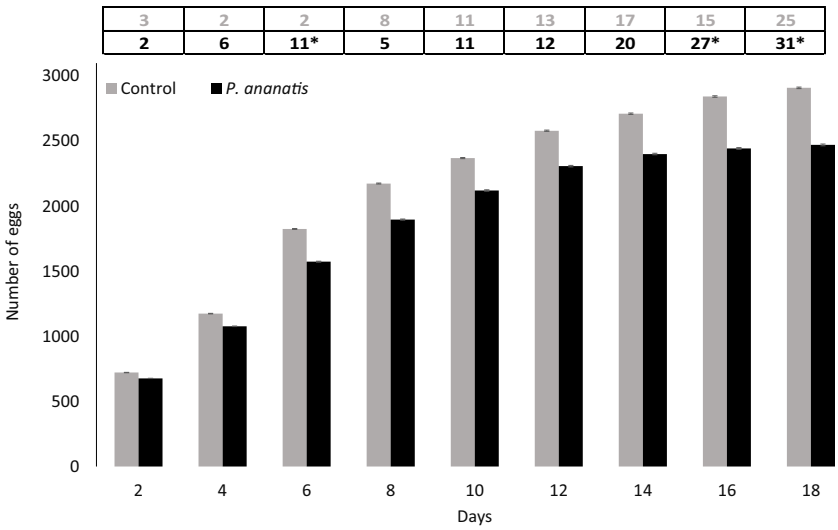

Fig. 8 Egg production (mean cumulative number of eggs laid per 2 days (± se) of adult females fed on bean leaf disks inoculated or not with *P. ananatis*. The number of females not laying any eggs in each observation day is shown in the boxes. Asterisks indicate significant differences ($P < 0.05$) according to pairwise *t* -test ($n = 40$)

process. Overall, our study shows that microbial effects vary with microbe identity and that more comparative microbiome studies in agricultural ecosystems are required to comprehend core players in immunity modifications that may be used in future agricultural applications to control herbivores.

Acknowledgments Irmgard Schäffler and Stefan Dötterl for providing the infrastructure and for help with GC-MS analyses.

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