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Avocado rhizobacteria emit volatile organic compounds with antifungal activity against *Fusarium solani*, *Fusarium* sp. associated with Kuroshio shot hole borer, and *Colletotrichum gloeosporioides*

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ABSTRACT

Recent studies showed that bacterial volatile organic compounds (VOCs) play an important role in the suppression of phytopathogens. The ability of VOCs produced by avocado (*Persea americana* Mill.) rhizobacteria to suppress the growth of common avocado pathogens was therefore investigated. We evaluated the antifungal activity of VOCs emitted by avocado rhizobacteria in a first screening against *Fusarium solani,* and in subsequent antagonism assays against *Fusarium* sp. associated with Kuroshio shot hole borer, *Colletotrichum gloeosporioides* and *Phytophthora cinnamomi*, responsible for Fusarium dieback, anthracnosis and Phytophthora root rot in avocado, respectively. We also analyzed the composition of the bacterial volatile profiles by solid phase microextraction (SPME) gas chromatography coupled to mass spectrometry (GC–MS). Seven isolates, belonging to the bacterial genera *Bacillus* and *Pseudomonas*, reduced the mycelial growth of *F. solani* with inhibition percentages higher than 20%. Isolate HA, related to *Bacillus amyloliquefaciens*, significantly reduced the mycelial growth of *Fusarium* sp. and *C. gloeosporioides* and the mycelium density of *P. cinnamomi*. Isolates SO and SJJ, also members of the genus *Bacillus*, reduced *Fusarium* sp. mycelial growth and induced morphological alterations of fungal hyphae whilst isolate HB, close to *B. mycoides*, inhibited *C. gloeosporioides*. The analysis of the volatile profiles revealed the presence of ketones, pyrazines and sulfur-containing compounds, previously reported with antifungal activity. Altogether, our results support the potential of avocado rhizobacteria to act as biocontrol agents of avocado fungal pathogens and emphasize the importance of *Bacillus* spp. for the control of emerging avocado diseases such as Fusarium dieback.

1. Introduction

Microorganisms occurring in the rhizosphere – the zone of soil under the influence of plant roots - are of utmost importance for plant health and productivity, since they can increase plant nutrient uptake, stimulate plant development through the production of plant growth regulators, degrade phytotoxic compounds and even protect the plant against the attack of phytopathogens (Peiffer et al., 2013; Philippot et al., 2013). The suppression of plant pathogens by rhizosphere microorganisms is especially relevant for agricultural crops growing in intensive monocultures and therefore highly susceptible to the occurrence of fungal diseases (Weller et al., 2002).

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The inhibition of fungal phytopathogens by rhizobacteria may occur through a variety of pathways: 1) competition for resources; 2) production of soluble antimicrobial compounds and 3) emission of antimicrobial volatile organic compounds (VOCs) (Cazorla et al., 2007; Kai et al., 2009; Vinodkumar et al., 2017; Guevara-Avendaño et al., 2018). Emitted VOCs are of particular importance in microbial interactions and pathogen suppression. Volatiles can diffuse easily in the air contained in soil pores and can therefore reach farther distances than bacterial soluble compounds in the soil (Tyc et al., 2017). Previous reports have shown, in *in vitro* tests, the antagonistic effects of volatiles produced by bacteria of the genus *Bacillus* against a wide range of fungal pathogens, such as *Alternaria alternata, A. solani, Botrytis cinerea, Cladosporium oxysporum, Fusarium oxysporum, Moniliophtora perniciosa, Paecilomyces lilacinus, P. variotii,* and the oomycete *Pythium afertile* (Chaurasia et al., 2005; Yuan et al., 2012; Chaves-López et al., 2015; Gao et al., 2017). A recent study also demonstrated that bacterial isolate A8a, closely related to *Bacillus acidiceler,* that was isolated from avocado rhizosphere, was able to inhibit the growth of the oomycete *Phytophthora cinnamomi* through the emission of ketones, sulfoxides, and pyrazines in form of volatiles (Méndez-Bravo et al., 2018). The bacterial genus *Pseudomonas* is also capable of emitting antifungal and anti-oomycete VOCs that have been reported to inhibit the mycelial growth of *Phytophthora vignae, Rhizoctonia solani* and *Sclerotinia sclerotiorum* (Fernando and Linderman, 1994; Fernando et al., 2005; Elkahoui et al., 2015). *In vivo* tests have also highlighted the potential of VOCs emitted by bacteria *Bacillus* spp. and *Streptomyces philanthi* to control postharvest anthracnose diseases in mango and chili fruits, respectively (Zheng et al., 2013; Boukaew et al., 2018). The ability of rhizobacteria to secrete a wide range of antifungal VOCs makes them useful in the search of potential biological control agents, which could be used to develop more sustainable agricultural practices (Bais et al., 2006; Umeda et al., 2016).

Mexico is the world's largest avocado (*Persea americana* Mill.) producer with approximately 35% of the global production (Food and Agriculture Organization of the United Nations (FAOSTAT), 2016(FAOSTAT, 2016). The United States of America are also among the world ten major producers with more than 172,000 tons of avocado produced per year (FAOSTAT, 2016). However, the productivity of avocado orchards in these two countries has been hindered by fast-spreading fungal diseases such as Fusarium dieback, caused by *Fusarium euwallaceae and F. kuroshium* among other fungi and vectored by invasive shot hole borers (*Euwallacea* spp. nr. *fornicatus*) in California (Lira-Noriega et al., 2018; Na et al., 2018), and avocado root rot caused by *Phytophthora cinnamomi* (Guevara-Avendaño et al., 2018). Other common fungal pathogens such as *Colletotrichum* spp. have also hampered avocado production and commercialization (Xoca-Orozco et al., 2017; Guardado-Valdivia et al., 2018). Research efforts need to consider biocontrol as an option to mitigate the negative impact of such fungal diseases, since the use of chemical pesticides is restricted for avocado export and consumption (Stout et al., 2004). The objective of this study was therefore to assess the antifungal activity of VOCs produced by avocado rhizobacteria against the common avocado fungal and oomycete pathogens *Fusarium* sp. associated with Kuroshio shot hole borer (KSHB), *C. gloeosporioides* and *P. cinnamomi*; antifungal activity was also evaluated against *Fusarium solani*, a phylogenetically close relative of *F. euwallaceae* and *F. kuroshium* and causal pathogen of post-harvest disease on avocado (Darvas and Kotze, 1987). Furthermore, we also aimed at identifying the chemical nature of the emitted bacterial VOCs by using solid phase microextraction (SPME) followed by gas chromatography and mass spectrometry (GC–MS).

2. Materials and methods

2.1. Isolation of avocado rhizobacteria

Rhizosphere soil samples were collected in an avocado orchard located in Escondido, San Diego County, California, where Phytophthora root rot and Fusarium dieback had been previously detected. Sampling was carried out as described in Guevara-Avendaño et al. (2018). Briefly, five non symptomatic avocado trees and five avocado trees with symptoms of Fusarium dieback were selected. Four soil and root samples were collected per tree with a disinfected shovel, at a depth of 5–10 cm and 50 cm away from the trunk, and were mixed in order to obtain one bulk sample per tree. Samples were transported in a cooler and immediately processed upon arrival at the laboratory (Eskalen Lab., University of California, Riverside). Each bulk sample (n = 10) was processed separately. Loose soil was removed from the roots and the soil that was strongly adhered to the roots was recovered as rhizosphere soil. Suspensions were prepared from 1 g rhizosphere soil and 99 ml distilled water and 100 µl of 1:10 and 1:100 dilutions were subsequently plated on Petri dishes containing solid Luria–Bertani medium (LB, Difco), in triplicate. The composition of solid LB medium was the following: tryptone (10 g/L), yeast extract (10 g/L), NaCl (5 g/L) and agar (15 g/L). Petri dishes were incubated at 30 °C for seven days. The obtained bacterial isolates were re-streaked onto LB until pure cultures were obtained and were named according to their provenance (S: "sick" tree; H: "healthy" tree; subsequent letters indicate the order in which pure strains were obtained). Bacterial isolates were then clustered into 45 morphotypes (independently from their provenance) based on colony and cell morphological criteria such as shape, edge, elevation, surface, texture, color, transmitted light, reflected light, and Gram staining results. One bacterial isolate per morphotype was then selected for subsequent antagonism assays, in order to encompass as much of the culturable diversity as possible (Hunziker et al., 2015). All isolates were preserved at −20 °C in LB and 20% glycerol.

2.2. In vitro evaluation of the antifungal activity of bacterial VOCs against F. solani

The selected bacterial isolates were re-streaked onto LB and incubated for 24 h at 30 °C prior to implementing the antagonism assays. Antifungal activities of bacterial VOCs were first evaluated against *F. solani*, a phylogenetically close relative to *F. euwallaceae* (O'Donnell et al., 2015) and *F. kuroshium* (Na et al., 2018), as access to *F. euwallaceae* was restricted due to quarantine measures. An isolate of *F. solani* (provided by Dr. Mauricio Luna-Rodríguez, Universidad Veracruzana) was placed onto potato dextrose agar medium (PDA, Difco) and incubated at room temperature for seven days. The antagonism assays were carried out in four different batches with the two-sealed-base-plates method described in Gotor-Vila et al. (2017), with some modifications. Briefly, each bacterial isolate (n = 45) was streaked to prepare a bacterial lawn onto a base plate containing LB medium (Chaurasia et al., 2005; Gao et al., 2017). Lids were removed and replaced by another base plate containing in its center a disc of 5 mm diameter of fungal mycelium on PDA. Both base plates were sealed with Parafilm® and incubated for seven days at 30 °C. Each bacterial isolate was tested for antifungal activity in triplicate. Three assays were set up without bacterial treatments (LB only) and used as controls.

After seven days, the percentage of inhibition of mycelial growth (PI) was calculated based on the formula proposed by Gotor-Vila et al. (2017): PI = (C–T/C) × 100, where **C** is the diameter of fungal mycelial growth in the control and **T** is the diameter of mycelial growth when fungus is exposed to bacterial VOCs. The antagonistic activity of VOCs produced by bacterial isolates that presented a PI higher than 25% in the first screening was further confirmed by repeating the antagonism assays as described above, but using five replicates.

2.3. In vitro antagonistic activity of bacterial VOCs against Fusarium sp., C. gloeosporioides and P. cinnamomi

The bacterial isolates that could inhibit *F. solani* mycelial growth by more than 20% in the second antagonism assay were selected for additional evaluation; the antifungal activity of their emitted VOCs was further tested against important avocado fungal pathogens: *Fusarium* sp.

associated with KSHB, *C. gloeosporioides*, and the oomycete *P. cinnamomi*. The same procedure as described before for antagonism assays against *F. solani* was implemented, using three replicates. Temperature and time of fungal incubation, for inoculum preparation and for monitoring the antagonism assays, were adjusted depending on fungal species as follows: *Fusarium* sp. was incubated at 25 °C for seven days, *C. gloeosporioides* was incubated at 28 °C for nine days and *P. cinnamomi* was incubated at 28 °C for seven days. Antagonism assays against *Fusarium* sp. were carried out at the quarantine facilities of SENASICA (Servicio Nacional de Sanidad, Inocuidad y Calidad Agroalimentaria), through the CNRF (Centro Nacional de Referencia Fotosanitaria, Tecámac, Mexico), where strain HFEW-16-IV-019 of *Fusarium* sp., isolated from the ambrosia beetle *Euwallacea* sp. nr. *fornicatus* (KSHB), was provided. The isolate of *P. cinnamomi* was provided by Alfonso Méndez-Bravo (Méndez-Bravo et al., 2018). Isolate A7 of *C. gloeosporioides*, which showed 99–100% of ITS sequence similarity with other sequences of *C. gloeosporioides*, was obtained from an avocado leaf sample collected in the avocado orchard "Rancho San Carlos", in Huatusco, Veracruz, Mexico.

2.4. Microscopic observation of Fusarium sp. mycelium exposed to bacterial volatiles

The mycelium of *Fusarium* sp., associated with KSHB, which was exposed to bacterial volatiles was observed with Confocal Laser Scanning Microscopy (CLSM) and Scanning Electron Microscopy (SEM). Mycelium samples for CLSM were fixed in 4% paraformaldehyde + 0.2% cacodylate in Phosphate-Buffered Saline (PBS). Samples were stained with 50 µl of calcofluor white (Sigma-Aldrich, USA) for 15 min. Images were acquired with a Leica TCS-SP8 + STED microscope (Leica Microsystems) using plan apochromatic 40× (NA 1.25, oil) and 63× (NA 1.4, oil) objectives. Calcofluor-stained samples were recorded in grayscale channel (410–440 nm wavelength emission; excitation wavelength 405 nm).

Samples for SEM were fixed in 4% paraformaldehyde + 0.2% cacodylate in PBS buffer, rinsed twice in the same buffer for 5 min, dehydrated in a graded ethanol series (30–100%) during 30 min for each concentration, dried in a Quorum K850 critical point drying with CO_2 and attached to aluminum stubs using a carbon adhesive prior to coating with gold in a sputtering Quorum Q150. The preparations were observed and photographed with a JEOL-IT300LV scanning electron microscope.

2.5. Analysis of bacterial VOCs by SPME-GC–MS

The composition of VOCs emitted by bacterial isolates with antifungal activity against *F. solani* (PI > 20%) was analyzed by solid phase microextraction (SPME) coupled to gas chromatography and mass spectrometry (GC–MS), following the procedure described in Méndez-Bravo et al. (2018). Bacterial isolates were re-streaked onto Petri plates containing LB agar and were immediately sealed with a quadruple layer of plastic wrap (Kleenpack®). As a control, LB agar plates without bacteria were used. Subsequently, Petri plates were incubated at 30 °C for seven days with SPME fibers (50-30 µm DVB/CAR/PDMS, Supelco, Inc, Bellefonte, PA) inserted into the headspace through a hole previously obtained by piercing the plastic wrap layer, in order to trap volatile compounds, as recommended by Raza et al. (2016a, 2016b). Trapping of VOCs was carried out during seven days to take into account the same time frame as that used in the antagonism assays. After seven days of incubation, SPME fibers were injected into the GC port and volatile compounds were thermally desorbed at 250 °C. The separation and detection of peaks were carried out in a gas chromatograph (Perkin Elmer, Clarus 680) coupled to a mass analyzer (Perkin Elmer, Clarus Single Quadrupole (SQ)8 T MS). Helium gas was used as carrier gas (1.0 ml/min, constant flow) and a Elite-5MS column (30 m length × 0.25 mm inner diameter × 0.25 µm film thickness;

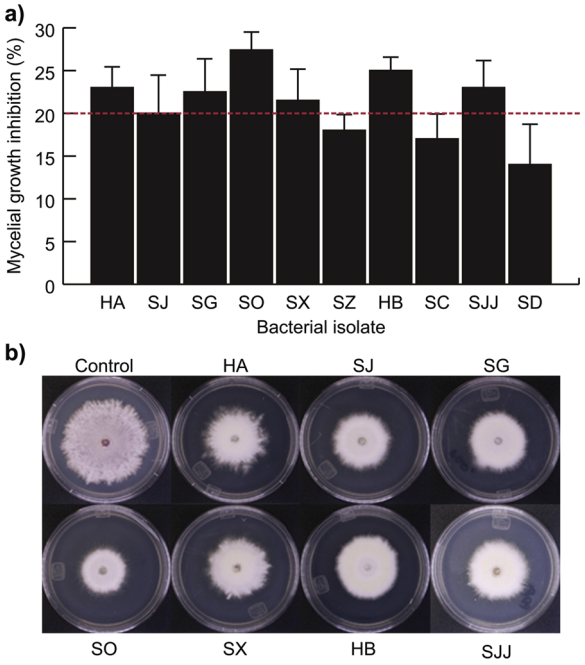

Fig. 1. Antifungal activity of VOCs produced by selected avocado rhizobacteria against *Fusarium solani*. (a) Percentage of inhibition of *F. solani* mycelial growth exposed to VOCs emitted by the ten bacterial isolates that were selected after the first screening. Values represent the average of five replicates ± standard error (s.e.). All isolates significantly inhibited mycelial growth in comparison with a control (Dunnett's test, $P \leq 0.05$). Black columns over intermittent red line represent bacterial treatments that showed a percentage of inhibition higher than 20%; (b) Mycelial growth inhibition of *F. solani* by VOCs produced by the bacterial isolates which presented a percentage of inhibition higher than 20%. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Perkin-Elmer Inc.) was used as a stationary phase. A splitless injector was used to introduce the sample to the GC column. The GC–MS was operated following the conditions described in Méndez-Bravo et al. (2018). Briefly, the GC oven temperature was adjusted: initial temperature of 40 °C was held for 3 min, increased at a rate of 15 °C/min to 160 °C, and further increased at a rate of 10 °C/min to 250 °C. The MS was operated in electron impact (70 eV) mode with a source temperature of 230 °C and a continuous scan from 35 *m/z* to 500 *m/z*. The mass spectrum data of volatile compounds were compared with those in the NIST/EPA/NIH Mass Spectrometry Library 2014 (National Institute of Standards and Technology, www.nist.gov), using a range of 65–100% similarity values, with the Turbomass ver 6.0.0 software (Perkin-Elmer Inc.). The relative abundances of the putatively annotated VOCs were expressed as adjusted peak area, and corrected taking into consideration the area of the compounds that were detected in agar controls. The trapping and analysis of VOCs were carried out in triplicate, using three different LB agar plates.

2.6. Antifungal activity of selected synthetic VOCs

Six representative VOCs, produced by at least three of the seven bacterial isolates with antifungal activity, were selected to confirm their capacity to inhibit fungal growth. The selected compounds were: 1) 2,3,5-Trimethylpyrazine; 2) 2-Nonanone; 3) 2-Decanone; 4) 2-Dodecanone; 5) Dimethyl disulfide; 6) Dimethyl trisulfide. Pure standards of these VOCs were purchased from Sigma-Aldrich (St. Louis, MO, USA). The evaluation of their antifungal activity was carried out by using the two-sealed-base-plates method as follows: a disc of 5 mm diameter of *F. solani* mycelium was placed in the center of a Petri dish containing PDA medium. A sterile paper filter with 200 µl of pure

Table 1
Sequenced bacterial isolates showing antagonism against *F. solani* and their closest matches based on the NCBI database "16S ribosomal RNA sequences (Bacteria and Archaea)".

Bacterial isolate	Tree health status	GenBank Accession number	NCBI best match		Identity %
			Taxonomy	Accession number	
SG	Sick	MG890317	*Pseudomonas frederiksbergensis*	NR_117177.1	99
SO	Sick	MG890318	*Bacillus stratosphericus*	NR_042336.1	99
HA	Healthy	MG890319	*Bacillus velezensis*	NR_075005.2	99
SX	Sick	MG890320	*Bacillus mycoides*	NR_113990.1	100
HB	Healthy	MG890321	*Bacillus mycoides*	NR_113990.1	99
SJ	Sick	MG890322	*Bacillus aerius*	NR_118439.1	99
SJJ	Sick	MG890323	*Bacillus acidiceler*	NR_043774.1	99

compound was placed in the center of another Petri dish with solid LB medium. The plates were immediately doubled sealed with Parafilm® and incubated at 30 °C for seven days. Each pure compound was evaluated in triplicate. Three controls were established with filter papers without pure compound. Inhibition of mycelial growth was calculated at day 7 with the formula previously described in Section 2.2.

2.7. Molecular identification of bacterial isolates with antifungal activity

The DNA from the seven selected bacterial isolates with antifungal activity against *F. solani* was extracted using DNeasy® Blood and Tissue kit (Qiagen, The Netherlands) following the manufacturer's instructions. The 16S rRNA region was amplified by PCR using universal primers 27 F (5′- AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′). The PCR reactions and amplification cycles were carried out as described in Guevara-Avendaño et al. (2018). The resulting PCR products were purified using Wizard® SV Gel and PCR Clean-Up System (Promega, U.S.A.) or Purelink® Quick Gel Extraction Kit (Qiagen, The Netherlands). Purified DNA samples were sent to Macrogen Inc. for sequencing. Sequences were deposited in GenBank (accession numbers MG890317 to MG890323).

2.8. Data analysis

Data from the antagonism assays were analyzed with the software STATISTICA version 10. Fungal growth data obtained in the antagonism assays against *F. solani* were analyzed with a one-way analysis of variance (ANOVA, $P \leq 0.05$). Subsequently, bacterial treatments were contrasted against control by Dunnett´s *post hoc* test ($P \leq 0.05$).

Sequences were manually checked in BioEdit 7.2.5. (Hall, 1999). An alignment was constructed in MEGA 7 (Kumar et al., 2016), using the multiple alignment program MUSCLE (Edgar, 2004) with the edited sequences and their best matches in GenBank nucleotide database (www.ncbi.nlm.nih.gov). The resulting alignment was manually checked. A Maximum-Likehood tree was constructed, using a Kimura 2 parameter model with Gamma distribution rates, and a Bootstrap method with 1000 replicates.

3. Results

3.1. Antifungal activity of bacterial VOCs against F. solani

In total, 45 bacterial isolates were first screened *in vitro* for the antifungal activity of their emitted VOCs against *F. solani*. Of these 45 isolates, only four inhibited fungal growth significantly (Dunnett's post hoc test, $P \leq 0.05$). Ten bacterial isolates reduced the mycelial growth of *F. solani* from 25.5% up to 54.6% (Supplementary Material 1). In the second antagonism assay, where the antifungal activity of these ten bacterial isolates against *F. solani* was confirmed by using five replicates instead of three, all isolates reduced mycelial growth significantly (Dunnett's post hoc test, $P \leq 0.05$; Supplementary Material 2), with inhibition percentages ranging from 14.8 to 28.2%. Seven bacterial isolates, identified as isolates HA, SJ, SG, SO, SX, HB and SJJ, were able to inhibit *F. solani* mycelial growth with percentages of inhibition higher than 20% (Fig. 1a, b) and were selected to be further evaluated against other avocado fungal pathogens. All bacterial isolates belonged to the genus *Bacillus*, except for isolate SG, which was identified as *Pseudomonas* sp. (Table 1; Fig. 2).

3.2. Antagonistic activity of bacterial VOCs against Fusarium sp., C. gloeosporioides and Phytophthora cinnamomi

Three bacterial isolates (isolates HA, SO and SJJ, all belonging to the genus *Bacillus*) emitted VOCs which were able to significantly inhibit the mycelial growth of *Fusarium* sp., associated with KSHB ($P \leq 0.05$), with percentages of inhibition ranging from 37.0% to 38.7% (Fig. 3a, b). Microscopic observations showed evidence of morphological alterations in fungal mycelium when *Fusarium* sp. was exposed to bacterial VOCs, such as shorter hyphal segments between septa (isolate HA, Fig. 4a), slightly distorted hyphae (isolate SO, Fig. 4a) and ramified and curved hyphae at the edge of colonies (isolate SJJ, Fig. 4a). A fourth isolate, isolate HB, reduced *Fusarium* sp. mycelial growth by 28.1%, although the inhibition was not significant ($P = 0.06$). Confocal microscopy revealed that volatiles emitted by isolate HB also induced shorter hyphal segments between septa and distorted hyphae (Fig. 4a). Isolates HA, SO, SJJ and HB all produced shriveling of hyphal surfaces, as shown by SEM images (Fig. 4b).

In the antagonism assays against *C. gloeosporioides*, only the volatiles emitted by bacterial isolates HA and HB significantly reduced mycelial growth ($P \leq 0.05$), with percentages of inhibition of 32.6% and 25.4%, respectively (Fig. 5a, b). No significant differences were observed in the diameter of mycelial growth of *P. cinnamomi* exposed to the VOCs emitted by the selected bacterial isolates. However, bacterial isolate HA, phylogenetically close to *B. velezensis,* visually reduced the density of *P. cinnamomi* aerial mycelium (Fig. 6). Isolate HA was the only bacterial isolate to show a negative effect on the mycelial growth and development of the four pathogenic microorganisms that were tested in this study (Table 2).

3.3. Chemical composition of bacterial VOCs analyzed by SPME-GC–MS

The analysis of the bacterial volatile profiles indicated that the seven bacterial isolates with significant antifungal activity against *F. solani* produced different chemical classes such as: ketones, hydrocarbons, pyrazines, alcohols and sulfides, among others. The chemical profiles of tentatively identified VOCs emitted by each bacterial isolate and detected by GC−MS are shown in Table 3. The VOCs belonging to the ketone chemical group were present in the volatile profile of all bacterial isolates and were usually amongst the most abundant chemical groups, representing 28.6% to 65.2% of all detected compounds emitted by each isolate. In addition, compounds belonging to the pyrazine family were also observed in the volatile profile from the seven

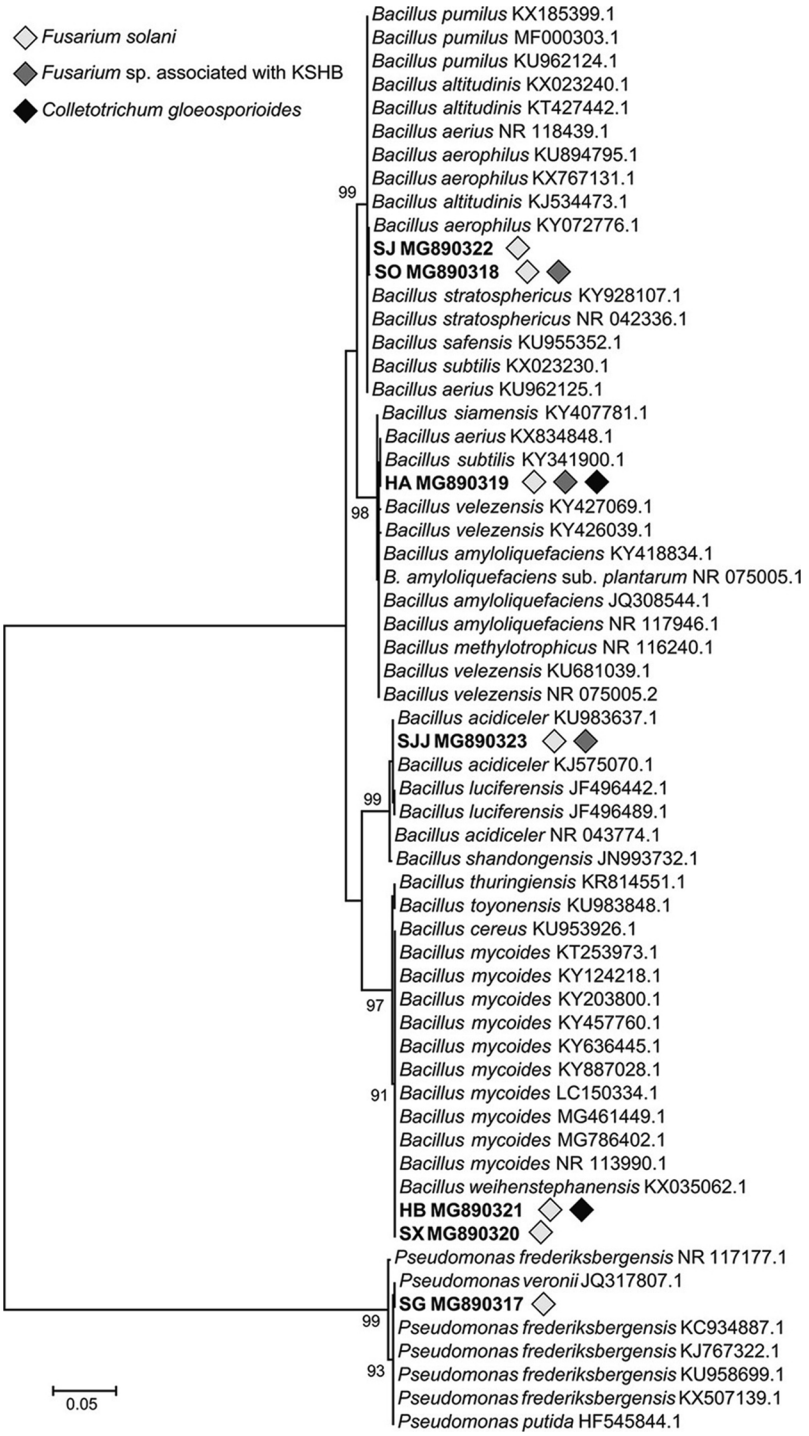

Fig. 2. Maximum-Likelihood tree of partially sequenced 16S rRNA genes. Bold letters indicate bacterial isolates that were obtained in this study and presented a percentage of inhibition higher than 20% against *F. solani*. Values above nodes correspond to bootstrap values obtained from 1000 replicates.

isolates presenting antifungal activities against *F. solani*. However, none of the detected specific compounds was present in all volatile profiles. Isolates HA, SJ and SJJ produced the largest number of VOCs with 23, 15 and 15 different compounds respectively, among which ketones were the most abundant (Table 3).

3.4. Antifungal activity of selected synthetic VOCs

The antifungal activity of the six selected compounds (2,3,5-Trimethylpyrazine; 2-Nonanone; 2-Decanone; 2-Dodecanone; Dimethyl disulfide; Dimethyl trisulfide) was verified through antagonism assays against *F. solani*. After seven days, the ketone 2-Dodecanone reduced *F. solani* mycelial growth by 38.5% (Table 4). The other tested VOCs completely inhibited mycelial growth of the fungus *in vitro*.

4. Discussion

Bacterial VOCs have been presented as a sustainable alternative to the use of synthetic pesticides, since reports of their antimicrobial activity against several important fungal phytopathogens suggest their potential to act as biocontrol agents of various plant diseases (Velázquez-Becerra et al., 2013; Munjal et al., 2016; Gotor-Vila et al.,

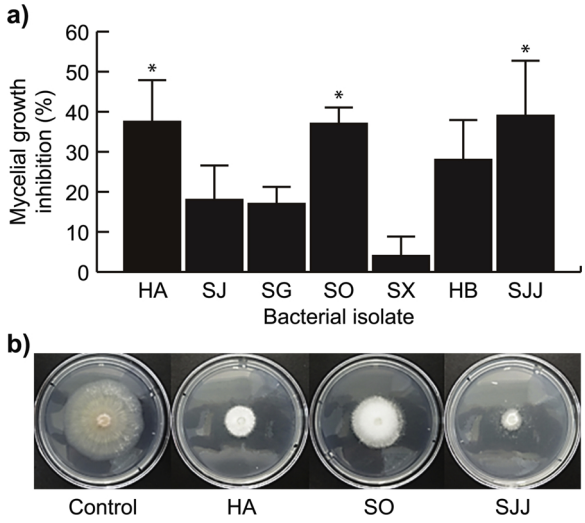

Fig. 3. Antifungal activity of bacterial VOCs against *Fusarium* sp., causal agent of Fusarium dieback. (a) Percentage of inhibition of *Fusarium* sp. mycelial growth exposed to VOCs emitted by the selected bacterial isolates. Values represent the average of three replicates ± standard error (s.e.). Asterisk (*) indicates significant inhibition in comparison with a control (Dunnett's test, $P \leq 0.05$); (b) Mycelial growth inhibition of *Fusarium* sp. by VOCs produced by the selected bacterial isolates.

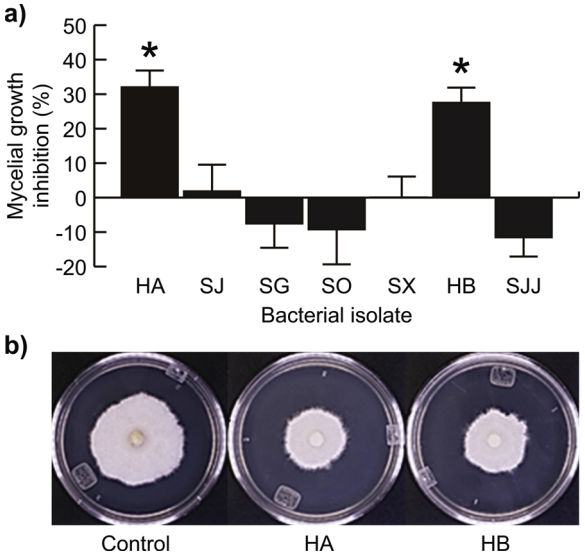

Fig. 5. Antifungal activity of bacterial VOCs against *Colletotrichum gloeosporioides*. (a) Percentage of inhibition of *C. gloeosporioides* mycelial growth exposed to VOCs emitted by the selected bacterial isolates. Values represent the average of three replicates ± standard error (s.e.). Asterisk (*) indicates significant inhibition in comparison with a control (Dunnett's test, $P \leq 0.05$); (b) Mycelial growth inhibition of *C. gloeosporioides* by VOCs produced by the selected bacterial isolates.

2017; Mu et al., 2017). Bacterial VOCs exhibit important advantages over the use of conventional fungicides, due to their long-distance range of action and the fact that they can be easily degraded, which makes them safer for the environment and human health (Elkahoui et al., 2015; Gao et al., 2017). In order to identify new biocontrol agents of important avocado diseases such as Fusarium dieback, anthracnose and Phytophthora root rot, we isolated bacteria from the avocado rhizosphere and evaluated the antagonistic activity of their emitted VOCs against *F. solani*, *Fusarium* sp. associated with KSHB, *C. gloeosporioides* and *P. cinnamomi*. In addition, we analyzed the chemical profile of the VOCs emitted by each bacterial isolate presenting antifungal activity.

In this study, six bacterial isolates (HA, SJ, SO, SX, HB, SJJ) belonging to the genus *Bacillus*, and one isolate (SG) identified as *Pseudomonas* sp., exhibited antifungal activity against *F. solani*, reducing mycelial growth up to 54.6%. In subsequent assays, some of these bacterial isolates reduced the mycelial growth of *Fusarium* sp. and *C. gloeosporioides* by up to 38.7% and 32.6% respectively. Previous works have indicated the strong antifungal activity of VOCs produced by *Bacillus* spp. against important fungal phytopathogens. Volatiles emitted by *B. subtilis*, which were mainly composed of ketones and alcohols as VOCs emitted by isolates HA, SO, SJJ and HB in the present work, were shown to induce the shriveling and stripping of hyphal surfaces of *Botrytis cinerea* (Mu et al., 2017), as observed in the present study for *Fusarium* sp. (Fig. 4b). *Bacillus pumilus* (strain TB09) and *B. thuringiensis* (strain TB72) were reported to reduce mycelial growth of *C. gloeosporioides* by 88.9% and 80.1% respectively in postharvest mangoes, due to the emission of 2-nonanone, β-benzeneethanamine and 2-decanone, among other VOCs (Zheng et al., 2013). The VOCs 2-nonanone, 2-decanone, 2-tridecanone and 2,3,6-trimethylphenol have also been reported to be emitted by *B. amyloliquefaciens* and to present antifungal activity against *F. oxysporum* f. sp. *cubense* (Yuan et al., 2012; Wang et al., 2013). All these compounds were detected in the volatile profile of isolate HA, which belongs to the subgroup *B. amyloliquefaciens / B. velezensis* within the *B. subtilis* species complex. The antifungal activities of representatives of the *B. amyloliquefaciens / B. velezensis* subgroup have been widely reported (Dunlap et al., 2017; Fan et al., 2017; Guevara-Avendaño et al., 2018). The VOCs emitted by *B. velezensis* (strain ZSY-1) have been shown to strongly inhibit the mycelial growth of *Alternaria solani*, *B. cinerea*, *Valsa mali*, *Monilinia fructicola*, *F. oxysporum* f. sp. *capsicum*, and *C. lindemuthianum* by more than 70% (Gao et al., 2017). In the present study, the VOCs emitted by isolate HA produced a reduction of hyphal density in *P. cinnamomi* (Fig. 6), as

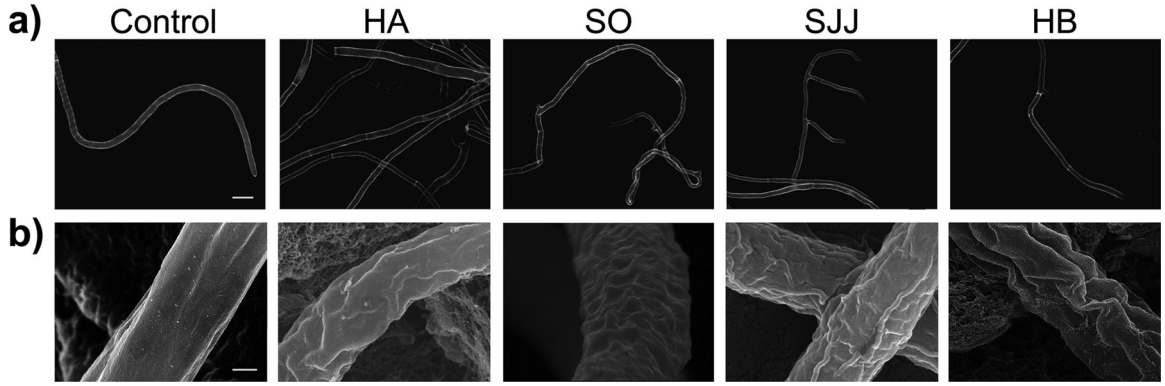

Fig. 4. Confocal (a) and Scanning Electron (b) microscopy images of the morphological alterations in the hyphal structure of *Fusarium* sp., causal agent of Fusarium dieback, induced by bacterial isolates HA, SO, SJJ and HB. Scale bars represent 10 µm (a) and 0.5 µm (b).

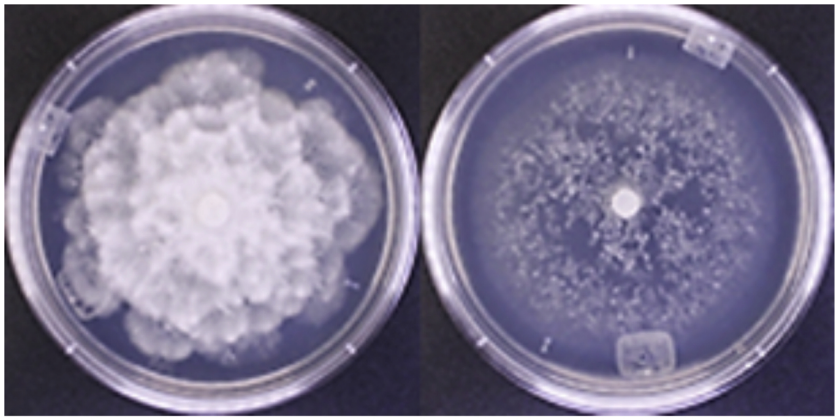

Fig. 6. Reduction in the density of *Phytophthora cinnamomi* aerial mycelium by VOCs emitted by bacterial isolate HA (*Bacillus* sp.).

Table 2
Antifungal activity of VOCs emitted by avocado rhizobacterial isolates against *Fusarium solani*, *Fusarium* sp., associated with Kuroshio shot hole borer, *Colletotrichum gloeosporioides* and *Phytophthora cinnamomi*.

Bacterial isolates	*F. solani*	*Fusarium* sp.	*C. gloeosporioides*	*P. cinnamomi*
HA (*Bacillus* sp.)	+	+	+	– / ~
SJ (*Bacillus* sp.)	+	–	–	–
SG (*Pseudomonas* sp.)	+	–	–	–
SO (*Bacillus* sp.)	+	+	–	–
SX (*Bacillus* sp.)	+	–	–	–
HB (*Bacillus* sp.)	+	–	+	–
SJJ (*Bacillus* sp.)	+	+	–	–

+ significant reduction of mycelial growth (Dunnett's test, $P \leq 0.05$); – no significant effect on mycelial growth; ~ visual negative effect on the density of aerial mycelium.

reported for *Bacillus* spp. against *S. sclerotiorum* (Vinodkumar et al., 2017).

Compounds 2-nonanone and 2-decanone were also detected in the volatile profile of isolate SO, identified as phylogenetically close to *B. stratosphericus,* which showed antifungal activity against *F. solani* and *Fusarium* sp. These two ketones were tested, as pure compounds, for antifungal activity against *F. solani*, and completely inhibited mycelial growth. Isolate SJJ, related to *B. acidiceler,* also showed antifungal activity against *Fusarium* spp. Interestingly, a close relative of *B. acidiceler* (isolate A8a), which was also isolated from the rhizosphere of avocado trees in Mexico, displayed antagonistic activity against the oomycete *P. cinnamomi* in addition to showing strong plant growth promoting abilities (Méndez-Bravo et al., 2018). The deformations that isolate SJJ induced on *Fusarium* sp. hyphal morphology were similar to those observed on *P. cinnamomi* hyphae (shriveled hyphal walls and lower hyphal density, Méndez-Bravo et al., 2018). However, in the present study, isolate SJJ was not able to inhibit the growth of *P. cinnamomi* through VOC emission. The volatile profiles of isolates SJJ and A8a markedly differed, 2,3,5-trimethylpyrazine being the only common compound, which suggest that this compound may not be responsible for the antagonistic activity displayed by isolate A8a. However, the antifungal activity of 2,3,5-trimethylpyrazine was confirmed in this study against *F. solani.*

The antifungal effect of VOCs emitted by several *Pseudomonas* species has also been reported. *Pseudomonas donghuensis*, for instance, showed strong antimicrobial activity against *Rhizoctonia solani*, *F. culmorum* and the oomycete *Pythium ultimum* through the emission of dimethyl sulfide, S-methyl thioacetate, methyl thiocyanate, dimethyl trisulfide and 1-undecene as main VOCs (Ossowicki et al., 2017). Sulfur-containing compounds produced by *Pseudomonas* spp., such as dimethyl disulfide (DMDS), dimethyl trisulfide (DMTS) and S-methyl methanethiosulfonate, were also found by De Vrieze et al. (2015) to block *P. infestans* growth and development. Nevertheless, in the present study, the VOCs emitted by the tested bacterial isolates did not reduce the mycelium growth of the oomycete *P. cinnamomi*, although DMDS and DMTS were tentatively identified in the volatile profile of isolate SG, a close relative of *P. frederiksbergensis*. The compounds DMDS and DMTS, however, completely inhibited the mycelial growth of *F. solani* when tested as commercial standards. In another study, the VOCs of cyanogenic *Pseudomonas* strains, including *P. frederiksbergensis,* inhibited the growth of *Phytophthora infestans* (Hunziker et al., 2015). The observed antagonistic activity was attributed by the authors to the emission of the alkene 1-undecene. This compound was scarcely found in the volatile profile of isolate SG, unlike DMDS and DMTS, which were widely detected. Altogether, these results suggest that a mixture of volatile compounds may be responsible for the antifungal and anti-oomycete properties exhibited by some *Pseudomonas* strains.

Many *Bacillus* and *Pseudomonas* species have also been reported to act as plant growth promoters (López-Bucio et al., 2007; Ossowicki et al., 2017; Méndez-Bravo et al., 2018). Bacterial VOCs may play a significant role in the plant growth promoting abilities of *Bacillus* and *Pseudomonas*, as evidenced by previous reports. Different strains of *Bacillus subtilis* and *B. amyloliquefaciens* have been shown to emit 2,3-butanediol among other compounds that could enhance plant growth and induce systemic resistance in *Arabidopsis thaliana,* tobacco and tomato (Ryu et al., 2003; Tahir et al., 2017a, 2017b). Similarly, *Pseudomonas fluorescens* could enhance tobacco growth through the emission of VOCs including 13-tetradecadien1-ol, 2-butanone and 2-methyl-*n*-1-tridecene (Park et al., 2015). The potential of the VOCs characterized in this study for plant growth promotion should therefore be investigated, as those bacterial isolates able to stimulate plant growth and inhibit the development of fungal pathogens would be good candidates to develop biologically active formulations.

5. Conclusion

In this work we studied the antifungal activity of VOCs emitted by avocado rhizobacteria. We identified seven bacterial isolates that reduced mycelial growth of *F. solani* with inhibition percentages higher than 20%. These isolates were also tested against *Fusarium* sp., associated with KSHB, *C. gloeosporioides* and *P. cinnamomi*. Isolate HA, close to *B. amyloliquefaciens,* significantly reduced the mycelial growth of all tested fungal pathogens and affected the aerial mycelium density of *P. cinnamomi*. In *Fusarium* sp., isolate HA was shown to induce the formation of shorter hyphal segments between septa. Isolates SO and SJJ,

Table 3
Chemical composition of VOCs produced by bacterial isolates with antifungal activity, analyzed by SPME-GC–MS.

Bacterial isolates	Compounds	RT (min)	RA (%)	S (%)	Chemical class
HA	2,5-Dimethyl pyrazine	4.86 ± 0.01	9.04 ± 1.20	91.2	Pyrazine
(*Bacillus* sp.)	6-Methyl-2-heptanone	5.56 ± 0.01	0.84 ± 0.19	91.9	Ketone
	5-Methyl-2-heptanone	5.70 ± 0.01	0.91 ± 0.16	88.4	Ketone
	2-Nonanone	7.38 ± 0.00	1.14 ± 0.29	92.0	Ketone
	4-Acetyl-1-methylcyclohexene	7.99 ± 0.01	3.07 ± 0.31	84.0	Ketone
	2-Propanone,1-cyclohexylidene-	8.03 ± 0.01	1.72 ± 0.24	83.7	Ketone
	2-Decanone	8.13 ± 0.01	2.05 ± 0.49	76.8	Ketone
	2-Decanol	8.25 ± 0.01	1.36 ± 0.35	85.7	Alcohol
	2-Undecanol	8.32 ± 0.00	0.86 ± 0.11	73.2	Alcohol
	2,3,6-Trimethylphenol	9.21 ± 0.02	7.18 ± 1.01	78.4	Phenol
	(3E)-4-(1-Cyclopenten-1-yl)-3-buten-2-one	9.24 ± 0.01	2.99 ± 0.20	73.7	Ketone
	2-Undecanone	9.53 ± 0.01	3.32 ± 0.41	88.0	Ketone
	2-Dodecanone	10.14 ± 0.01	12.82 ± 0.98	85.5	Ketone
	2-Tetradecanone	10.21 ± 0.01	10.36 ± 0.80	72.3	Ketone
	2-Dodecanol	10.24 ± 0.01	6.49 ± 0.71	72.8	Alcohol
	(3Z)-9-Methyl-3-undecene	10.30 ± 0.01	7.70 ± 0.22	70.4	Hydrocarbon
	2-Tridecanone	10.47 ± 0.01	2.89 ± 0.20	73.8	Ketone
	2,6-di-tert-butyl-4-hydroxy-4-methoxycyclohexa-2,5-dien-1-one	11.05 ± 0.01	6.82 ± 0.94	72.5	Ketone
	9-({[2-(Dimethylamino)ethyl]amino}methyl)-2,5a-dimethyloctahydro-2H-oxireno[4,4a] naphtho[2,3-b]furan-8(9 H)-one	11.92 ± 0.01	3.39 ± 0.39	69.4	Furanone
	2-Nonadecanone	11.99 ± 0.01	4.11 ± 0.60	75.9	Ketone
	2-Hexadecanone	13.78 ± 0.01	1.19 ± 0.21	82.7	Ketone
	1-Phenyl-1,2,3,4-tetrahydronaphthalene	13.85 ± 0.01	2.05 ± 0.29	77.4	Tetralin
	Octadecane	14.15 ± 0.01	1.22 ± 0.21	83.4	Hydrocarbon
SJ	Isobutylamine	0.95 ± 0.05	2.64 ± 1.37	94.9	Amine
(*Bacillus* sp.)	Isopentylamine	1.47 ± 0.02	2.68 ± 1.39	92.7	Amine
	6-Methyl-2-heptanone	5.54 ± 0.03	2.31 ± 0.13	91.9	Ketone
	5-Methyl-2-heptanone	5.68 ± 0.03	1.09 ± 0.24	79.9	Ketone
	Trimethylpyrazine	6.21 ± 0.02	2.58 ± 0.89	79.1	Pyrazine
	Benzyl methyl ketone	7.79 ± 0.01	2.96 ± 0.63	89.7	Ketone
	2-Decanone	8.13 ± 0.02	2.12 ± 0.06	75.6	Ketone
	2-Undecanone	8.19 ± 0.01	2.76 ± 0.17	72.3	Ketone
	2,3-Dimethyl-5-sec-butylpyrazine	8.51 ± 0.02	51.32 ± 6.82	70.2	Pyrazine
	Benzeneacetic acid, ethyl ester	9.04 ± 0.01	3.02 ± 0.88	87.9	Ester
	2-Methyl-5,6-diethylpyrazine	9.34 ± 0.01	7.47 ± 1.71	67.5	Pyrazine
	2-Isobutyl-3-isopropylpyrazine	9.40 ± 0.01	4.47 ± 0.71	82.7	Pyrazine
	2-Dodecanone	10.15 ± 0.01	8.44 ± 1.78	86.6	Ketone
	6,10-Dimethyl-5,9-undecadien-2-one	10.95 ± 0.01	3.12 ± 1.25	84.1	Ketone
	3-Tridecanone	11.00 ± 0.01	3.02 ± 1.53	67.2	Ketone
SG	2-Butanone	1.04 ± 0.00	2.02 ± 0.25	92.9	Ketone
(*Pseudomonas* sp.)	Dimethyl disulfide	1.94 ± 0.01	28.98 ± 4.72	95.6	Sulfide
	2,5-Dimethyl pyrazine	4.85 ± 0.03	2.48 ± 0.16	92.7	Pyrazine
	Dimethyl trisulfide	5.72 ± 0.02	4.53 ± 1.31	93.0	Sulfide
	2,5-Dimethyl-3-ethylpyrazine	7.21 ± 0.02	1.07 ± 0.19	71.1	Pyrazine
	Phenylethyl alcohol	7.64 ± 0.01	7.79 ± 0.99	93.9	Alcohol
	Benzyl methyl ketone	7.80 ± 0.01	10.29 ± 0.52	95.8	Ketone
	Dimethyl tetrasulphide	8.77 ± 0.01	1.23 ± 0.84	84.5	Sulfide
	o-Aminoacetophenone	9.62 ± 0.02	6.02 ± 2.09	83.7	Others
	Methyl phenylthiolacetate	10.16 ± 0.02	8.09 ± 0.97	74.2	Others
	2-Dodecanone	10.20 ± 0.01	7.26 ± 0.20	80.5	Ketone
	6,10-Dimethyl-5,9-undecadien-2-one	10.95 ± 0.02	5.74 ± 0.51	89.6	Ketone
	2-Tetradecanone	12.00 ± 0.01	8.64 ± 0.43	81.3	Ketone
	Farnesyl acetone	14.95 ± 0.02	5.85 ± 1.01	89.9	Ketone
SO	Isobutylamine	1.10 ± 0.01	4.61 ± 4.13	92.2	Amine
(*Bacillus* sp.)	2-Methyl-6-heptanone	5.53 ± 0.00	4.95 ± 0.07	90.4	Ketone
	5-Methyl-2-heptanone	5.66 ± 0.00	1.89 ± 0.71	86.6	Ketone
	2-Ethyl-3-methylpyrazine	6.19 ± 0.00	4.02 ± 1.96	78.9	Pyrazine
	2-Nonanone	6.89 ± 0.00	1.60 ± 0.12	66.4	Ketone
	2-Ethyl-3,6-dimethylpyrazine	7.15 ± 0.00	1.25 ± 0.32	78.8	Pyrazine
	Benzyl methyl ketone	7.73 ± 0.01	2.91 ± 2.02	86.4	Ketone
	2-Decanone	8.12 ± 0.01	2.47 ± 0.62	77.4	Ketone
	5-Sec-butyl-2,3-dimethyl- pyrazine	8.43 ± 0.02	63.48 ± 8.38	71.5	Pyrazine
	2-Methyl-5,6-diethylpyrazine	9.25 ± 0.01	6.05 ± 1.71	67.1	Pyrazine
	2-Isobutyl-3-isopropylpyrazine	9.31 ± 0.01	3.00 ± 0.62	81.4	Pyrazine
	2-Dodecanone	10.05 ± 0.01	3.77 ± 2.44	81.7	Ketone
SX	2-Butanone	1.04 ± 0.00	4.58 ± 0.93	91.7	Ketone
(*Bacillus* sp.)	Dimethyl disulfide	1.93 ± 0.00	41.08 ± 17.65	95.8	Sulfide
	2-Methyl-6-heptanone	5.50 ± 0.00	2.06 ± 0.31	90.0	Ketone
	Dimethyl trisulfide	5.66 ± 0.01	3.41 ± 1.50	90.4	Sulfide
	Trimethylpyrazine	6.14 ± 0.01	13.12 ± 1.27	80.9	Pyrazine
	N-benzylidene-N-phenethylamine	13.79 ± 0.01	18.80 ± 24.45	89.9	Amine
	Phenol, 2,6-di(tert-butyl)-4-bis(2-hydroxyethyl)aminomethyl-	14.24 ± 0.01	16.95 ± 6.65	75.7	Phenol

(*continued on next page*)

Table 3 (*continued*)

Bacterial isolates	Compounds	RT (min)	RA (%)	S (%)	Chemical class
HB	2-Butanone	1.05 ± 0.02	6.57 ± 0.58	90.2	Ketone
(*Bacillus* sp.)	Dimethyl disulfide	1.95 ± 0.03	50.62 ± 10.69	95.3	Sulfide
	2-Methyl-6-heptanone	5.56 ± 0.02	2.65 ± 0.97	89.8	Ketone
	Dimethyl trisulfide	5.72 ± 0.02	6.20 ± 4.25	92.4	Sulfide
	Trimethylpyrazine	6.22 ± 0.01	8.65 ± 1.58	85.9	Pyrazine
	2-Ethyl-1-hexanol	6.63 ± 0.01	2.40 ± 0.82	86.4	Alcohol
	2-Nonanone	6.94 ± 0.02	3.01 ± 1.02	70.6	Ketone
	2-Ethyl-3,5-dimethylpyrazine	7.27 ± 0.01	5.87 ± 1.76	88.0	Pyrazine
	2-Tetradecanone	11.91 ± 0.01	7.83 ± 2.58	84.2	Ketone
	2-Pentadecanone	12.84 ± 0.02	8.02 ± 3.01	81.3	Ketone
SJJ	Acetone	0.87 ± 0.02	4.07 ± 0.45	94.7	Ketone
(*Bacillus* sp.)	2-Butanone	1.05 ± 0.02	7.41 ± 0.88	93.1	Ketone
	1-Butanol	1.32 ± 0.02	1.14 ± 0.24	84.7	Alcohol
	Tetrahydropyridine	2.05 ± 0.01	2.16 ± 0.72	88.3	Amine
	Trimethylpyrazine	6.22 ± 0.01	5.46 ± 1.76	88.2	Pyrazine
	Phenylethyl alcohol	7.65 ± 0.01	11.76 ± 2.25	93.2	Alcohol
	Citronellol	8.90 ± 0.01	5.26 ± 0.69	87.0	Alcohol
	Benzeneacetic acid, ethyl ester	9.04 ± 0.01	14.23 ± 9.62	91.6	Ester
	2-Dodecanone	10.14 ± 0.01	3.61 ± 0.34	70.6	Ketone
	2-Tetradecanone	10.20 ± 0.01	5.37 ± 0.58	78.8	Ketone
	Geranylacetone	10.95 ± 0.01	4.73 ± 1.49	72.2	Ketone
	2-Tridecanone	12.00 ± 0.01	12.28 ± 1.94	69.5	Ketone
	Citronellyl valerate	12.05 ± 0.01	4.28 ± 0.75	85.0	Terpene
	2-Pentadecanone	12.85 ± 0.02	8.96 ± 2.11	84.8	Ketone
	[(2E)-4-Phenyl-2-butenyl]benzene	13.40 ± 0.02	9.26 ± 2.55	72.4	Benzene

RT represents the retention times in minutes. RA represents the relative peak area (relative area concentration) of the different compounds detected for each bacterial isolate, expressed as a percentage. Data are presented as means of three replicates ± standard deviation (s.d.). S (%) = Similarity percentage. The tentative names of compounds were annotated according to the information provided by NIST/EPA/NIH Mass Spectrometry Library 2014.

Table 4
Mycelial growth of *Fusarium solani* exposed to pure standard volatile compounds after seven days.

Standard compound	Diameter of mycelial growth (mm)	Percentage of inhibition (%)
2,3,5-Trimethylpyrazine	No growth	100 ± 0.00
2-Nonanone	No growth	100 ± 0.00
2-Decanone	No growth	100 ± 0.00
2-Dodecanone	45.33 ± 2.52*	38.49 ± 2.22
Dimethyl disulfide	No growth	100 ± 0.00
Dimethyl trisulfide	No growth	100 ± 0.00

Data represent the mean of three replicates ± standard deviation (s.d.). Asterisk (*) indicates significant differences in mycelial growth as compared with the control (Student's *t*-test test, $P \leq 0.05$).

identified as close relatives of *B. stratosphericus* and *B. acidiceler* respectively, also reduced *Fusarium* sp. mycelial growth and induced slight distortions of fungal hyphae. Analysis of bacterial volatile profiles by SPME and GC–MS revealed the presence of ketones, pyrazines and sulfur-containing compounds, previously reported with antifungal activity. The capacity of some of these pure volatile compounds to inhibit *F. solani* mycelial growth was verified *in vitro*. Further studies should aim at evaluating the potential of these *Bacillus* isolates to control Fusarium dieback, anthracnosis and Phytophthora root rot *in planta*. Altogether, our results support the potential of avocado rhizobacteria to reduce the growth of pathogenic fungi and report for the first time the effect of bacterial volatiles against the causal agent of Fusarium dieback. Our findings are especially relevant since Fusarium dieback has been causing important damages to avocado production in the U.S.A. and is now expanding geographically towards Mexico.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2018.11.009.

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