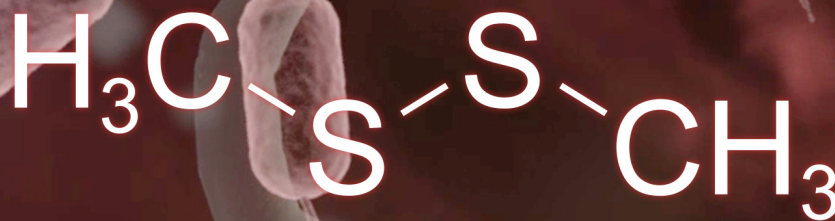# SMELLY FUMES: VOLATILE-MEDIATED COMMUNICATION BETWEEN BACTERIA AND OTHER ORGANISMS

EDITED BY : Laure Weisskopf, Choong-Min Ryu, Jos M. Raaijmakers and Paolina Garbeva

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SMELLY FUMES: VOLATILE-MEDIATED COMMUNICATION BETWEEN BACTERIA AND OTHER ORGANISMS

Topic Editors:

Laure Weisskopf, University of Fribourg, Switzerland
Choong-Min Ryu, Korea Research Institute of Bioscience and Biotechnology, South Korea
Jos M. Raaijmakers, Netherlands Institute of Ecology, Netherlands
Paolina Garbeva, Netherlands Institute of Ecology, Netherlands
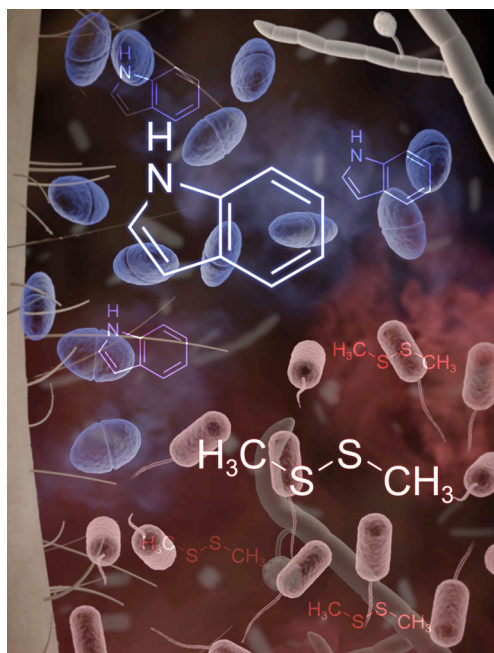
Volatile organic compounds emitted by microbes, such as indole or dimethyl-disulfide, mediate diverse types of interspecific and interkingdom interactions.
Cover image by Paolina Garbeva and Froukje Riensk, produced using iTZiT BV visual technology

This e-book summarizes recent advances in the young and rapidly developing field of microbial volatiles. Articles included here reveal novel information about the chemical diversity of bacterial and fungal volatiles, their functions, their roles in inter-specific and inter-kingdom interactions and the metabolic and physiological changes their exposure causes in the target organisms. The e-book is divided in three chapters: (1) Natural Functions of Microbial Volatiles; (2) Volatile Production and Ecosystem Functioning and (3) Volatile Detection and Identification.

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Editorial: Smelly Fumes: Volatile-Mediated Communication between Bacteria and Other Organisms

Laure Weisskopf[1]*, Choong-Min Ryu[2], Jos M. Raaijmakers[3,4] and Paolina Garbeva[3]*

[1] CHANGINS, Viticulture and Oenology, University of Applied Sciences and Arts Western Switzerland, Nyon, Switzerland, [2] Molecular Phytobacteriology Laboratory, Infectious Disease Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon, South Korea, [3] Department of Microbial Ecology, Netherlands Institute of Ecology (NIOO-KNAW), Wageningen, Netherlands, [4] Molecular Biotechnology, Institute of Biology, Leiden University, Leiden, Netherlands

Editorial on the Research Topic

Smelly Fumes: Volatile-Mediated Communication between Bacteria and Other Organisms

Volatiles are small (<300 Da), smelly molecules emitted by all organisms. They have very diverse roles for the producing organism (e.g., as infochemicals or antimicrobial compounds) and fulfill important ecosystem functions. While the importance of plant volatiles has been recognized for more than 30 years, research on microbial volatiles attracted attention only in the last decades. This special issue focuses on several new findings and recent developments in the field of microbial (fungal and bacterial) volatiles, their biological functions and chemical identification, which are highlighted in this editorial.

NATURAL FUNCTIONS OF MICROBIAL VOLATILES

Already at the very start of this research field, it became apparent that several microbial volatiles can modulate plant growth and have both plant growth-promoting and disease-suppressing activities (Ryu et al., 2003, 2004; Bailly and Weisskopf, 2012; Li et al., 2016). In this special issue, two papers describe the role of fungal volatiles on plant growth and defense (Bitas et al.; Kottb et al.): Bitas and colleagues studied volatile-mediated signaling between fungi and plants using a nonpathogenic *Fusarium oxysporum* and *Arabidopsis thaliana* as model organisms. They showed that fungal volatiles can enhance root and shoot biomass production through an auxin-dependent mechanism (Bitas et al.). In contrast, *Trichoderma* volatiles did not induce growth promotion in *A. thaliana* but triggered enhanced expression of defense-related genes and accumulation of phytoalexins, suggesting that plants can discriminate between different types of microbial volatiles (i.e., between those produced by *Trichoderma* and those produced by *Fusarium* strains) and induce different responses (Kottb et al.). 6-pentyl-alpha-pyrone (6PP) was identified as the main volatile in the headspace of *Trichoderma* and exposure of *A. thaliana* to pure 6PP mimicked the effect of the whole blend with respect to the increased expression of defense-related genes involved in the salicylic acid- and ethylene-mediated pathways (Kottb et al.). In the study of Song et al., the treatment with the plant volatile 3-pentanol led to an increased expression of defense-related genes involved in both the salicylic acid and the jasmonic acid-mediated pathways in *A. thaliana*, which in turn triggered resistance to the bacterial leaf pathogen *Pseudomonas syringae* pv. tomato (Song et al.). Similar induction of genes involved in both plant immune systems was observed in *A. thaliana*

upon exposure to bacterial volatiles, as reported by Sharifi and Ryu and reviewed by Liu and Zhang.

Beyond stimulation of the plant immune system, many papers in this special issue addressed the direct role of bacterial volatiles in disease protection, i.e., through direct inhibition of pathogens: *Streptomyces* strains isolated from disease-suppressive soils emitted volatiles that reduced the growth of *Rhizoctonia solani*, which was mediated, at least partly, by 2-methylpentanoate and 1,3,5-trichloro-2-methoxy benzene. Additionally, the same isolates also demonstrated volatile-mediated plant growth promotion of *A. thaliana* (Cordovez et al.). Along the same lines, six rhizobacteria isolated from common bean, able to protect bean plants from the common bacterial blight (CBB) causal agent, were evaluated *in vitro* for their potential antifungal effects toward different plant pathogenic fungi (Giorgio et al.). The six rhizobacteria caused strong volatile-mediated inhibition of mycelial growth of *Sclerotinia sclerotiorum*. Volatile-mediated effects on the target fungus were further investigated by electron microscopy, which revealed multifaceted effects of bacterial volatiles on the fungal cells, including alteration of membranes, mitochondria and endoplasmic reticulum (Giorgio et al.). Natural enemies of fungi might also be a source of antifungal volatiles, as shown by the work of Lo Cantore et al.. They investigated the effects of volatiles from *Pseudomonas tolaasii*, a major bacterial pathogen of mushrooms and observed volatile-mediated inhibition of mycelium growth of different basidiomycetes. These volatiles also affected plant growth negatively or positively depending on compound and dose (Lo Cantore et al.), highlighting the need for testing different concentrations within the biologically relevant range when assessing the bioactivity of volatiles. Sulfur compounds and 1-undecene detected, among other volatiles, in the blends of potato-associated *Pseudomonas* strains showed adverse effects on the oomycete pathogen *Phytophthora infestans*. In this work, small sulfur containing volatiles proved most efficient in inhibiting different life stages of the late blight pathogen *in vitro* and *in planta* (De Vrieze et al.). Volatile-mediated bacteria-fungal interactions have mainly focused on suppression of fungal pathogens. The reverse effect of fungal volatiles on bacteria, however, has been largely ignored. In this special issue, Schmidt et al. revealed that rhizosphere bacteria can distinguish between different fungi and oomycetes based on their volatile blends. Bacterial volatiles also affect other bacteria as reviewed in Audrain et al. and as highlighted in the work of Tyc et al., who showed that the production of volatiles such as indole was significantly affected by interspecific bacterial interactions.

VOLATILE PRODUCTION AND ECOSYSTEM FUNCTIONING

The vast majority of studies on microbial volatiles performed to date have used strains growing in isolation on artificial media. However, little is known about volatile emission in an ecosystem context. Van Agtmaal et al. investigated the role of microbial volatiles in suppressiveness of soils against the oomycete pathogen *Pythium intermedium*. They observed that anaerobic soil disinfestation used to kill soil-borne pathogens caused significant changes in soil community composition and temporary reduction of volatile emission. Another study also performed in soil with synthetic communities reported that interspecific interactions have a strong effect on volatile production and that the slow-growing and low-abundant strains significantly affected the emission of volatiles by the whole microbial community. Moreover, this study revealed that volatiles emitted by strains with direct access to nutrients may activate the growth of distantly located dormant bacteria (Schulz-Bohm et al.).

VOLATILE DETECTION AND IDENTIFICATION

Microbial volatiles are chemically highly diverse (Schenkel et al.; Kanchiswamy et al.) as they derive from various biosynthetic pathways. The technical developments in mass spectrometry that have been made in the recent years have led to the improvement of volatile compound detection and identification. However, the main challenge in volatile metabolomics, also referred to as "volatolomics," is the ability to identify and quantify the blends of emitted volatiles produced *in situ*. These blends are usually highly complex and often contain a significant proportion of yet unidentified compounds. This makes the identification of biologically relevant volatiles a challenging task. This special issue contains several reports where detailed workflows for volatile analysis are presented (Tyc et al.; Schmidt et al.; Cordovez et al.), including the application of freely available software packages (such as MetAlign, mzMine, MetaboAnalyst, AMDIS) suitable for metabolomic analysis of volatile compounds. One additional challenge of working with microbial volatiles is the experimental design that allow high through-put analysis of the biological effects they have on target organisms while excluding effects mediated by non-volatile compounds (Cernava et al.).

OUTLOOK

The prominent role of microbial volatiles in the interaction with eukaryotes and in particular with plants has become more evident in the past decade. In contrast to above-ground interactions, exchange of volatile signals in the rhizosphere is largely understudied, mainly because of the physical-chemical and (micro)biological complexity of the root-soil interface. New methods such as those described by Kai et al. will help to tackle this challenge and will significantly improve our understanding of the biological significance of volatile-mediated plant-microbe interactions, both below- and above-ground. Ultimately, this knowledge can be translated into innovative strategies for a more sustainable crop production by applying volatiles as alternatives to deleterious pesticides or as environmentally friendly gaseous biofertilizers (Kanchiswamy et al.). For example, dimethyl disulfide, a volatile frequently emitted by many bacteria has been used in the recent years as the novel soil fumigant PALADIN[®] against nematodes and soil-borne pathogens. The research on

the application of microbial volatiles in agriculture is still in its infancy. Further studies are needed to harness the potential of volatiles and to bring the knowledge from laboratory to field conditions.

AUTHOR CONTRIBUTIONS

All authors LW, C-MR, JR, and PG, contributed in writing this Editorial article for the research topic "Smelly fumes: volatile-mediated communication between bacteria and other organisms".

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Fusarium Oxysporum Volatiles Enhance Plant Growth Via Affecting Auxin Transport and Signaling

Vasileios Bitas[1], Nathaniel McCartney[2,3], Ningxiao Li[4], Jill Demers[1], Jung-Eun Kim[1], Hye-Seon Kim[1], Kathleen M. Brown[4,5] and Seogchan Kang[1,3,4*]

[1] *Department of Plant Pathology and Environmental Microbiology, The Pennsylvania State University, University Park, PA, USA,* [2] *Department of Entomology, The Pennsylvania State University, University Park, PA, USA,* [3] *Center for Chemical Ecology, The Pennsylvania State University, University Park, PA, USA,* [4] *Intercollege Graduate Degree Program in Plant Biology, The Pennsylvania State University, University Park, PA, USA,* [5] *Department of Plant Science, The Pennsylvania State University, University Park, PA, USA*

Volatile organic compounds (VOCs) have well-documented roles in plant-plant communication and directing animal behavior. In this study, we examine the less understood roles of VOCs in plant-fungal relationships. Phylogenetically and ecologically diverse strains of *Fusarium oxysporum*, a fungal species complex that often resides in the rhizosphere of assorted plants, produce volatile compounds that augment shoot and root growth of *Arabidopsis thaliana* and tobacco. Growth responses of *A. thaliana* hormone signaling mutants and expression patterns of a GUS reporter gene under the auxin-responsive DR5 promoter supported the involvement of auxin signaling in *F. oxysporum* volatile-mediated growth enhancement. In addition, 1-naphthylthalamic acid, an inhibitor of auxin efflux, negated *F. oxysporum* volatile-mediated growth enhancement in both plants. Comparison of the profiles of volatile compounds produced by *F. oxysporum* strains that differentially affected plant growth suggests that the relative compositions of both growth inhibitory and stimulatory compounds may determine the degree of plant growth enhancement. Volatile-mediated signaling between fungi and plants may represent a potentially conserved, yet mostly overlooked, mechanism underpinning plant-fungus interactions and fungal niche adaption.

Keywords: auxin signaling, chemical effector, fungal ecology, plant growth and development, volatile organic compounds

INTRODUCTION

Organisms in multiple kingdoms use volatile metabolites as semio-chemicals for intra- and inter-species communication and manipulation. Such function of various volatiles produced by animals and plants has been well-documented (Baldwin et al., 2002; Herrmann, 2010). Although available data on microbial semio-volatiles, especially those produced by fungi, is rather limited, they also seem to participate in modulating sexual reproduction, controlling physiology and growth within and between species, antagonizing other organisms, and coordinating symbiosis (Tsavkelova et al., 2006; Young, 2009; Bennett et al., 2012; Morath et al., 2012; Bitas et al., 2013). Volatile compounds produced by certain plant growth promoting rhizobacteria enhanced plant growth, induced systemic resistance against pathogens and/or increased abiotic stress tolerance (Ryu et al., 2003, 2004; Han et al., 2006; Zhang et al., 2007; Kwon et al., 2010). In contrast, bacteria, such as

strains of *Serratia* spp., *Pseudomonas* spp. and *Stenotrophomonas* spp., produce volatiles that inhibit the growth of *Arabidopsis thaliana* (Vespermann et al., 2007; Kai and Piechulla, 2009). Volatiles from *Trichoderma viride*, a biocontrol fungus, augmented *A. thaliana* growth (Hung et al., 2013), and volatiles produced by *Phoma* sp. enhanced tobacco growth (Naznin et al., 2013). Similarly, a biocontrol strain of *Fusarium oxysporum* that carries ectosymbiotic bacteria produced β-caryophyllene, a volatile sesquiterpene that appeared to enhance lettuce growth (Minerdi et al., 2011). However, when its symbionts were removed, the strain became pathogenic, ceased producing β-caryophyllene, and failed to enhance plant growth. Volatiles produced by ectomycorrhizal truffles (*Tuber* spp.) inhibited *A. thaliana* root growth and development (Splivallo et al., 2007).

In this study, we characterized how volatile compounds produced by diverse isolates of *F. oxysporum* affect plant growth using *A. thaliana* and tobacco. Collectively, members of this cosmopolitan soilborne fungal species complex cause diseases in >100 plant species (Kang et al., 2014) by invading through the roots and subsequently blocking water and mineral movement through the xylem (Czymmek et al., 2007; Michielse and Rep, 2009; Rispail and Di Pietro, 2009). Pathogenic strains typically exhibit narrow host specificity, causing disease only in a single, or closely related plant species (Kang et al., 2014); such host-specialized groups of pathogen isolates are classified as formae speciales. They also asymptomatically colonize a wide spectrum of plants (Michielse and Rep, 2009). Strains that belong to diverse *formae speciales* were screened to assess how their volatiles affect plant growth. We demonstrate here that volatiles from *F. oxysporum* can stimulate growth of plants and that auxin participates in the responses to these volatiles. How such volatiles contribute to *F. oxysporum* rhizosphere competency and pathogenesis is also discussed.

MATERIALS AND METHODS

Fungal Cultures and Plant Materials

Strains used in this study and their origins are listed in **Table 1**. They were stored as conidial suspension in 20% glycerol at −80°C and were revitalized by inoculating on half-strength Potato Dextrose Agar (PDA) at room temperature. Seeds of *A. thaliana* ecotypes were acquired from Lehle Seed Co. (Round Rock, TX, USA). The mutants of Col-0 used in this study, obtained from the Arabidopsis Biological Resource Center at Ohio State University, included *etr1* (Bleecker et al., 1988; Chang et al., 1993), *ga3ox1* and *aux1* (Marchant et al., 1999), *axr5* and *tir1* (Yang et al., 2004), and *eir1* (Luschnig et al., 1998). Transgenic Col-0 containing *DR5::GUS* (Jefferson et al., 1987) was provided by Darrell Desveaux at University of Toronto. Sara May at Penn State provided seeds of *Nicotiana tabacum* variety Samsun.

I-plate Assay for Plant Growth Enhancement by Fungal Volatiles

Surface-sterilized seeds (soaked for 1 min in 95% ethanol, rinsed three times with sterile distilled water, soaked for 15 min in 5% sodium hypochlorite solution, and rinsed three times with sterile

TABLE 1 | *F. oxysporum* strains screened in this study.

Accession #[a]	f. sp.[b]	Geographical origin
7802	*ciceris*	Spain
8012	*ciceris*	Spain
9605	*ciceris*	Tunisia
W6-1	*ciceris*	California, USA
NRRL 38272	*conglutinans*	Australia
NRRL 38340	*conglutinans*	Unknown
FRC O-210	*conglutinans*	New York, USA
NRRL 38341	*conglutinans*	Unknown
NRRL 38342	*conglutinans*	Unknown
NRRL 38491	*conglutinans*	Unknown
FRC O-1115	*conglutinans*	California, USA
NRRL 36364	*conglutinans*	Unknown
NRRL 34936	*lycopersici*	Spain
NRRL 36467	*lycopersici*	Unknown
NRRL 38499	*lycopersici*	Unknown
NRRL 26383	*lycopersici*	Florida, USA
NRRL 38445	*lycopersici*	Unknown
NRRL 38550	*lycopersici*	Unknown
NRRL 36570	*radicis-lycopersici*	Unknown
NRRL 36572	*radicis-lycopersici*	Unknown
NRRL 26379	*radicis-lycopersici*	Florida, USA
NRRL 38343	*mathioli*	Unknown
NRRL 22545	*mathioli*	Germany
NRRL 22553	*raphani*	Germany
NRRL 38337	*raphani*	Unknown
NRRL 37616	*pisi*	Unknown
NRRL 37611	*pisi*	Australia
NRRL 36573	*phaseoli*	Unknown
NRRL 38282	*medicaginis*	N. Carolina, USA
NRRL 26411	*fabae*	Unknown
NRRL 36118	*cubense*	Unknown
NRRL 26029	*cubense*	Unknown
NRRL 36113	*cubense*	Unknown
NRRL 22519	*melonis*	Unknown
NRRL 36472	*melonis*	Unknown
NRRL 22557	*vasinfectum*	Unknown
NRRL 32599	*vasinfectum*	Australia
NRRL 36385	*cucurbitacearum*	Unknown
NRRL 36470	*dianthi*	Unknown
NRRL 26874	*spinaciae*	Unknown
NRRL 38507	*zingiberi*	Unknown
NRRL 38335	*tuberosi*	Korea
NRRL 38275	*caladium*	Florida, USA
NRRL 28973	*asparagi*	Korea
NRRL 26844	*lactucum*	Unknown
NRRL 39464	*carnation*	Korea

[a]*NRRL corresponds to ARS Culture Collection at the National Center for Agricultural Utilization Research and FRC indicates Fusarium Research Center at Penn State. The first four strains were provided by Dr. Maria Jimenez-Gasco at Penn State.*
[b]*Forma specialis or source of isolation.*

distilled water) were kept in sterile distilled water for 2 days at 4°C in the dark. Sterilized seeds were inoculated on half-strength Murashige and Skoog (MS) salts medium supplemented with 0.8% (w/v) agar and 2.5% (w/v) sucrose (Murashige and Skoog,

1962) in 110 × 110 mm square Petri dishes (VWR, Radnor, PA, USA). These plates were placed in a growth chamber (Conviron PGR15, Winnipeg, MB, Canada) set at 22°C, 12 h light (4500 lux, 60 μmol photons m^{-2} s^{-1}) and 60% relative humidity.

One compartment of the I-plate contained half-strength PDA for culturing *F. oxysporum*, and the other had the MS medium described above for growing plants. One day before seedling transplantation, one plug of fungal culture (5 mm in diameter) was inoculated on the PDA side, and a 0.5 cm-wide strip of PDA along the center partition was removed (**Figure 1**) to prevent fungal overgrowth to the MS side. Five *A. thaliana* seedlings with similar size and growth stage were transplanted to the MS side by cutting and transferring 1 cm^2 agar blocks containing one seedling each. The control treatment for all experiments consisted of PDA alone. Inoculated I-plates were sealed with Parafilm and placed in a growth chamber under the conditions described above.

Measurement of Changes Caused by *F. oxysporum* Volatiles

After cocultivation, each plant was removed, and its roots were detached from the shoot. After removing any excess moisture on the leaves using a paper tissue, each shoot was weighed immediately. Leaf area, root length and the lateral root density were measured using the image analysis software WinRHIZO (Regent Instruments Inc., Ch Ste-Foy, Quebec, Canada).

Chlorophyll content was measured spectrophotometically as previously described (Hiscox and Israelstam, 1979). After measuring shoot fresh weight, they were placed in a sterile 15 mL tube containing 5 mL dimethyl sulfoxide (DMSO). This tube was incubated at 65°C for 8 h and then stored in the dark at 0 to −4°C until absorbance measurement. Absorbance was measured at 645 and 663 nm, and the total chlorophyll content (mg per gram of shoots) was calculated using the following formula: $(A_{645} \times 0.0202) + (A_{663} \times 0.00802) \times V/W$, where V is the volume of DMSO and W corresponds to the fresh weight of the seedlings analyzed. Relative water content (RWC) was calculated as previously described (Smart and Bingham, 1974): RWC = (fresh weight – dry weight)/(turgid weight – dry weight). After floating harvested leaves on sterile distilled water for 6 h, their turgid weight was recorded. Leaves were weighed after drying in an oven at 85°C. All experiments were conducted at least twice, with three biological replicates for each treatment and five seedlings per replicate.

Indirect Measurement of Fungal CO$_2$ and its Effect on Plant Growth

We used a tripartite Petri plate (Y-plate)-based assay (Kai and Piechulla, 2009) to determine whether CO$_2$ produced by fungal culture contributed to plant growth. Cocultivation was performed as described above, except that only three *A. thaliana* seedlings per plate were grown due to space constraint. One compartment contained 8 mL 0.1 M Ba(OH)$_2$, which reacts with CO$_2$ to form BaCO$_3$ precipitate. After 14 days of cocultivation, fresh shoot weight was measured, and the dry weight of BaCO$_3$ was measured by filtering the solution through a filter paper

followed by drying for 4 days at 50°C. Experiments were conducted twice, with three biological replicates.

GUS Staining

Histochemical staining of *in planta* GUS (β-glucuronidase) activity was performed as described in Jefferson et al. (1987). After cocultivation, *DR5::GUS* seedlings removed from MS were immersed in GUS staining solution, which consists of 50 mM sodium phosphate (pH 7.0), 10 mM EDTA (pH 8.0), 2 mM K$_4$Fe(CN)$_6$, 2 mM K$_3$Fe(CN)$_6$, 0.1% (v/v) Triton X-100, and 2 mM X-Gluc (5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid, cyclohexylammonium salt). After applying vacuum for 5 min, they were incubated overnight at 37°C in the dark with gentle agitation (75 rpm). These seedlings were washed sequentially with 70, 80, and 90% ethanol solutions until all chlorophylls were removed and were stored in 90% ethanol at 4°C until imaging. Staining patterns were observed using a stereomicroscope (Olympus SZ60, Center Valley, PA, USA) and imaged using the Olympus DP26 camera.

NPA Treatment

A stock solution of 1-naphthylthalamic acid (NPA; Sigma-Aldrich, St. Louis, MO, USA) in DMSO was used to produce MS containing 1, 5, and 10 μM NPA. Control plants were cultivated on MS containing DMSO only. A same volume of DMSO was used for all treatments. Experiments were conducted twice, with five seedlings per replicate.

Analysis of *F. oxysporum* Volatile Compounds

Individual strains were cultured on half-strength PDA in 1 L glass flask for 7 days at room temperature. Charcoal purified air was introduced into these flasks and headspace volatiles were collected using SuperQ volatile collection trap (ARS Inc., Micanopy, FL, USA) at a rate of 0.5 L/min by applying vacuum for 24 h. Captured compounds were eluted using 120 μL dichloromethane. As internal standards, 200 ng of octane and 200 ng of nonyl-acetate, dissolved in 5 μL dichloromethane, were added to the elution. Eluted compounds were analyzed by gas chromatography-mass spectroscopy (GC-MS) in electron impact (EI) mode using a Hewlett-Packard 6890 GC equipped with an HP-5MS bonded phase capillary column (0.25 mm × 0.25 μm × 30 m; Agilent Technologies, Little Falls, DE, USA) interfaced to a HP 5973 MS (Hewlett-Packard, Palo Alto, CA, USA). The column temperature was programmed from an initial temperature of 40°C, with a 1 min hold time, 8°C min^{-1} to 240°C, and 40°C min^{-1} to 300°C with a 3-min hold at 300°C. Injections of 1 μL were made with the inlet in splitless mode at 250°C with a split time of 0.75 min and helium carrier gas flow rate of 0.7 mLmin^{-1}. EI analysis used the default settings (ion source: 230°C, quadrupole: 150°C, and with spectra generated at 70 eV), and identification was performed using retention indices, the NIST 08 and Adams 09 libraries, as well as authentic standards.

To determine the amount of ethylene produced by *F. oxysporum*, individual strains were cultured in 50 mL flask containing 25 mL half-strength Potato Dextrose Broth (PDB),
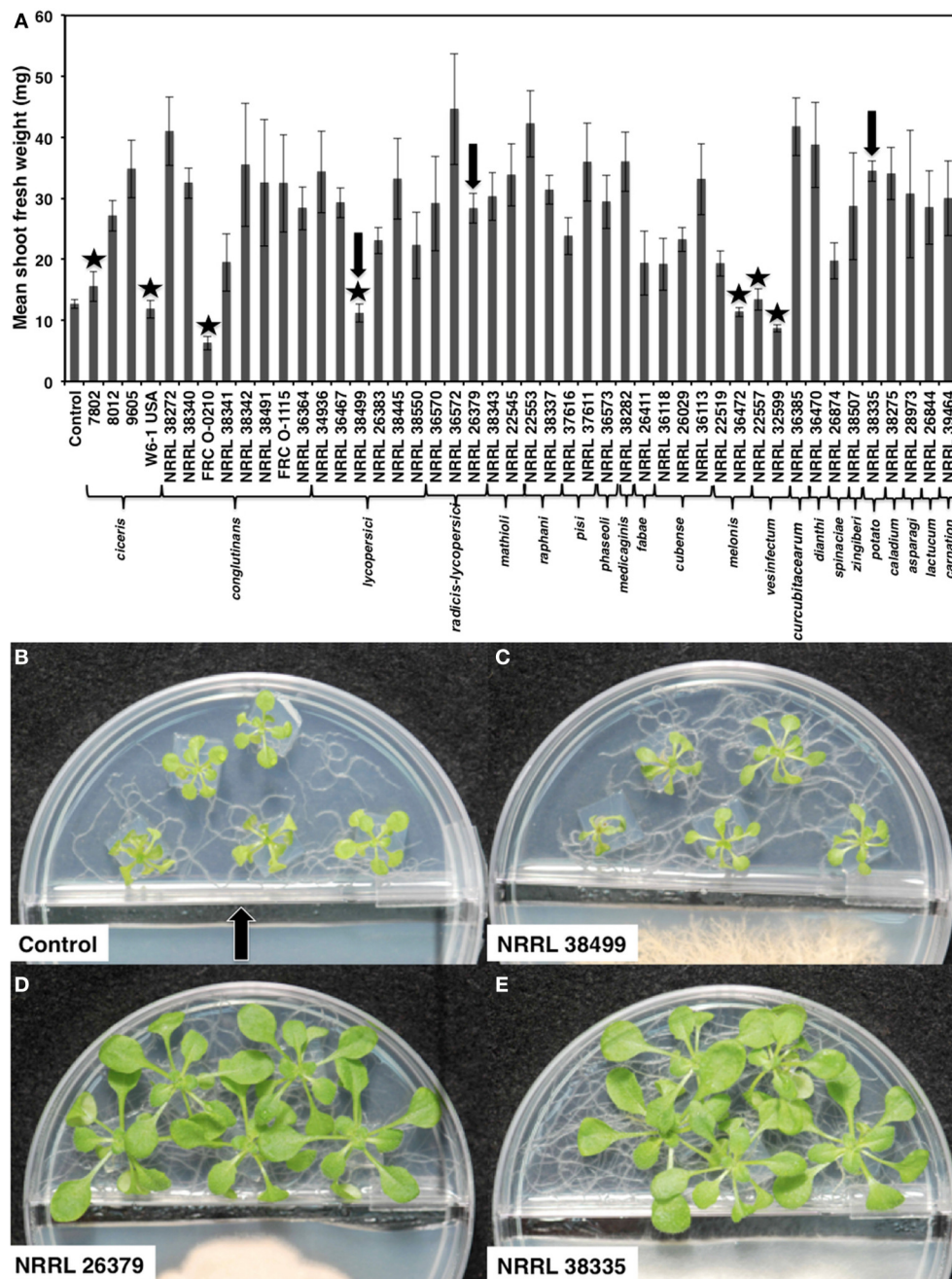
FIGURE 1 | Growth response of *A. thaliana* to *F. oxysporum* volatiles. (A) Mean shoot fresh weights of ecotype Col-0 cocultivated with no fungus (control) and 46 *F. oxysporum* strains individually for 2 weeks are shown. Means and standard errors for nine biological replicates per treatment, with five seedlings per replicate, are shown. Isolates that did not cause statistically significant growth enhancement compared to control (Tukey's test, α < 0.05) are marked with star. Arrows denote the three strains used for subsequent analyses. Images of seedlings after cocultivation with these three strains are shown in **(B–E)**. The center partition, marked by an arrow in **(B)** prevents physical contact between two compartments. A strip of fungal culture medium along the center partition was removed at the time of fungal inoculation to prevent fungal overgrowth to the plant side.

on an orbital shaker (75 rpm) at room temperature for 3 days. Control was un-inoculated PDB. Prior to ethylene measurement, the flasks were opened inside a laminar hood for 5 min to release any previously produced ethylene, resealed with sterile rubber stopper and incubated for 24 h. A 1 mL of headspace air was collected from each flask using a sterile syringe, then injected into a GC with a flame ionization detector (HP 6890). Fungal biomass was measured after filtering the culture in each flask through 25 μm filter paper (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) and dried at 65°C for 12 h. Ethylene production rate was

calculated as ethylene produced (μ L per liter) per hour per g of fungal mycelia. The experiment was conducted twice, with three biological replicates for each strain.

Statistical Analysis

Collected data were analyzed by one-way ANOVA analysis using SAS 9.3 statistical analysis software (SAS Institute, Cary, NC). Means were compared using a Tukey's test and statistical significance was evaluated using the alpha value ($\alpha < 0.05$).

RESULTS

Volatile Compounds Produced by Genetically and Phenotypically Diverse *F. oxysporum* Strains Enhanced the Growth of *A. thaliana* and Tobacco

We cocultivated 46 strains, representing 22 different *formae speciales* and phylogenetic lineages (O'Donnell et al., 2009), (**Table 1**) with *A. thaliana* ecotype Col-0 for 14 days to assess their ability to affect *A. thaliana* growth via volatile production (**Figure 1**). Compared with plants grown in the absence of fungal culture (control), 39 isolates (85%) significantly enhanced growth (Tukey's test with alpha value < 0.05). Two isolates, NRRL 32599 and FRC O-210, reduced shoot fresh weight by 50 and 25%, respectively, while five isolates caused no statistically significant effect. Two growth-enhancing isolates that exhibited the least variation, NRRL 26379 and NRRL 38335, and one neutral isolate, NRRL 38499, were chosen for further analyses.

As with Col-0, volatiles from NRRL 38499 did not enhance tobacco (*N. tabacum*) growth, but volatiles from NRRL 26379 and NRRL 38335 resulted in 2.5- and 3-fold shoot weight increases, respectively (**Figure 2**). Growth responses of nine additional *A. thaliana* ecotypes to volatiles from NRRL 26379 and NRRL 38335 were similar to those observed in Col-0 with a few exceptions (**Figure 3**). Ecotype C24 responded positively to NRRL 26379 but negatively to NRRL 38335 (**Figure 3** and Supplementary Figure 1). Ecotype Mh-0 was neutral to NRRL 26379 volatiles, but volatiles from NRRL 38335 inhibited its growth, whereas Ecotype Nd-0 responded positively to NRRL 26379 but appeared neutral to NRRL 38335.

Since elevated atmospheric CO_2 affects plant growth (Poorter and Navas, 2003; Baldwin, 2010), CO_2 produced by microbial culture could also enhance plant growth (Kai and Piechulla, 2009). To determine whether CO_2 produced by *F. oxysporum* culture contributed to plant growth, we compared Col-0 growth in the presence and absence of $Ba(OH)_2$, which removes CO_2 by forming $BaCO_3$ precipitate (Kai and Piechulla, 2009). We also compared the amounts of CO_2 produced by NRRL 38499, NRRL 26379, and NRRL 38335 by measuring the quantity of $BaCO_3$ formed, and found no significant differences among them (**Figure 4**). Col-0 seedlings grown without fungal culture but in the presence of $Ba(OH)_2$ displayed reduced shoot weight (by 55%) compared to those grown in the absence of $Ba(OH)_2$. The magnitude of reduction was similar (51%) for those cocultivated with NRRL 38499, whereas only slight reduction was recorded

in the seedlings cocultivated with NRRL 26379 and NRRL 38335 (**Figure 4** and Supplementary Figure 2).

F. oxysporum Volatiles Caused Multiple Changes in *A. thaliana*

To determine the time course of increase in shoot fresh weight, we harvested Col-0 seedlings after 5, 7, 10, and 14 days of cocultivation with NRRL 38499, NRRL 26379, and NRRL 38335 (Supplementary Figure 3). The growth response to NRRL 26379 and NRRL 38335 was not significantly different from that of the control and NRRL 38499 treatments up to day 10, but then growth of plants co-cultivated with NRRL 26379 and NRRL 38335 accelerated. To test if the degree of growth enhancement correlates with the duration of fungal volatile exposure, fungal culture was removed after cocultivation with these strains for 5, 7, and 10 days, but the seedlings were not harvested until day 14. For NRRL 38335, significant growth enhancement compared to control plants was noted at 7–10 days of cocultivation, while for NRRL 26379, it took 10–14 days of cocultivation (Supplementary Figure 4).

Compared to control plants, cocultivation with NRRL 26379 and NRRL 38335 increased the total leaf area by 2.7- and 4-fold, respectively, and the chlorophyll content by 3-fold (**Figure 5** and Supplementary Figure 5). However, RWC did not significantly differ amongst the treatments (Tukey's test with alpha value < 0.05). Cocultivation with NRRL 26379 and NRRL 38335 also increased the mass (4.8- and 4-fold, respectively) and total length (3.6- and 5.2-fold, respectively) of roots (**Figure 6** and Supplementary Figure 6). The lateral root density was more than doubled (**Figure 6**). The root to shoot ratio in plants cocultivated with NRRL 26379 and NRRL 38335 nearly doubled compared to control plants.

Growth Responses of *A. thaliana* Hormone Signaling Mutants Suggest the Involvement of Auxin Signaling in Mediating Growth Enhancement by *F. oxysporum* Volatiles

To elucidate the mechanism underlying *F. oxysporum* volatile-mediated growth enhancement, selected *A. thaliana* mutants that are defective in hormone signaling were exposed to *F. oxysporum* volatiles (**Figure 7**). Based on a previous study showing the involvement of auxin in bacterial volatile-mediated plant growth enhancement (Zhang et al., 2007), most of the chosen mutants are defective in auxin signaling. The *ETR1* gene encodes an ethylene receptor (Schaller et al., 1995; Chen et al., 2002). The *EIR1* gene, encoding a root specific auxin efflux carrier, is involved in gravitropism (Luschnig et al., 1998). The *AXR5* and *AUX1* genes encode a repressor of auxin response (Yang et al., 2004) and an auxin influx transporter (Yang et al., 2006), respectively. The *TIR1* gene encodes an F-box protein, a component of the ubiquitin ligase complex that degrades the AUX/IAA transcriptional repressor proteins, and is thus required for activating auxin-responsive genes (Dharmasiri et al., 2005; Kepinski and Leyser, 2005). The *GA3OX1* gene product catalyzes the first step in gibberellic acid biosynthesis (Mitchum
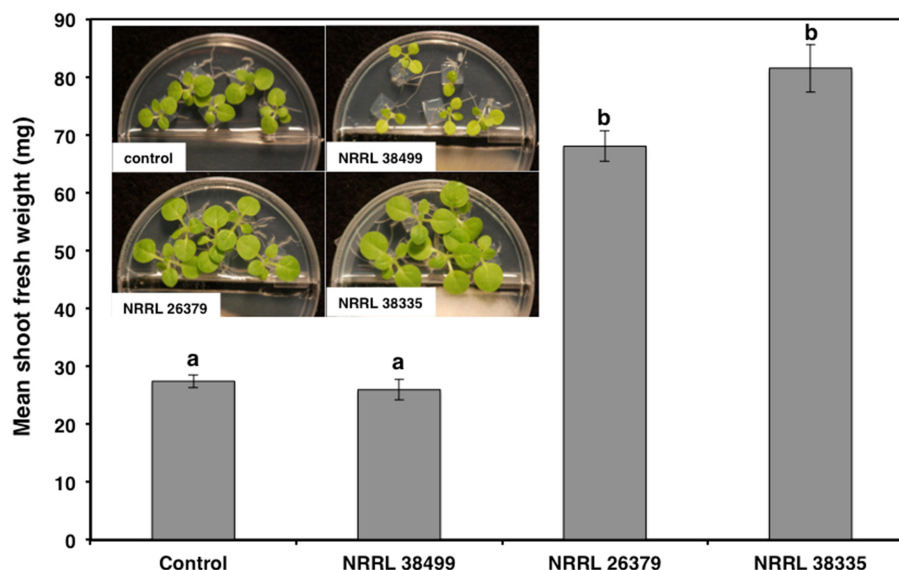
FIGURE 2 | Effect of *F. oxysporum* volatiles on *N. tabacum* growth. Shoot fresh weight was measured after 2 weeks of cocultivation with no fungus (control), NRRL 38499, NRRL 26379, and NRRL 38335. Representative plates are shown in the insert. Means and standard errors for nine biological replicates per treatment, with five seedlings per replicate, are shown. Different letters on columns represent statistically significant differences by one-way analysis of variance.
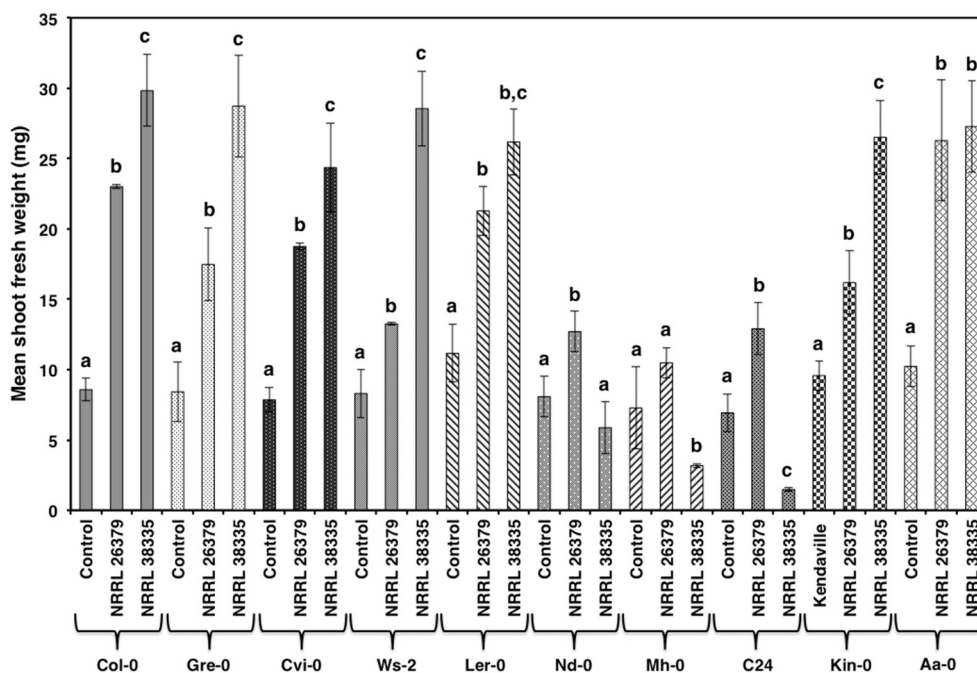
FIGURE 3 | Growth response of 10 *A. thaliana* ecotypes to *F. oxysporum* volatiles. The mean shoot fresh weight of each ecotype was measured after 2 weeks of cocultivation with no fungus (control), NRRL 26379, and NRRL 38335. Means and standard errors for six biological replicates per treatment, with five seedlings per replicate, are shown. Different letters on columns represent statistically significant differences within each ecotype by one-way analysis of variance. Photos of ecotype C24 after this experiment are shown in Supplementary Figure 1.

et al., 2006). The mutants *etr1, eir1, ga3ox1,* and *axr5* looked comparable to Col-0, upon cocultivation with NRRL 26379 and NRRL 38335 (**Figure 7**). However, *aux1* and *tir1* did not exhibit significant growth enhancement (**Figure 7**).

We also used transgenic Col-0 carrying the *DR5::GUS* reporter, which has been widely used for monitoring the spatial and temporal patterns of auxin response (Dubrovsky et al., 2008; Lucas et al., 2008; Péret et al., 2009). The stain permitted easy
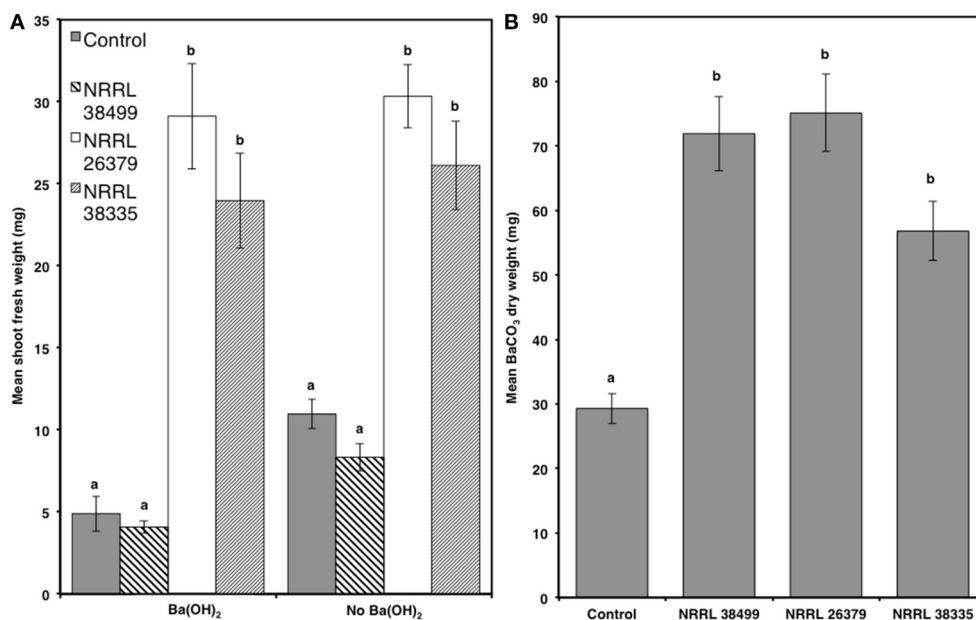
FIGURE 4 | Effect of CO_2 produced by fungal culture on plant growth. (A) Mean shoot fresh weights of Col-0 plants after 2 weeks of cocultivation with NRRL 38499, NRRL 26379, and NRRL 38335 in Y-plate in the presence and absence of $Ba(OH)_2$ are shown. **(B)** Quantities of CO_2 produced by these strains wereestimated by measuring the amount of dried $BaCO_3$ precipitant formed during cocultivation. Means and standard errors for six biological replicates per treatment, with three seedlings per replicate, are shown. Different letters on columns represent statistically significant differences by one-way analysis of variance.
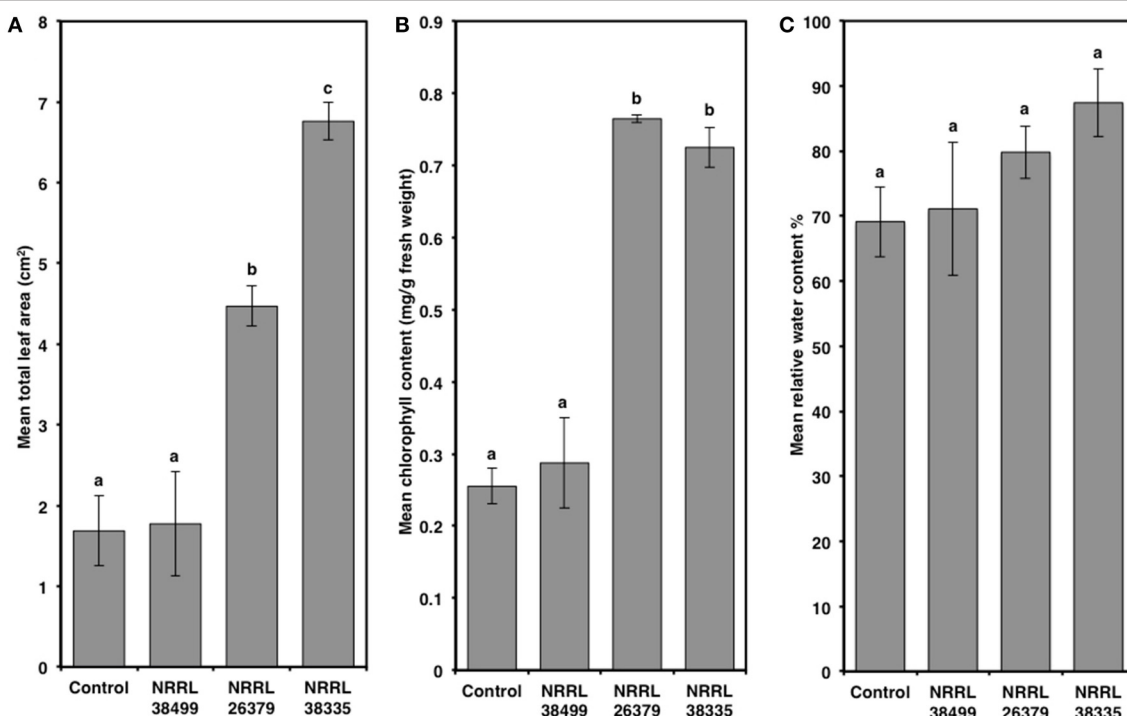
FIGURE 5 | Alterations of leaves caused by *F. oxysporum* volatiles. (A) total leaf area, **(B)** chlorophyll content, and **(C)** relative water content in Col-0 leaves after 2 weeks of cocultivation with no fungus (control), NRRL 38499, NRRL 26379, and NRRL 38335 are shown. Means and standard errors for four biological replicates per treatment, with five seedlings per replicate, are shown. Different letters on columns represent statistically significant differences by one-way analysis of variance. Scanned images of representative leaves used for **(A)** are shown in Supplementary Figure 5.
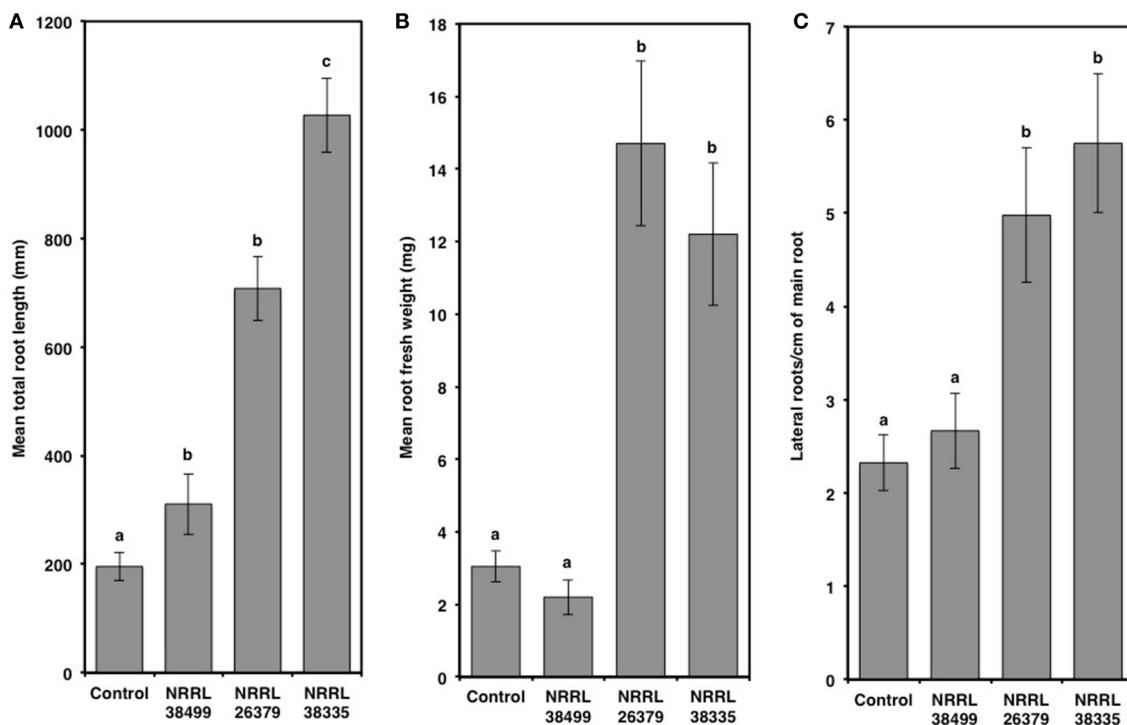
FIGURE 6 | Root alterations caused by *F. oxysporum* volatiles. (A) total root length, **(B)** root fresh weight, and **(C)** lateral root density of Col-0 were quantified after 14 days of cocultivation with no fungus (control), NRRL 38499, NRRL 26379, and NRRL 38335. Means and standard errors for three biological replicates per treatment, with five seedlings per replicate, are shown. Different letters on columns represent statistically significant differences by one-way analysis of variance. Supplementary Figure 6 shows representative roots collected after this experiment.
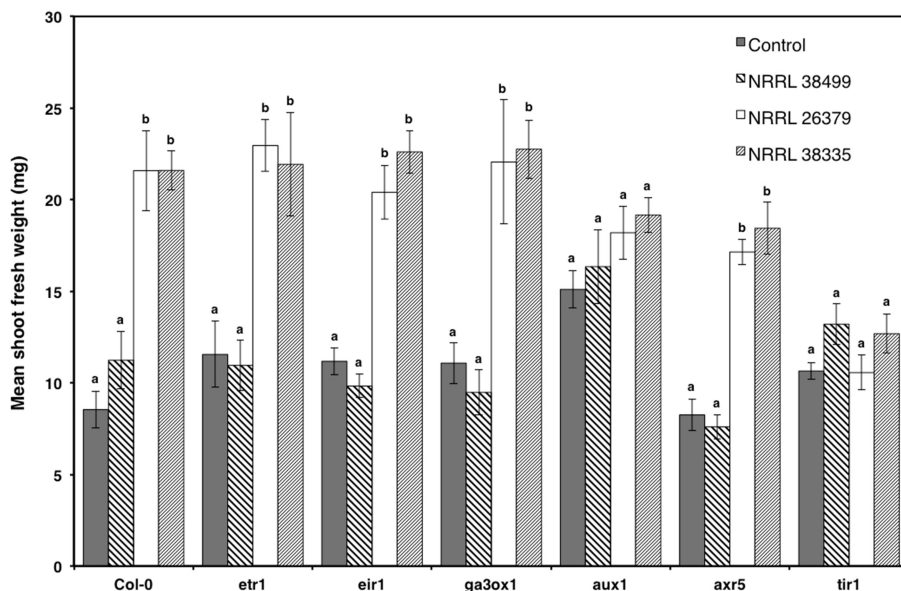
FIGURE 7 | Growth response of *A. thaliana* hormone signaling mutants to *F. oxysporum* volatiles. Growth response of *six* mutants to volatiles produced by NRRL 38499, NRRL 26379, and NRRL 38335 are shown. Means and standard errors for two biological replicates per treatment, with five seedlings per replicate, are shown. Different letters on columns represent statistically significant differences within each mutant line by one-way analysis of variance.

counting of lateral root tips and primordia. Consistent with the data shown in **Figure 6**, the density of lateral roots in *DR5::GUS* plants became significantly higher when cocultivated with NRRL 26379 and NRRL 38335 for 7 and 14 days (**Figure 8** and Supplementary Figure 7). GUS activity was primarily localized at root tips and lateral root primordia in all treatments, but the magnitude of resulting GUS staining varied among strains (Supplementary Figures 7, 8). At 7 days after cocultivation with NRRL 26379 and NRRL 38335, straining was stronger and more extensive than that observed in plants cocultivated with no fungus and NRRL 38499 (Supplementary Figure 8). However, at 14 days after cocultivation, no obvious difference was observed between these groups. Shoot growth, root development and GUS activity were also analyzed in the presence of different concentrations of 1-naphthylphthalamic acid (NPA), an auxin efflux inhibitor (Keller et al., 2004). In the presence of $1\,\mu$ M NPA, the lateral root density of plants cocultivated with NRRL 26379 and NRRL 38335 still remained significantly higher than that observed in the other two treatments at both days 7 and 14 (**Figure 8** and Supplementary Figure 9). However, in the presence of $5\,\mu$ M NPA, lateral root formation was drastically reduced in all treatments, and GUS staining was reduced compared to that observed with no or $1\,\mu$ M NPA (**Figure 8** and Supplementary Figure 9). Growth enhancement by NRRL 26379 and NRRL 38335 volatiles was not affected by $1\,\mu$ M NPA, but at higher concentrations (5 and $10\,\mu$ M), the growth enhancement effect was abolished (**Figure 9**). We also determined whether growth enhancement of tobacco plants by *F. oxysporum* volatiles was affected by NPA (Supplementary Figure 10). Although tobacco plants were less sensitive to NPA than *A. thaliana*, in the presence of 5 and $10\,\mu$ M NPA, growth enhancement by NRRL 26379 and NRRL 38335 volatiles was significantly reduced.

Volatile Compounds Produced by *F. oxysporum*

Analysis of volatile compounds produced by NRRL 38499, NRRL 26379 and NRRL 38335, using GC-MS, led to the tentative identification of several compounds (**Table 2** and **Figure 10**), but many others remain to be identified. Interestingly, NRRL 38499, which did not enhance plant growth, produced more diverse compounds and higher quantities of many compounds than NRRL 26379 and NRRL 38335. The most abundant classes of volatile compounds were sesquiterpenes and diterpenes, most of which remain unidentified. NRRL 38499 produced many more sesquiterpenes and diterpenes than NRRL 26379 or NRRL 38335. All three isolates produced compounds that have been shown to inhibit plant growth, such as 3-methyl-1-butanol, 1-hexanol, 1-octen-3-ol and 3-octanone. Even though no compounds uniquely produced by both NRRL 26379 and NRRL 38335 were detected, NRRL 26379 produced 1-2 diterpenes that appeared absent in NRRL 38335. These patterns suggested the possibility that all three strains produced plant growth enhancing volatile(s), but NRRL 38499 might also produce compounds that inhibit plant growth, thus negating growth enhancement effect or even stunting plant growth. Comparison of ethylene production among the three strains did not reveal significant differences

**TABLE 2 | Identified *F. oxysporum* volatile compounds.**

Volatile compounds	Strains		
	NRRL 38499	NRRL 38335	NRRL 26379
*3-methyl-1-butanol	✓	✓	✓
2-methyl-1-butanol	✓	✓	✓
E-2-hexanal	✓	✓	✓
*1-hexanol	✓	✓	✓
3-methyl-1-butanol acetate	✓	✗	✗
2-methyl-1-butanol acetate	✓	✗	✗
Benzaldehyde	✓	✓	✓
ethyl 2-hydroxy-3-methyl butyrate	✓	✗	✗
*1-octen-3-ol	✓	✓	✓
*3-octanone	✓	✓	✓
Hexyl Acetate	✓	✗	✗
D-limonene	✗	✓	✓
1-ethyl-4-methoxy-benzene	✓	✓	✓
1-ethyenyl-4-methoxybenzene	✓	✗	✗
2-undecanone	✓	✗	✗
Acora-3,7 (14)-diene	✓	✗	✗
α-cedrene	✓	✓	✗
*β-caryophylene	✓	✓	✗
β-cedrene	✓	✓	✗
γ-curcumene	✓	✓	✗
cis-β-guaiene	✓	✓	✓
3-(Z)-cembrene A	✓	✗	✗

✓ and ✗ *denote the presence and absence, respectively, of each compound.*
* *Compounds with known/suspected effect on plant growth.*

(Supplementary Figure 11), suggesting that ethylene is not one such compound.

DISCUSSION

Both plants and microbes employ multiple strategies to coordinate or take control of their interactions, resulting in diverse forms of association ranging from mutualistic to pathogenic. One strategy that has evolved convergently in multiple microbial kingdoms is secreting or injecting a variety of effectors that manipulate plant defense machineries. Secretion of effectors is not limited to pathogens, e.g., symbiotic associations also require suppression of host defense by microbial partners (Evangelisti et al., 2014). Effectors include proteins (Deslandes and Rivas, 2012; Okmen and Doehlemann, 2014; Rovenich et al., 2014), metabolites (Collemare and Lebrun, 2011), and nucleic acids (Weiberg et al., 2014), but compared to research on protein effectors, work on the other types of effectors lags considerably behind. In addition to effectors, microbes also secrete various molecules that affect plant health both directly and indirectly (Bonfante and Anca, 2009; Bednarek et al., 2010), such as siderophores for iron acquisition, enzymes for nutrient mobilization and antibiotics. Because volatile metabolites can travel through air and porous soils, they can potentially function as signals for both short- and long-range organismal interactions even in the absence of water as medium. We tested the hypothesis
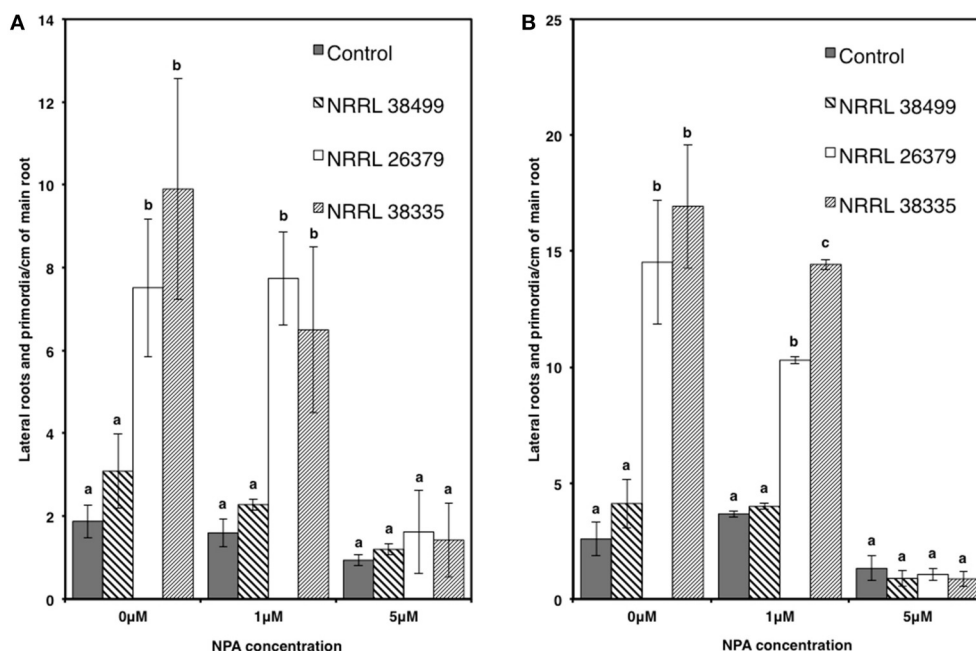
FIGURE 8 | Effect of *F. oxysporum* volatiles on *A. thaliana* lateral root development in the presence of NPA. Lateral root density (both lateral roots and lateral root primordia) of Col-0 containing *DR5::GUS* in the presence of 0, 1, and 5 μ M NPA was determined after cocultivation with no fungus (control), NRRL 38499, NRRL 26379, and NRRL 38335 for 7 **(A)** and 14 **(B)** days. Means and standard errors for two biological replicates per treatment, with five seedlings per replicate, are shown. Different letters on columns denote statistically significant differences within each treatment by one-way analysis of variance.
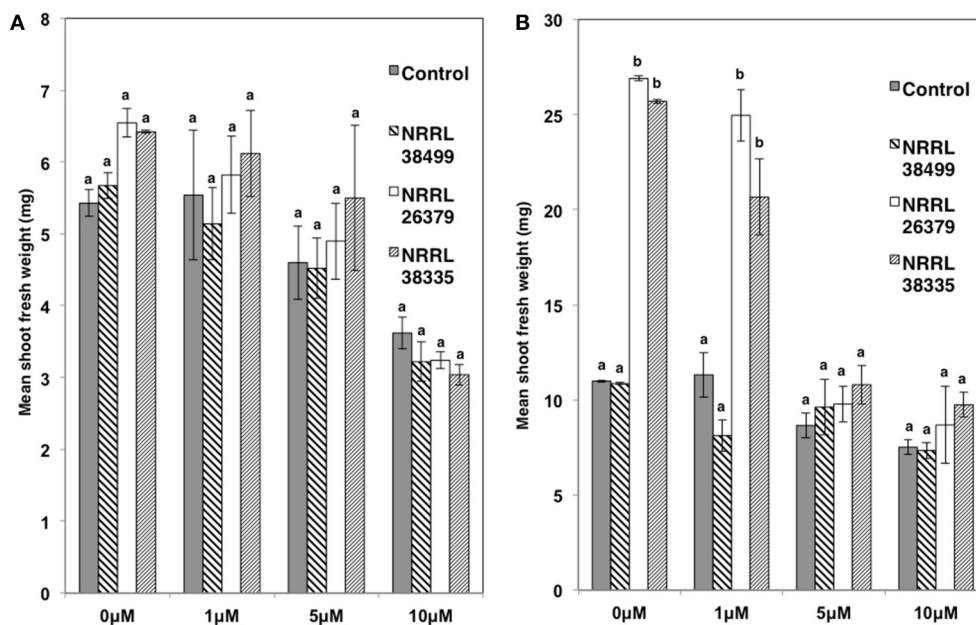
FIGURE 9 | Effect of NPA on volatile-mediated growth enhancement. The shoot fresh weight of Col-0 containing *DR5::GUS* was measured after cocultivation with no fungus (control), NRRL 38499, NRRL 26379, and NRRL 38335 for 7 **(A)** and 14 **(B)** days in the presence of NPA. Means and standard errors for three biological replicates per treatment, with five seedlings per replicate, are shown. Different letters on columns denote statistically significant differences within each NPA treatment by one-way analysis of variance.
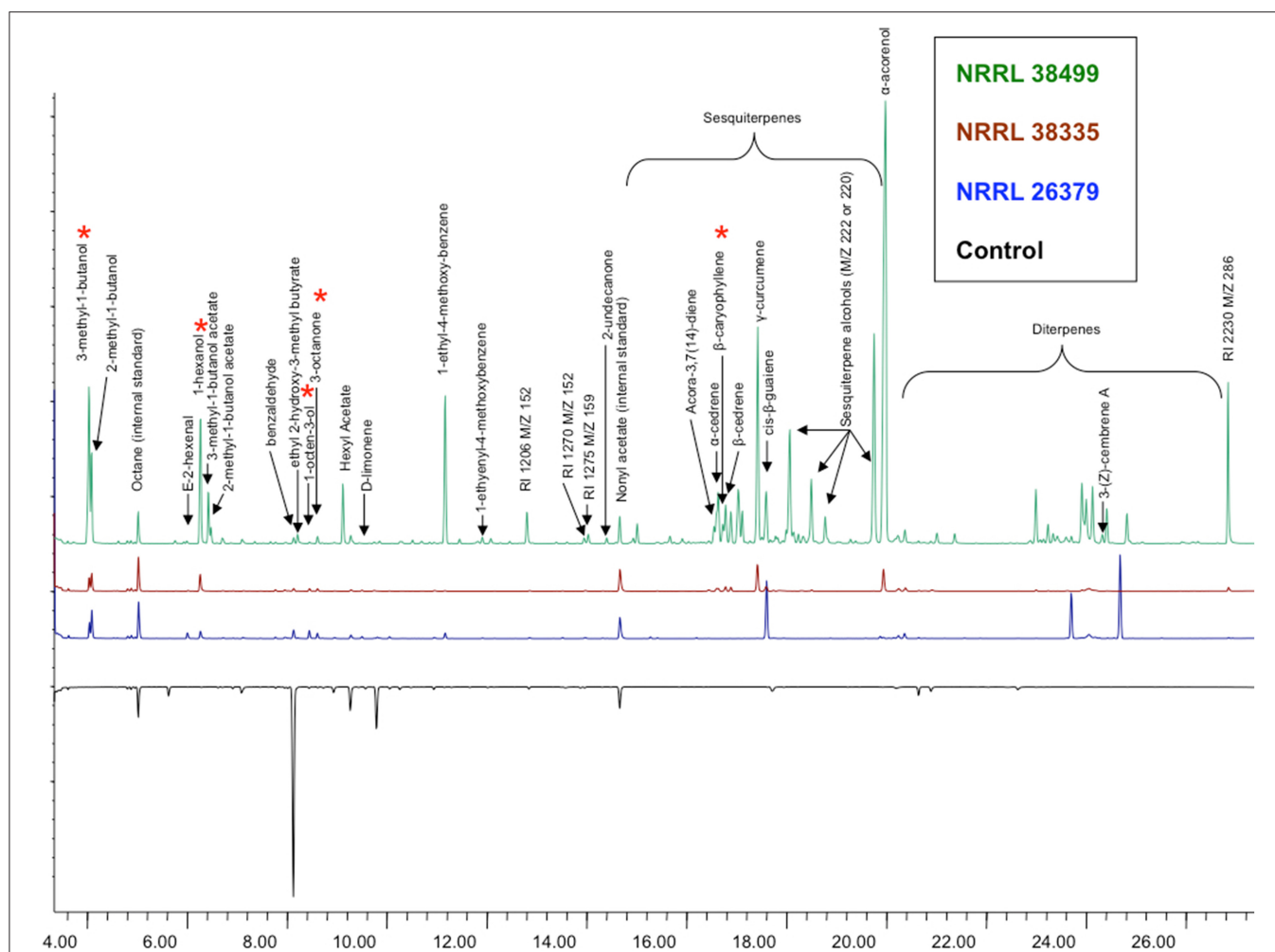
FIGURE 10 | Volatile compounds produced by *F. oxysporum*. Headspace volatiles from no fungus (bottom, control), NRRL 38499, NRRL 26379, and NRRL 38335 cultured on PDA were analyzed using GC-MS. Asterisks denote compounds that have been shown to affect plants in previously published studies.

that certain volatiles produced by plant-associated fungi affect their interaction with plants.

Multifaceted Roles of *F. oxysporum* Volatiles?

Potential benefits of producing volatiles that enhance plant growth are likely to be found in *F. oxysporum*'s main ecological niches, the rhizosphere and within plants. The rhizosphere offers rich nutrient sources for microflora (e.g., root exudates, mucilage, dead root cells). Accordingly, the rhizosphere harbors diverse groups of microbes and serves as their "playground and battlefield" (de Weert and Bloemberg, 2006; Raaijmakers et al., 2009). Due to competition among rhizosphere dwellers and antimicrobials released by plants, the membership to this community (i.e., rhizosphere competency) requires a few prerequisites (de Weert and Bloemberg, 2006). The frequent presence of *F. oxysporum* in the rhizosphere of diverse plants, including those that are not considered as their hosts (Malcolm et al., 2013), indicates its broad rhizosphere competency. In this

study we showed that volatiles from genetically and ecologically diverse strains (**Table 1**) significantly enhanced plant growth (**Figures 1–3**). This ability likely contributes to its rhizosphere competency by increasing nutrient availability, since enhanced root growth (e.g., **Figure 6**) would be expected to increase plant-derived exudates as well as root tissue for potential colonization.

Another potential benefit comes from the root developmental change caused by its volatiles. Volatiles from NRRL 26379 and NRRL 38335 not only increased the root biomass but also doubled the density of lateral roots (**Figures 6**, **8** and Supplementary Figure 7). Root penetration by *F. oxysporum* occurs primarily through the meristematic region of primary and lateral roots (Bishop and Cooper, 1983; Turlier et al., 1994; Czymmek et al., 2007). For pathogenic strains, increased formation of lateral roots will likely facilitate infection by presenting more potential entry points to the vascular system. NRRL 38272, a previously characterized pathogenic strain of *A. thaliana* (Ospina-Giraldo et al., 2003; Czymmek et al., 2007), as well as most other strains that are likely to be pathogenic

to crucifer plants (formae speciales *conglutinans, mathioli,* and *raphani*), enhanced *A. thaliana* growth (**Figure 1**). Since the integrity of adjacent cortical cells is disrupted during hyphal growth of *F. oxysporum* through the vascular system (Czymmek et al., 2007), another possible role of volatile compounds is to act as phytotoxic virulence factors.

Which Compounds Affect Plant Growth?

Volatile profiles (**Figure 10** and **Table 2**) present an enigma, since both growth-promoting and growth-inhibiting compounds were detected, along with compounds with unknown effects. NRRL 38499, but not growth-enhancing strains NRRL 26379 and NRRL 38335, seems to produce β-caryophyllene, a sesquiterpene that was previously reported to enhance lettuce growth (Minerdi et al., 2011). In addition, NRRL 38499 produced more compounds in both the number and quantity than NRRL 26379 and NRRL 38335, and no compounds appear unique to the latter two. These patterns raised the possibility that NRRL 38499 might produce both growth-inhibitory and -stimulatory compounds, thus making their net effect on plants neutral. Although specific compound(s) that are inhibitory remain to be confirmed, previous studies suggest some candidates, such as 3-methyl-1-butanol, 1-hexanol, 1-octen-3-ol, and 3-octanone (**Figure 10**). Volatiles produced by truffles, which inhibited *A. thaliana* growth, included these compounds, and their synthetic versions also inhibited *A. thaliana* growth (Splivallo et al., 2007). Additional candidates include volatile terpenes. Some terpenes have been shown to inhibit the growth of other organisms, including plants (Collado et al., 2007; Kramer and Abraham, 2012). More abundant production of volatile sesquiterpenes and diterpenes by NRRL 38499 than NRRL 26379 and NRRL 38335 (**Figure 10**) suggests that some of these terpenes may inhibit plant growth. An attempt to test this supposition by disrupting the production of these groups of terpenes in NRRL 38499 via targeted mutagenesis of their biosynthetic genes is in progress.

It was suggested that promotion of plant growth by microbial volatiles could simply be a result of CO_2 enrichment during cocultivation (Kai and Piechulla, 2009). We found no significant difference in CO_2 production among growth-promoting and neutral strains of *F. oxysporum*. Removal of CO_2 using $Ba(OH)_2$ reduced the growth of *A. thaliana* without fungus and cocultivated with NRRL 38499 (**Figure 4**), indicating that carbon fixation via photosynthesis still limits plant growth even when plants are cultured on sucrose-containing medium. However, no significant reduction in growth was observed for plants cocultivated with NRRL 26379 and NRRL 38335 (**Figure 4**), arguing against the proposition that increased CO_2 production solely drove plant growth enhancement. Higher chlorophyll in the plants exposed to volatiles from NRRL 26379 and NRRL 38335, but not by volatiles from NRRL 38499 suggests more active photosynthesis in plants cocultivated with the growth-promoting strains.

Mechanism Underpinning Growth Enhancement by *F. oxysporum* Volatiles

Manipulation of auxin transport and signaling by *F. oxysporum* volatiles seems to underpin growth enhancement (**Figures 7–9**

and Supplementary Figures 7–9). Mutation of either *AUX1*, encoding an auxin influx transporter (Yang et al., 2006) or *TIR1*, encoding an auxin receptor that regulates the expression of auxin-responsive genes (Dharmasiri et al., 2005; Kepinski and Leyser, 2005), negated growth promotion by *F. oxysporum* volatiles (**Figure 7**). In addition, chemical inhibition of auxin efflux blocked volatile-mediated growth promotion in both *A. thaliana* and tobacco (**Figures 8, 9**). Temporal and spatial patterns of GUS activity in *DR5::GUS* plants indicated earlier (before shoot biomass enhancement became visible) and stronger activation of lateral root development via auxin signaling (Supplementary Figures 7–9). Volatiles from other microbes that enhanced plant growth similarly affected root growth and development (Zhang et al., 2007; Gutierrez-Luna et al., 2010; Hung et al., 2013). *Bacillus subtilis* volatile-mediated growth enhancement of *A. thaliana* was also blocked by NPA (Zhang et al., 2007), suggesting that manipulation of auxin transport and signaling using volatiles is a mechanism employed by diverse microbes. As discussed above, this manipulation likely contributes to the fitness of plant-associated microbes.

Kidd et al. (2011) showed that several genes involved in auxin signaling were differentially regulated in response to *F. oxysporum* infection. In addition, mutants in some of these genes, as well as several other mutants defective in auxin transport or signaling, were more resistant to *F. oxysporum*. Inhibition of auxin efflux by NPA and 2,3,5-trioidobenzoic acid also enhanced resistance. Collectively, these results suggest that *F. oxysporum* exploits auxin transport and signaling to facilitate infection in *A. thaliana*. Clearly, *F. oxysporum* volatiles are candidate signals for this manipulation.

FUTURE DIRECTIONS

Exploration of plant-microbe communication through volatile compounds will likely reveal novel mechanisms underpinning plant-microbe interactions and offer potential applications (Morath et al., 2012; Bitas et al., 2013). Given the vast diversity of fungi associated with plants and their seemingly critical, yet mostly underexplored, roles in plant health, concerted efforts are needed to understand if and how volatiles produced by such fungi affect plant growth and how such molecules are perceived and processed by plants. Another key question is whether volatiles from *F. oxysporum* and other fungi also affect other traits. Several studies showed that volatiles produced by several bacterial species enhanced both plant growth and stress resistance (Bitas et al., 2013). Since increased plant growth supports microbial growth in the rhizosphere, enhanced resistance to biotic and abiotic stress also provides improves potential habitat for the fungus under unfavorable environmental conditions. In addition, as discussed above, manipulation of plant growth and development via volatile production may also facilitate infection. Identification of specific volatile compound(s) that affect plant growth and their biosynthetic pathways in *F. oxysporum* is a key step to address these questions. Exposure of plants to synthetic versions of candidate compounds has been employed to identify bacterial and fungal volatiles compounds that enhance plant growth (e.g., Ryu et al., 2003; Splivallo et al., 2007). Although this approach

is not sufficient for proving their involvement in affecting plant growth in the field, it helps identify candidate compounds for further research. Knowledge of the identity and biosynthetic pathways for fungal volatile semio-chemicals, in combination with targeted genetic manipulation, will help confirm their involvement in plant growth via disruption of their biosynthesis. Ryu et al. (2003) used this approach to show the involvement of two compounds, acetoin and 2,3-butanediol, in causing growth enhancement by *Bacillus* spp.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.01248

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Trichoderma volatiles effecting Arabidopsis: from inhibition to protection against phytopathogenic fungi

Metwally Kottb[1][†], Tamara Gigolashvili[2], Dominik K. Großkinsky[3, 4] and Birgit Piechulla[1]*

[1] Institute for Biological Sciences, University of Rostock, Rostock, Germany, [2] Biocenter, Botanical Institute and Cluster of Excellence on Plant Sciences, University of Cologne, Cologne, Germany, [3] Department of Plant and Environmental Sciences, Copenhagen Plant Science Centre, University of Copenhagen, Taastrup, Denmark, [4] Institute of Plant Sciences, University of Graz, Graz, Austria

Trichoderma species are present in many ecosystems and some strains have the ability to reduce the severity of plant diseases by activating various defense pathways via specific biologically active signaling molecules. Hence we investigated the effects of low molecular weight volatile compounds of *Trichoderma asperellum* IsmT5 on *Arabidopsis thaliana*. During co-cultivation of *T. asperellum* IsmT5 without physical contact to *A. thaliana* we observed smaller but vital and robust plants. The exposed plants exhibit increased trichome numbers, accumulation of defense-related compounds such as H_2O_2, anthocyanin, camalexin, and increased expression of defense-related genes. We conclude that *A. thaliana* perceives the *Trichoderma* volatiles as stress compounds and subsequently initiates multilayered adaptations including activation of signaling cascades to withstand this environmental influence. The prominent headspace volatile of *T. asperellum* IsmT5 was identified to be 6-pentyl-α-pyrone (6PP), which was solely applied to *A. thaliana* to verify the growth and defense reactions. Most noticeable is that *A. thaliana* preexposed to 6PP showed significantly reduced symptoms when challenged with *Botrytis cinerea* and *Alternaria brassicicola*, indicating that defense-activated plants subsequently became more resistant to pathogen attack. Together, these results support that products that are based on *Trichoderma* volatiles have the potential being a useful biocontrol agent in agriculture.

Keywords: *Trichoderma asperellum* IsmT5, *Botrytis cinerea*, *Alternaria brassicicola*, *Arabidopsis thaliana*, mVOCs, 6-pentyl-α-pyrone, glucosinolates, camalexin

Introduction

The genus *Trichoderma* (telemorph Hypocrea) includes cosmopolitan soil-borne species, some of them are saprophytes and are frequently isolated from soil and wood as well as plant litter (Błaszczyk et al., 2011). Other species were detected inside of root tissues of many plants as opportunistic, avirulent symbionts (Harman et al., 2004). *Trichoderma longibraciatum,* an example

Abbreviations: VOCs, Volatile organic compounds; 6PP, 6-pentyl-α-pyrone; gus, glucoronidase; ROS, reactive oxygen species, SA, salicylic acid; JA, jasmonic acid; ABA, abscisic acid.

of a human pathogen, was isolated from infected tissues of immunocompromised individuals, who suffered opportunistic infections (Kuhls et al., 1999). Together, this reflects a large distribution and a pronounced adaptability of this genus to live in different habitats. Furthermore, *Trichoderma* species play an important role in the health of an ecosystem (Klein and Eveleigh, 1998) and since at least the 1930s species are known and used as biocontrol agents (plant growth promoting fungi) to reduce the severity of plant diseases (Weindling, 1932) and subsequently increase yields (Harman et al., 2004; Lorito et al., 2010). These beneficial effects were related to the control of deleterious soil microflora, the degradation of toxic compounds, the direct stimulation of root development by the production of phytohormones, enhanced solubility and subsequent increase in the availability of phosphorus and several micronutrients due to the presence of *Trichoderma* (Altomare et al., 1999; Gravel et al., 2007; Bae et al., 2009; Contreras-Cornejo et al., 2009; Martínez-Medina et al., 2011; Vos et al., 2015). Some effective *Trichoderma* strains were shown to produce a variety of microbe-associated molecular patterns (MAMPs) (Vinale et al., 2012). The first recognized MAMP was identified as an ethylene induced xylanase 2 (Xyn2/Eix) which is a potent elicitor of plant defense responses in specific tobacco and tomato cultivars (Rotblat et al., 2002). Plants colonized by *Trichoderma* species, or treated by e.g., cellulases, 18-mer peptaibols, harzianolide, and harzianopyridone, provide resistance to a wide variety of pathogenic microorganisms (Hermosa et al., 2012). According to Harman et al. (2004) each of the above mentioned activation process begins with the colonization of the plant roots by *Trichoderma* spp. *Trichoderma* species that are able to establish such interactions, induce massive changes in their transcriptome and metabolism (Reino et al., 2008; Brotman et al., 2010) and such metabolites have been found not only to directly inhibit the growth of pathogenic microorganisms but also increase disease resistance by triggering the defense system in plants (induced systemic resistance, ISR) (overview Vos et al., 2015). Furthermore, metabolite-pretreated plants responded to a pathogen attack much faster or more intensively (Verhagen et al., 2004; Shoresh et al., 2010; Verhage et al., 2010; Hermosa et al., 2012), a mechanism known as priming (Conrath, 2011).

The application of compounds originating from biological sources (biological control agents) and the development of novel sustainable crop protection strategies to reduce the usage of pesticides, bactericides and fungicides in agriculture are increasingly demanded by consumers. *Trichoderma* has the potential to find broad application, because it is already used due to its high mycoparasitic and antibiotic potential against different plant pathogens (review Vos et al., 2015). More than 60% of the registered biopesticides are based on *Trichoderma* (Verma et al., 2007).

We propose in a new and innovative hypothesis that volatile compounds emitted by *Trichoderma* facilitate the distribution of beneficial effects over long distances and that they may contribute to the improvement of plant growth. In the past decade progress has been made in understanding the role of microbial volatiles in multitrophic interactions and their potential functions (e.g., Kai et al., 2007, 2009; Vespermann et al.,

2007; Minerdi et al., 2009; Wenke et al., 2010; Blom et al., 2011; Junker and Tholl, 2013; Naznin et al., 2013; D'Alessandro et al., 2014; Piechulla and Degenhardt, 2014). At present ca. 10,000 procaryots were identified, but only less than 500 bacterial and fungal species have been investigated regarding their potential to emit VOCs (Effmert et al., 2012; Lemfack et al., 2013). VOCs belong to different chemical classes, e.g., mono- and sesquiterpenes, alcohols, ketones, lactones, esters, thioalcohols, thioesters, and cyclohexanes (Splivallo et al., 2011; Kramer and Abraham, 2012; Lemfack et al., 2013). Due to their economical importance the volatile profiles of the prominent truffles were studied in detail and it was shown that the volatiles of *Tuber borchii*, *Tuber indicum*, and *Tuber melanosporum* inhibit leaf growth and root development of *A. thaliana* (Splivallo et al., 2007). Many fungi produce 1-octeno-3-ol, which enhances plant resistance to the necrotrophic fungus *Botrytis cinerea* by inducing defense signaling cascades (Kishimoto et al., 2007; Contreras-Cornejo et al., 2014). Volatiles of *Alternaria alternata*, *Penicillium charlesii,* and *Penicillium aurantiogriseum* promote growth and starch accumulation in several plant species (Ezquer et al., 2010).

As mentioned above, *Trichoderma* has numerous ways of indirectly enhancing plant growth (Vos et al., 2015) however so far very limited information is available upon volatile-based interactions in the context of plant growth promotion. *Trichoderma viride* for example stimulated the growth of *A. thaliana* in the absence of direct physical contact and increased lateral root formation and established early-flowering phenotypes (Hung et al., 2013). It was also shown that volatiles of *Trichoderma* act antibiotically against pathogenic fungi and thereby confer plant growth promotion (Vinale et al., 2008a). These hints stimulated us to screen several *Trichoderma* species for VOCs-mediated effects and that subsequently one specific strain was chosen to investigate thoroughly the morphological, physiological and molecular alterations in *A. thaliana* upon co-cultivation with *Trichoderma asperellum* IsmT5. These studies included the monitoring of growth and defense reactions in the plants under normal and challenged conditions.

Material and Methods

Biological Materials

The wild type *Arabidopsis thaliana* (Col-0) was used in all experiments. Seeds were kindly provided by Dr. Zhonglin Mou (Microbiology Department, University of Florida, Gainesville, FL, USA). Transgenic lines were obtained from: DR5::GUS from Dr. Zsuzsanna Kolbert (Department of Plant Biology, Faculty of Science and Informatics, University of Szeged, Szeged, Hungary), PDF1.2::GUS from Dr. Anja van Dijken (Utrecht University, The Netherlands), pYUC8::GUS from Dr. Stephan Pollmann (Metabolomics Unit at the Center of Plant Biotechnology and Genomics, Madrid, Spain), and PR1::GUS from the European Arabidopsis Stock Centre (UK; http://arabidopsis.info/).

Trichoderma species used in this study were isolated from different locations in Egypt during January 2005–January 2006. *T. asperellum* IsmT5 was isolated from the rhizosphere of maize cultivated in Ismailia, *Trichoderma harzianum* was isolated from okra roots cultivated in Serabium village, and *Trichoderma spp.*

was isolated from soil in Sinai (Wadi El Arbeen). *T. asperellum* IsmT5 Samuels, Lieckf. and Nirenberg was identified and deposited at The Centraalbureau voor Schimmelcultures (Applied and Industrial Mycology/Identification Service CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands) under accession number CBS 137093 (Data sheets 1 and 2 in Supplementary Material).

Alternaria brassicicola was obtained from Dr. Eckehard Koch (Julius-Kühn-Institute, Braunschweig, Germany) and *Botrytis cinerea* from Dr. Andreas v. Tiedemann (University of Göttingen, Germany).

Trichoderma—Plant Co-cultivation

The effects of volatiles of *Trichoderma* sp. on plant growth were tested in a closed and open co-cultivation system (Figure S1A). *A. thaliana* seeds were surface sterilized (1 min 70% ethanol, 5 min 5% calcium hypochlorite, rinsed four times with sterilized distilled water) and cultivated on MS medium (Murashige and Skoog, 1962). The seeds were vernalized for 3 days at 4°C in the absence of light and then four seedlings were transferred to glass jars containing solidified MS medium in slant position for 3 days. At this time a disc ($\varnothing$ 0.5 cm) of a 7 days old *Trichoderma* culture grown on nutrient broth agar (30 g glucose; 2 g $NaNO_3$; 1 g KH_2PO_4; 1 g yeast extract; 2 g peptone; 0.5 g KCl; 0.5 g $MgSO_4 \cdot 7H_2O$; 8 mg $CaCl_2 \cdot 6H_2O$; 1 mg $ZnSO_4 \cdot 7H_2O$; 10 mg $FeSO_4 \cdot 7H_2O$ per liter) (Bonnarme et al., 1997) at 20°C was introduced into a small beaker containing 20 ml broth agar. The small beaker was placed into the jar without any physical contact to the plants or MS agar. The jar was placed in 3 cm distance to the plants. The seedlings were exposed to *Trichoderma* volatiles for 9 days at 24°C and 84 $\mu mol m^{-2} s^{-1}$ of light at a 16/8 h light/dark cycle. Under these growth conditions *Trichoderma* did not produce spores and fungal growth was not observed in control experiments in MS agar. Fresh weight, leaf area and root length were determined four times (technical replicates). The experiments were repeated three times (biological replicates).

Analysis of Anthocyanins

The accumulation of anthocyanins in *Arabidopsis* seedlings was determined after 9 days of co-cultivation with *T. asperellum* IsmT5 applying the method of Neff and Chory (1998) with some modifications. At least two samples of 100 mg VOCs-exposed seedlings in comparison with control plants were incubated overnight in 150 μL of methanol acidified with 1% HCl (w/v). After the addition of 100 μL of distilled water, anthocyanins were separated from chlorophylls with 250 μL of chloroform. The absorbance of the aqueous phase was measured at 535 and 657 nm. The relative amount of anthocyanins per 100 mg of fresh weight was calculated by the equation $A_{535} - A_{657} \times 100$.

Analysis of Chlorophyll Content

Total chlorophylls were extracted in 1 mL of 80% aqueous acetone containing 2.5 mM sodium phosphate buffer (pH 7.8) to minimize conversion of chlorophylls into phaeophytins. The suspension was centrifuged at 4°C at 2500 rpm for 10 min. For each experiment, at least two groups of 100 mg of seedlings (after 9 days of co-cultivation) were used and fluorescence was

determined using a spectrometer (Ultrospec 3000, Pharmacia). The absorbance at different wavelength (480, 646, 647, 652, 663, 664, and 750 nm) was measured. The absolute amount of chlorophyll was calculated using the extinction coefficient indicated by Porra et al. (1989). Concentration of chlorophyll, expressed as μg/mg was determined by the following equations:

$$\text{Chl a} = 12.25 (A_{663-750}) \times 2.55 (A_{647-750})$$

$$\text{Chl b} = 20.31 (A_{647-750}) \times 4.91 (A_{663-750})$$

$$\text{Chl a} + \text{b} = 17.76 (A_{647-750}) + 7.34 (A_{663-750})$$

Determination of Trichome Density

Trichome number was measured upon the appearance of the first true fully expanded rosette leaf produced by treated and untreated plants (after 9 days of co-cultivation). The target leaf was first removed from the plant and traced. The leaf area was measured using a leaf area meter (AM 300, ADC BioScientific, Hoddesdon Herts, UK) and the adaxial trichome number was determined under a dissecting microscope. The trichome density was calculated as trichome number per leaf area (number/cm^2).

Reactive Oxygen Species (ROS)
H_2O_2

Hydrogen peroxide content of treated and untreated *A. thaliana* seedlings (after 9 days of co-cultivation) was measured spectrophotometrically after reaction with potassium iodide (KI) according to Chakrabarty et al. (2009). The reaction mixture consisted of 0.5 ml supernatant of 0.1% trichloroacetic acid (TCA) seedling extract, 0.5 ml of 100 mM K-phosphate buffer (pH 7), and 2 ml reagent (1 M KI, w/v in fresh double-distilled water). 0.5 ml of 0.1% TCA served as control. After 1 h of incubation in darkness at room temperature, the absorbance was measured at 390 nm. The amount of hydrogen peroxide was calculated using a standard curve prepared with known concentrations of H_2O_2.

Viability of Roots

Root activity is an indirect indicator of tissue viability, and can be determined by using 2, 3, 5-triphenyl tetrazolium chloride (TTC) (Shen et al., 1991). Viable (respiring) tissue reduces TTC to red-colored triphenyl formazan by accepting electrons from the mitochondrial electron transport chain (Comas et al., 2000). Thus, a decrease in root activity is an indication of reduced respiration and reduced viability often resulting from tissue damage. Briefly, 50 mg of freshly harvested root tissue (after 9 days of co-cultivation) was treated with 5 ml of 0.4% TTC solution (w/v) and 5 ml of 0.067 M phosphate buffer (pH 7.4). This mixture was incubated at 40°C for 3 h followed by the addition of 2 ml 2 M H_2SO_4. Thereafter, roots were ground in 10 ml ethyl acetate to extract red triphenyl formazan. Its concentration was measured spectrophotometrically at 485 nm and expressed as A_{485} g^{-1} h^{-1}.

Quantification of Phytohormones and Camalexin

Determination/quantification of the phytohormones ABA, indole-3-acetic acid (IAA), JA, SA, and the phytoalexin camalexin was performed after 9 days of co-cultivation

according to Großkinsky et al. (2014) with slight modifications. Briefly, volatile exposed and control seedlings were frozen and ground in liquid nitrogen; 200 mg per sample were extracted with 80% methanol and internal standards were added for the quantification of camalexin (6-fluoroindole-3-carboxaldehyde; Sigma-Aldrich, Steinheim, Germany) and the phytohormones (deuterium-labeled hormones; Olchemim Ltd, Olomouc, Czech Republic). The extracts were directly subjected to HPLC analysis for camalexin (Ultimate 3000; Dionex, Sunnyvale, USA). For phytohormone determination, methanol extracts were passed through Chromafix C18-columns (Macherey-Nagel, Düren, Germany), completely dried (Integrated SpeedVac® Concentrator System AES1000; Savant Instruments Inc., Holbrook, USA), resuspended in 20% methanol, passed through Chromafil PES-20/25 filters (Macherey-Nagel, Düren, Germany) and subjected to UHPLC-MS/MS (Thermo Scientific; Waltham, USA) analyses.

Quantification of Glucosinolates

Freeze-dried exposed (after 9 days of co-cultivation) and control seedlings (100 mg) were transferred into 2 mL reaction tubes and lyophylised. The isolation and analysis of GSL content was performed by using the desulpho-GSL method on an ultra-performance liquid chromatography (UPLC) device (Waters, Eschborn) as described in Frerigmann et al. (2012).

GUS Assay

Transgenic *Arabidopsis* lines carrying a GUS reporter system were used in this study. After 9 days of co-cultivation, the expression patterns of the ß-glucuronidase activity were elucidated by histochemical staining using at least 10 exposed and control seedlings, each (Jefferson et al., 1987). In order to visualize the GUS activity, whole seedlings were incubated in a solution of 1 mM X-gluc (5-bromo-4-chloro-3-indolyl-ß-D-glucuronic acid), 0.1 M phosphate buffer (pH 7.0), 10 mM EDTA, 0.1% (v/v) Triton X-100, and 1 mM $K_3Fe(CN)_6$ overnight at 37°C. Subsequently, seedlings were incubated in buffer and fixed for at least 20 min in a mixture of 5% (v/v) formaldehyde, 5% (v/v) acetic acid, and 20% ethanol (v/v), followed by an additional incubation in 50% (v/v) ethanol. The seedlings were preserved in formaldehyde containing 80% (v/v) ethanol till microscopic examination. Images of the plants were recorded with a digital camera Ricoh Cx4 (Ricoh, Tokyo, Japan).

Head Space Collection and GC/MS Analysis of *Trichoderma* VOCs

In a glass Petri dish of 9 cm diameter, a disc (⌀ 0.5 cm) from a 7 days old *T. asperellum* IsmT5 culture was inoculated on 20 ml of broth agar (2.2). The Petri dish was placed into the incubation chamber of a slightly modified airflow collection system (Figure S1B) (Kai et al., 2007). Charcoal-purified, sterile humidified air was pushed through a closed system. Volatiles present in the headspace were carried along and finally trapped in a column containing 40 mg Super Q as trapping material (Alltech Associates, Deerfield, Illinois, USA). The airstream of the pump (Gardner Denver, Puchheim, Germany) was adjusted to a constant flow of 0.6 l min^{-1}. The volatiles were collected

in 24 h intervals during the incubation period of up to 9 days. Volatiles were eluted from the trapping material with 300 µl dichloromethane. For quantitation, 10 µl of nonyl acetate (5 ng) was added as an internal standard. Samples were analyzed using the Shimadzu GC/MSQP5000 (Kyoto, Japan) equipped with a DB5-MS column (60 m × 0.25 mm × 0.25 µm; J&W Scientific, Folsom, California, USA). Splitless injection of 1 µl sample was performed at 200°C with a sampling time of 2 min using a CTC autosampler (CTC Analytics, Zwingen, Switzerland). The initial column temperature was set at 35°C, followed by a ramp of 10°C min^{-1} up to 280°C with a final hold for 15 min at 280°C. Helium was used as the carrier gas at a flow rate of 1.1 ml min^{-1}. Ionization was performed at 70 eV and mass spectra were obtained using the scan modus (2 scans per second, total ion count, 40–280 *m/z*). Confirmation of structure assignments was done by comparison of mass spectra and retention times with those of available standards, with literature data, as well as by comparison with spectra covered by the NIST107 (version 1998) library, and by comparison of Kovats indices. Experiments were replicated at least three times.

Effect of 6PP on *Arabidopsis*

To investigate effects of 6PP (Sigma-Aldrich, Steinheim, Germany) on *Arabidopsis*, three concentrations (0.5, 1, and 2 mM) of the pure compound were applied. Briefly, 1 ml of 6PP was dissolved in sterilized distilled water and then applied to a small glass tube that was fixed in a 9 cm Petri dish distant to 3 days old seedlings. Petri dishes were sealed and incubated for 9 days at 24°C in vertical position. Fresh weight and root length were measured. The experiment was replicated four times.

The potential of 6PP in reducing disease severity of phytopathogenic fungi such as *A. brassicicola* and *B. cinerea* on *A. thaliana* was also tested. 2 mM 6PP (concentration equals the concentration emitted by *T. asperellum* IsmT5) were applied into the soil where 4 weeks old *A. thaliana* grew. After 24 h, four plant leaves in each glass jar were challenged with the pathogens. 5 µl of a spore suspension (5 × 10^6 spores/ml) of *B. cinerea* or *A. brassiciola* were spotted onto the leaves. The percentage of disease severity (lesion size) was measured 5 days post infection.

RT–PCR

Total RNA was extracted from leaves (100 mg FW) of exposed (after 9 days of co-cultivation) and control plants. After 9 days of volatile treatment with 2 mM 6PP (see Effect of 6PP on *Arabidopsis*) or co-cultivation with *T. asperellum* IsmT5 (see *Trichoderma*—Plant Co-cultivation) seedlings were harvested, cells were homogenized by grinding with mortar and pestle in liquid nitrogen and RNA was enriched using the Nucleospin kit (Machery-Nagel, Düren, Germany). mRNA was reverse-transcribed into cDNA using the primers (Table S1) and SuperScript reverse transcriptase (Thermo Scientific Maxima Reverse Transcriptase) according to the protocol of the manufacturer. Gene-specific primers (Table S1, obtained from Life Technologies, Carlsbad, USA) were used to amplify respective genes via PCR (Hippauf et al., 2010). The ubiquitin gene (AT4G05320) served as internal control.

Statistics

F-statistics of ANOVA was used to calculate significance in all experiments. Each F-statistic is a ratio of mean squares. The numerator is the mean square for the term. The denominator is chosen such that the expected value of the numerator mean square differs from the expected value of the denominator mean square only by the effect of interest. The effect for a random term is represented by the variance component of the term. The effect for a fixed term is represented by the sum of squares of the model components associated with that term divided by its degrees of freedom. Therefore, a high F-statistic indicates a significant effect (Moreira et al., 2013).

Results

Trichoderma Volatiles Alter Morphology and Physiology of *Arabidopsis*

To investigate the impact of *Trichoderma* volatile compounds on plant growth, a simple and reliable co-cultivation system was established (**Figure S1A**). After 9 days of co-cultivation and concurrent exposure various parameters were investigated. First, we determined how growth and development of *A. thaliana* were influenced by three different *Trichoderma* isolates (**Figure 1**). The three isolates originated from different locations in Egypt. While *Trichoderma* sp. and *T. harzianum* volatiles did not influence the morphology and habitus of *A. thaliana*, exposure of seedlings to *T. asperellum* IsmT5 volatiles reduced fresh weight, root length, and leaf area by ca. 40, 60, and 50%, respectively (**Figures 1A–C**). Thus, *T. asperellum* IsmT5 volatiles were investigated in more detail.

The plants treated with *T. asperellum* IsmT5 volatiles appeared robust and showed no wilt or other detrimental symptoms (**Figures 2A,B**). Interestingly, the leaves of such exposed seedlings appeared to be much darker. Spectrophotometric analyses revealed a ca. three-fold higher accumulation of anthocyanin pigments in the co-cultivated seedlings compared to controls (**Figure 2C**). Since anthocyanin pigmentation plays a role in plant protection, e.g., forming a photoprotective screen in vegetative tissues and functioning as antimicrobial agent and feeding deterrent in the defense responses (Winkel-Shirley, 2001; Steyn et al., 2002) we further investigated morphological and physiological parameters of plant defense in *A. thaliana*. Microscopic examination of the leaf surface showed that trichome density was significantly increased by 47% in the leaves exposed to *T. asperellum* IsmT5 volatiles (**Figure 3**).

Reactive oxygen species (ROS) are small molecules such as hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\bullet-}$), and hydroxyl radical ($^{\bullet}OH$) and are one of the earliest signals that activate plant defense responses (Singh et al., 2010). We examined H_2O_2 accumulation after 9 days of co-cultivation of *T. asperellum* IsmT5 with *A. thaliana*. The level of H_2O_2 was increased four-fold in the exposed plants compared to control plants (**Figure 4A**). Camalexin is a phytoalexin, which usually accumulates after pathogen attack or after treatment with abiotic elicitors such as UV or silver nitrate (Glawischnig, 2007). Camalexin accumulation increased by 97%

FIGURE 1 | Effect of volatiles of different *Trichoderma* isolates on the growth of *Arabidopsis thaliana*. Seedlings and fungi were co-cultivated for 9 days at 24°C and 84 µmol m^{-2}s^{-1} of light at a 16/8 h light/dark cycle. Fresh weight **(A)**, root length **(B)**, and leaf area **(C)** were measured (four technical replicates). The experiments were repeated three times (biological replicates). Non-exposed control plants were also investigated, $n = 12$, error bars indicate SD, *$P < 0.05$.

in *A. thaliana* upon fungal volatile exposure (**Figure 4B**, Figure S2). Furthermore, tissue viability of volatile exposed seedlings of *A. thaliana* was investigated by determining mitochondrial respiration activity. **Figure 4C** documents a ca. 40% higher respiration activity in plants treated with *T. asperellum* IsmT5 volatiles referring to an increased viability in exposed seedlings. In summary, *A. thaliana* plants exposed to *Trichoderma* volatiles manifest improved survival strategies and defense responses.

Trichoderma Volatiles Modulate Phytohormone Levels in *Arabidopsis*

Phytohormones are well known to regulate growth and development of plants and several plant hormones also act as central players in triggering the plant immune signaling network (Howe and Jander, 2008; Bari and Jones, 2009; Katagiri and Tsuda, 2010; Vos et al., 2015). Very little is known how the presence of volatiles is translated or mediated via phytohormone depending signaling cascades. Here we addressed the main signaling pathways. The levels of three prominent

FIGURE 2 | Phenotype and anthocyanin accumulation in *Arabidopsis thaliana* exposed to *Trichoderma asperellum* volatiles. Co-cultivation was performed for 9 days at 24°C and 84 μmol m^{-2}s^{-1} of light at a 16/8 h light/dark cycle. Stunted but robust *Arabidopsis* seedlings were obtained after *Trichoderma* volatile exposure, control **(A)**, and exposed seedlings **(B)**. **(C)** Accumulation of anthocyanin in *Arabidopsis* seedlings co-cultivated with *Trichoderma asperellum* IsmT5: Anthocyanins were extracted from control (left) and exposed plants (right) and measured at 657 nm. Concentrations were calculated from three independent experiments and four technical replicates, n = 12, error bars indicate SD, *P < 0.05.

FIGURE 3 | Trichomes on leaves of *Arabidopsis thaliana* exposed to *Trichoderma asperellum* volatiles. Co-cultivation was performed for 9 days at 24°C and 84 μmol m^{-2}s^{-1} of light at a 16/8 h light/dark cycle. Trichomes were counted and leaf area was measured to determine trichome density (number per cm^2) of control (left) and exposed plants (right). Density was calculated from five leaves, error bars indicate SD, *P < 0.05.

phytohormones were examined in control and *T. asperellum* IsmT5 volatile-exposed seedlings. The results showed that in co-cultured plants salicylic acid (SA) was increased by 61% and abscisic acid (ABA) by 40% (**Figures 5A,C**). No significant differences were recorded for other hormones, e.g., jasmonic acid (JA) (**Figure 5B**). Apparently the signal transduction chains that stimulate SA and ABA accumulation were selectively activated by the fungal volatiles leading to increased phytohormone levels.

Trichoderma Volatiles Stimulate Gene Expression in *Arabidopsis thaliana*

In order to investigate whether *T. asperellum* IsmT5 volatiles cause gene activation in *Arabidopsis*, we used four transgenic lines with promoter-*uidA* chimeric gene constructs related: (i) to IAA responses (DR5::GUS and YUC8::GUS), (ii) to the transcriptional activation of indolic glucosinolates (MYB51::GUS), (iii) plant defensin (PDF1.2::GUS), and (iv) pathogenesis related protein 1 (PR1::GUS). In exposed seedlings all four promoters were activated and a distinct blue coloration compared to control plants was observed (**Figure 6**, Figure S3). The promoter of the DR5 gene was primarily activated at the rim of the leaves (**Figure 6A**), while the PR1 promoter revealed gus activation in a zone near the base of the leaf (**Figure 6B**). Interestingly, the MYB51 promoter was activated in the trichomes and epidermal tissue (**Figure 6C**). PDF 1.2::GUS and YUC8::GUS were expressed in whole leaves of the exposed seedlings (Figures S3A,B). In summary, *Arabidopsis* plants respond to *T. asperellum* IsmT5 volatiles by activating genes that are involved in phytohormone biosynthesis and plant defense.

Analysis of Volatile Profiles of *Trichoderma* Species

The results described above demonstrate that volatiles released by *T. asperellum* IsmT5 caused changes at the morphological, physiological, and transcriptional level of *A. thaliana*. Since the volatile profile of *T. asperellum* IsmT5 was so far unknown, we collected and analyzed headspace volatiles (Figure S1B). In the GC chromatogram three peaks could be identified (Figure S4A). The contribution of the peak area of peak number #4 reflects ca. 90% of the total volatile emission. By comparison of mass spectra, retention times, and Kovats indices, the compound of peak #1 was tentatively identified as 1-octen-3-ol, #2 as nonanal and #4 as 6PP (**Table 1**). Because of its abundance, we focused our future work on compound #4. Its chemical identity was verified

FIGURE 4 | Determination of defense molecules in *Arabidopsis thaliana* exposed to *Trichoderma asperellum* volatiles. Co-cultivation was performed for 9 days at 24°C and 84 μmol m^{-2}s^{-1} of light at a 16/8 h light/dark cycle. H$_2$O$_2$ **(A)**, camalexin **(B)**, and activity of respiration chain in roots **(C)** were measured in control and volatile exposed seedlings. Parameters were obtained from two independent experiments and five leaves, error bars indicate SD, *$P < 0.05$.

FIGURE 5 | Determination of phytohormones in *Arabidopsis thaliana* exposed to *Trichoderma asperellum* volatiles. Co-cultivation was performed for 9 days at 24°C and 84 μmol m^{-2}s^{-1} of light at a 16/8 h light/dark cycle. Salicylic acid **(A)**, jasmonic acid **(B)**, and abscisic acid **(C)** were measured in control (left column) and volatile exposed (right column) seedlings. Parameters were obtained from two independent experiments and five leaves, error bars indicate SD, *$P < 0.05$.

by comparison with the commercially available 6PP (Figures S4C,D). The dynamics of volatile emission was recorded in 24 h intervals throughout of 10 days (Figure S5). A maximum of 6PP accumulation (450 ng/μl = 2.7 mM) was reached at day 8 of cultivation.

Effects of 6-pentyl-α-pyrone on *Arabidopsis thaliana*

After identification of the major volatile compound released by *T. asperellum* IsmT5, we determined which concentration of 6PP caused morphological alterations in *A. thaliana*. Two milliliters of 0.5 mM, 1 mM, and 2 mM were placed in a vial next to *Arabidopsis* seedlings (**Figure 7A**). Exposure to 2 mM 6PP revealed ca. 50% reduction of fresh weight and root length (**Figures 7B,C**, respectively). Surprisingly, four aliphatic and three indole glucosinolates were also reduced upon 6PP and *Trichoderma* volatile application (**Figure 7D**; Figures S6A,B). To

our knowledge it was shown for the first time that a volatile compound influenced glucosinolate levels in plants.

We furthermore investigated the effects of *T. asperellum* IsmT5 volatiles and 6PP on the expression (RT-PCR) of defense related genes in exposed *Arabidopsis* seedlings. Gene expression of the SA-induced pathogen related protein PR-1, the transcription factor involved in trichome formation GL3, and VSP2 activated by ethylene was clearly induced upon both treatments indicating that various defense genes were up-regulated upon volatile exposure (**Figure 7E**).

To further investigate whether the volatile compound 6PP is a plant defense inducer, we performed the following experiment: *Arabidopsis* plants were pretreated with 2 mM 6PP for 24 h followed by either the application of a spore suspension of the phytopathogenic fungus *A. brassicicola* or *B. cinerea*. After 5 days of incubation the lesion sizes were recorded. In both treatments the symptom sizes were reduced by ca. 40% and ca.
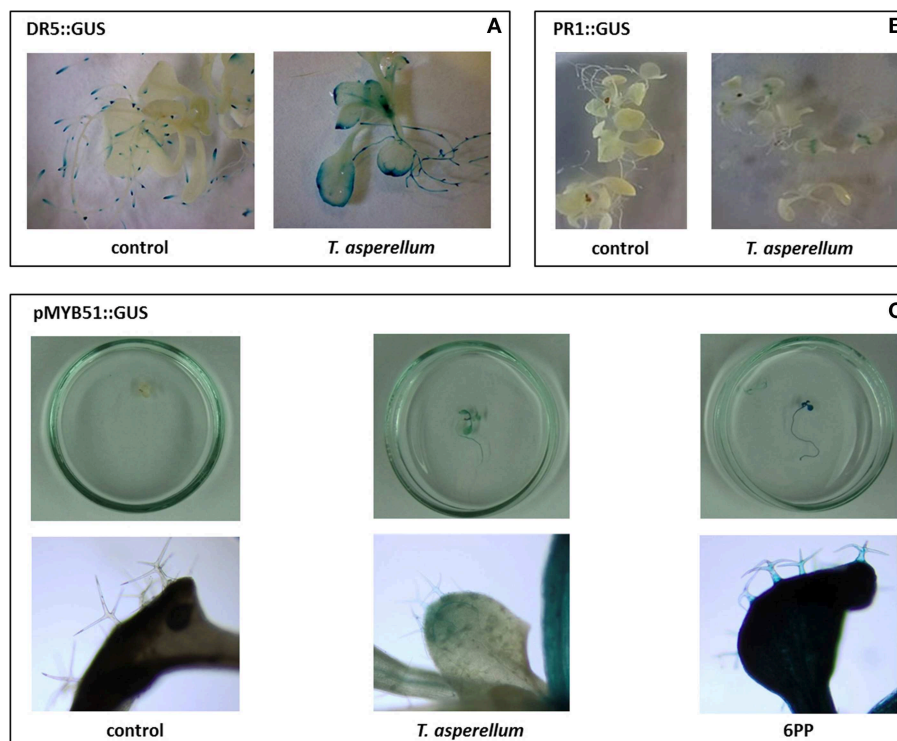
FIGURE 6 | Expression of genes in leaves of transgenic *Arabidopsis thaliana* lines exposed to *Trichoderma asperellum* volatiles. Transgenic cell lines equipped with promoter gus constructs were co-cultivated with *Trichoderma asperellum* IsmT5. Promoters originated from the DR5 (indole biosynthesis) **(A)** and PR1 (pathogenesis related protein 1) **(B)**, and MYB51 (transcription factor involved in trichome production) **(C)**. Co-cultivation was performed for 9 days at 24°C and 84 μmol m^{-2}s^{-1} of light at a 16/8 h light/dark cycle. Glucuronidase assay was performed in control and volatile exposed seedlings. Blue color indicates the expression of the *uid* gene in the tissue.

TABLE 1 | Volatile organic compounds in the headspace of *Trichoderma asperellum* IsmT5.

Number in Figure 8A	Volatile compound	Retention time (min)	Kovats index	% Similarity to database
#1	1-octen-3-ol	12.733	967	87
#2	nonanal	15.142	1090	87
#3	n-nonyl acetate (internal standard)	18.517	1287	89
#4	6-pentyl-2-pyrone	21.042	1453	not available in NIST 107, version 1998

60%, respectively (**Figure 8**). We concluded that the exposure to 6PP induced resistance against fungal pathogens in *Arabidopsis*. A direct effect of 6PP on *A. brassicicola* was also investigated and spore germination was significantly reduced by 2 mM 6PP (Figure S7).

Discussion

The goal was to study morphological and physiological reactions of *A. thaliana* upon exposure to volatiles of four *Trichoderma* species. Only *T. asperellum* IsmT5 showed significant effects on plant growth and was therefore selected for further investigations. The fungal volatiles did not kill the plant but initiated several plant defense responses like the production of ROS, increased trichome number, accumulation of anthocyanins,

and the phytoalexin camalexin. Furthermore, the expression of e.g., SA pathway depending defense genes were increased. We analyzed the headspace volatiles of *T. asperellum* IsmT5 and identified the most prominent VOC in the spectrum to be 6PP, which has a strong coconut-like aroma. Here we demonstrated a dual function of 6PP, (i) preincubation of *A. thaliana* with 6PP improved its resistance to the phytopathogenic fungi *Botrytis* and *Alternaria* (indirect action) and (ii) reduction of spore germination of *Alternaria* (direct action). These results support that *Trichoderma* volatiles have the potential being a useful biocontrol agent in agriculture.

Test System

Several co-cultivation systems were developed to test effects of fungal volatiles on plant growth. Naznin et al. (2013) and
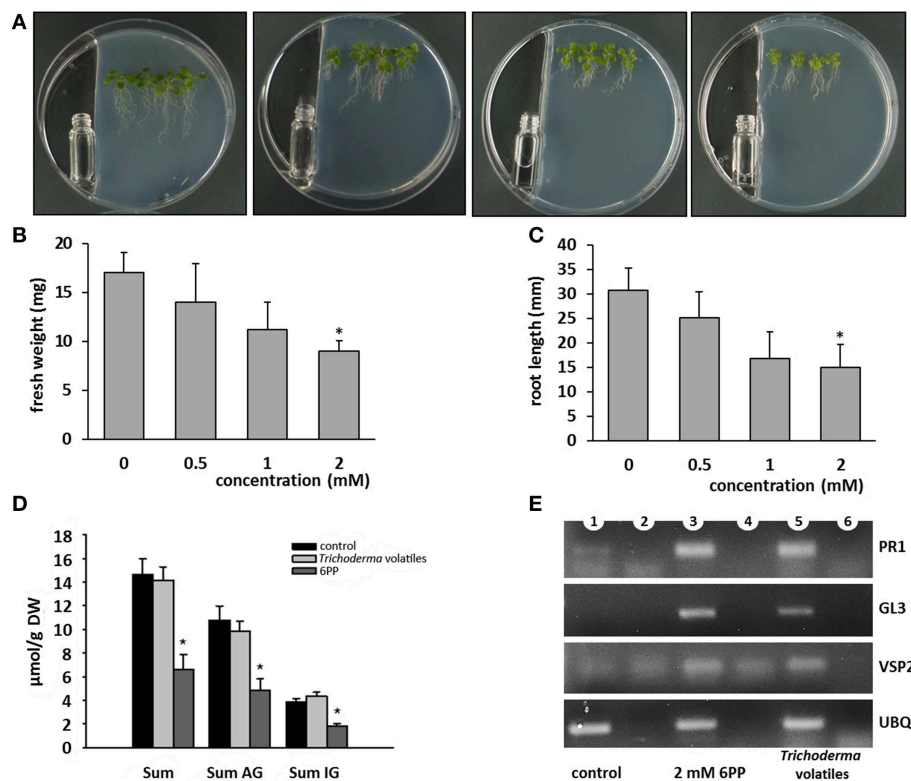
FIGURE 7 | Effects of 6-pentyl-α-pyrone (6PP) on the growth, glucosinolate level, and defense gene expression of *Arabidopsis thaliana*. One ml of 6PP of different concentrations were filled into sterile glass vials and positioned next to 3 days old *A. thaliana* seedlings growing on MS medium **(A)**. After 9 days fresh weight **(B)** and root length **(C)** were recorded. The experiment was repeated for three times and four seedlings were placed on the agar, $n = 3$, error bars indicate SD, $*P < 0.05$. **(D)**: 3 days old *A. thaliana* seedlings were co-cultivated with *T. asperellum* IsmT5 (see *Trichoderma*—Plant Co-cultivation) or exposed to 6PP (see Effect of 6PP on *Arabidopsis*). After 9 days of co-cultivation or after 9 days of 6PP application whole seedlings were harvested and glucosinolates were extracted and analyzed by HPLC (see Quantification of Glucosinolates). AG, aliphatic glucosinolates; IG, indolic glucosinolates. $n = 3$, error bars indicate SD, $*P < 0.05$. **(E)**: Via RT-PCR the expression of defense genes were analyzed: PR1, pathogenesis related protein 1; GL3, transcription factor GLABRA 3; VSP2, vegetative storage protein. Expression of ubiquitin (UBQ) was used for standardization. Controls in lane 2, 4, 6: RT-PCR without polymerase.

Hung et al. (2013) used airtight cultivation systems and observed growth promotion of tobacco and *A. thaliana* by *Phoma* and *T. viride*, respectively. We prepared slanted agar medium in glass jars for plant growth and inserted a beaker with fungi to avoid direct contact of both organisms (Figure S1A). This method had several advantages, e.g., easy and rapid inspection of the phenotypic responses of the roots and shoots during exposure to microbial volatiles, and no limitation to use only seedlings. We tested two alternative ways to cover the jars, either laying the lid on the jar however without sealing with Parafilm (closed system), or a funnel with sterile glass wool at the tip was placed upside down (open system) (Figure S1A). We preferred to use the open system (i) because it reflects the natural situation more closely, and (ii) to overcome the problem of CO_2 accumulation due to metabolic activity of the microorganism, since it was recently shown that CO_2 accumulated to 10-fold higher levels compared to ambient levels in closed containers (Kai and Piechulla, 2009).

Three different *Trichoderma* strains/isolates were investigated regarding their effects on *Arabidopsis* seedlings. Interestingly, the strains induced different phenotypic alterations in *A. thaliana*. The volatiles of *T. asperellum* IsmT5 influenced the plants

negatively, which was manifested in the inhibition of primary root, reduction in size of leaves and fresh weight. In contrast, plant growth promotions due to *Trichoderma* volatiles have been observed by Hung et al. (2013) and Contreras-Cornejo et al. (2014). These contradictory results are most likely due to the different (open vs. closed) test systems used.

Headspace Analysis and 6-pentyl-α-pyrone Application

The defense responses initiated in *A. thaliana* upon *Trichoderma* volatile exposure prompted us to investigate the nature of the bioactive compound. Identification of headspace volatiles indicated different chemical classes i.e., alcohols, alkanes, and pyrones (**Table 1**). Similar compound diversity (25 different compounds) was also previously reported for *Trichoderma atroviride* (Stoppacher et al., 2010). Very prominent levels of 6PP were detected in the headspace of *T. asperellum* IsmT5. This compound and other α-pyrone analogs have been detected in cultures of *T. viride* (Collins and Halim, 1972), *T. harzianum* (Claydon et al., 1987), *T. koningii* (Simon et al., 1988), *T. atroviride* (Reithner et al., 2005), *T. citrinoviride* and

FIGURE 8 | Development of disease symptoms in *Arabidopsis thaliana* leaves after preincubation with 6-pentyl-α-pyrone (6PP). Four weeks old *Arabidopsis* plants were grown in glass containers (see Effect of 6PP on *Arabidopsis*). Twenty milliliters of 2 mM 6PP were applied to the soil of each glass jar. The jar was covered with a lid and sealed with Parafilm®. Twenty-four hours later 50 μl of *Botrytis cinerea* spore suspension or of *Alternaria brassicicola* (each 5×10^6 spores/ml) were applied to the leaves. Representative examples of *A. thaliana* plants treated with a spore suspension of *B. cinerea* are shown in the upper panel **(A)**: without preexposure to 6PP = control; **(B)**: with 6PP preexposure. Lesions are visible as brown spots on the leaves. At the 5th day after inoculation the diameter of the lesions were recorded and calculated **(C)**. The experiment was repeated for three times, each with four plants, $n = 3$, error bars indicate SD, *$P < 0.05$.

T. hamatum, (Jeleň et al., 2013), and recently in *T. asperellum* 328 (Wickel et al., 2013).

Lactones are generally very pleasant and potent flavor compounds (Kapfer et al., 1989) and a variety of microorganisms perform *de novo* lactone biosynthesis (Tressl et al., 1978). 6PP has a coconut-like aroma and it was known for long time that this odor appeared during *Trichoderma* cultivation (Bisby, 1939; Rifai, 1969). The emission of 6PP by *T. asperellum* IsmT5 reached levels of up to 450 ng/μl (=2.7 mM), similar concentrations were reported by Kalyani et al. (2000) and Serrano-Carreón et al. (2004). A systematic optimization of growth conditions improved the production of 6PP by three orders of magnitude in *T. atroviride* (Oda et al., 2009). This was a success because of its use as a perfume in food and cosmetic industries. Beside the technological application it is well known that many natural lactones have antibacterial, antifungal or anti-inflammatory biological activity (Claydon et al., 1987; Simon et al., 1988; Cooney and Lauren, 1999; Pezet et al., 1999; Romero-Guido et al., 2011).

Plant Defense Reactions
The interaction of *Trichoderma* with a plant was thought to start by colonization of the outer root layers, resulting in the induction of resistance mechanisms to prevent further colonization (Yedidia et al., 1999; Harman et al., 2004; Mukherjee et al., 2013; Vos et al., 2015). Here we demonstrate that a volatile based interaction between *A. thaliana* and *T. asperellum* IsmT5

also exists, which might occur prior to physical contact. Although many evidences exist that *Trichoderma* activates plant immunity and development through different mechanisms, it was so far unknown whether microbial VOCs play a role in any of these plant defense processes (Contreras-Cornejo et al., 2014).

Here it was shown for the first time that *Arabidopsis* exposed to *T. asperellum* IsmT5 volatiles doubled its trichome number (**Figure 3**). Trichome formation in plants is a general defense strategy primarily developed to hinder landing, moving, and penetration of insects and other organisms on plant surfaces. The presented results indicate that the volatiles are apparently perceived as an intervening organismal interaction. While detailed investigations are needed to understand the underlying mechanisms related to these morphological alterations *T. asperellum* IsmT5 volatiles also induced typical plant defense responses at the physiological level, such as increasing the H_2O_2 level in leaves (**Figure 4A**). Similar observations were recorded by Splivallo et al. (2007). They found that truffle volatiles induced an oxidative burst in *Arabidopsis*. Increase of ROS in *A. thaliana* was also found upon bacterial volatile exposure (Wenke et al., 2012). Contradictory results of H_2O_2 production induced by *Trichoderma* volatiles were reported by Hung et al. (2013) and Contreras-Cornejo et al. (2014). Another early response in pathogen defense is the oxidation by peroxidases, subsequently reducing the amount of oxygen diffusing from the roots into the surrounding environment (Tiwari et al., 2002). This mechanism helps plant roots to avoid the uptake of toxic materials and thereby provides protection (Singh et al., 2007). The roots of volatile exposed plants showed increase in such root activity (**Figure 4C**). Similar results were recorded in rice and of seedlings of *Ageratina adenophora* (Zhang et al., 2012). Since ROS trigger many downstream processes leading to a dynamic defense responses characterized by inhibition of the growth of invaders through phytoalexin formation, callose deposition, strengthening of cell walls, synthesis of secondary metabolites and pathogenesis related (PR) proteins (Xu et al., 2008; Vinale et al., 2008b; Shoresh et al., 2010), we hypothesize that *T. asperellum* IsmT5 volatiles are perceived as oxidative stress and thus inducing alterations in the antioxidant enzyme machinery and accumulation of other protective substances in *A. thaliana*. A striking observation was the dark coloration of *Trichoderma* volatiles exposed leaves (**Figure 2C**) resulting from anthocyanin accumulation. Such accelerated anthocyanin accumulation due to volatile stress has to our knowledge not yet been reported although these results fit very well to the known function of anthocyanin to act as antimicrobial agents and feeding deterrents (Winkel-Shirley, 2001; Steyn et al., 2002; Shin et al., 2013). Furthermore, camalexin known as an important phytoalexin of *Arabidopsis* and an integral part of the plant defense system in *A. thaliana* (Glawischnig, 2007) was significantly up-regulated during volatiles exposure (**Figure 4B**). As other phytoalexins, camalexin production can be elicited by bacterial and fungal phytopathogens (as well as abiotic stress) and possesses antimicrobial activity (Großkinsky et al., 2012). Contreras-Cornejo et al. (2011) proved that *Arabidopsis* seedlings colonized with *T. virens* or *T. atroviride* accumulated high levels of camalexin. Camalexin deficient mutants such as

pad3 (encodes last step in phytoalexin biosynthesis) displayed enhanced susceptibility to *B. cinerea* (Ferrari et al., 2003), and in *A. thaliana* treated with *T. atroviride pad3* was up-regulated in roots and leaves (Salas-Marina et al., 2011). In this study the accumulation of camalexin due to *T. asperellum* IsmT5 volatile exposure highlights a new aspect and showed that not only by direct contact between plant and *Trichoderma* camalexin is induced but also by air borne signals. Glucosinolates play central roles in plant/biotic interactions and they are important determinants for plant fitness in the field. Hydrolysis products of glucosinolates are active against a wide variety of organisms, such as insects, plants, fungi, and bacteria (Vaughn, 1999). In this study we analyzed the levels of glucosinolates in *Arabidopsis* plants co-cultivated with *T. asperellum* IsmT5 or exposed to the fungal volatile 6PP. Our results demonstrate that 6PP caused a significant reduction in the accumulation of glucosinolates, while levels were not significantly altered in plants co-cultivated with *T. asperellum* IsmT5 (**Figure 7D**). Whereas the catabolism of glucosinolates and *de novo* biosynthesis is well balanced in plants co-cultivated with *T. asperellum* IsmT5, the degradation seems to be more dominant in 6PP-treated plants. Apparently, other volatiles of *T. asperellum* IsmT5 spectrum prevent a reduction of the essential glucosinolates and consequently without weakening the plant defense system. The function of Trp-derived indole glucosinolates in *Arabidopsis* immunity was validated with infection phenotypes of *cyp81F2* and *penetration2* (*pen2*) mutants. Analyses of loss-of-function mutant of *pen2* (an alternative myrosinase) suggested that PEN2-mediated glucosinolate metabolism is important for pre-invasive resistance, while camalexin is contributing at the post-invasive stage of immunity (Lipka et al., 2005; Hiruma et al., 2010; Sanchez-Vallet et al., 2010; Schlaeppi et al., 2010). These observations are also in agreement with the activation of the SA pathway of *T. asperellum* Ism5 co-cultivated plants as shown in (**Figure 5**) and MYB51 has been previously reported to be important regulator of glucosinolates at SA-signaling (Frerigmann and Gigolashvili, 2014). We therefore expected the increase in the levels of pathogen related genes such as DR5::GUS, YUC8::GUS, PDF1.2::GUS, PR1::GUS, MYB51::GUS (**Figure 6** and Figure S3). These results further supported that fungal volatiles were perceived as stress, which initiated defense processes and improved plant immunity.

The defense network in plants is regulated by the action of plant hormones via two main mechanisms, systemic acquired resistance (involvement of SA) and induced systemic resistance (regulated by ethylene and JA). It became clear that an intensive interplay between hormone signaling pathways exists, which effectively determines the response to specific types of invader. The activation of phytohormone signaling cascades by direct interactions between *Trichoderma* spp. and *A. thaliana* or other plants was already demonstrated to trigger JA or SA dependent systemic resistance (Contreras-Cornejo et al., 2011; Salas-Marina et al., 2011; Velázquez-Robledo et al., 2011; Yoshioka et al., 2012; Vos et al., 2015), however volatile based activations add a new facette to such strategies. While SA and ABA accumulated in *A. thaliana* exposed to *T. asperellum* IsmT5 volatiles, levels of JA were not altered (**Figure 5**). Why, in contrast, the marker gene

for the JA/ethylene mediated signaling in *A. thaliana*, PDF 1.2a, was expressed in transgenic lines (Figure S3) could be explained by a species-specific activation of the signaling cascades as found in *T. hamatum* T382 and *T. asperelloides* (Mathys et al., 2012; Brotman et al., 2013).

The presented results show that *A. thaliana* perceives the *Trichoderma* volatiles as stress compounds and subsequently initiates multilayered (morphological, physiological, and gene expression level) adaptations and activations of signaling cascades to withstand this environmental influence. This hypothesis was supported when the major volatile compound of the VOC spectrum of *T. asperellum* Ism5 was identified and *A. thaliana* preincubated with the pure compound 6PP and challenged with the phytopathogenic fungi *Botrytis* and *Alternaria*. The leaves showed significantly less necrotic symptoms compared to untreated plants (**Figure 8**). Consequently it was concluded that the volatiles of *T. asperellum* IsmT5 or 6PP activate the accumulation of typical defense molecules such as ROS, camalexin, anthocyanins, and the SA dependent plant hormone pathways and subsequently defense-activated plants become more resistant to pathogen attack and exhibited smaller lesions. Vinale et al. (2008a) also reported a reduction of disease symptoms in pea, tomato, and canola seedlings after addition of purified secondary metabolites. This effect correlated with elevated expression of chitinase, PR1 protein, and endochitinase. Maize plants growing in soil that was drenched with 6PP for 4 days prior to inoculation with *Fusarium moniliforme* showed considerable suppression of seedling blight compared to untreated controls (El-Hasan and Buchenauer, 2009). Cutler et al. (1986) were the first who recorded inhibitory effects on wheat coleoptiles. In addition, 6PP acts directly on spore germination of *A. brassicicola* (Figure S7), also demonstrated by Intana and Chamswarng (2007) and Yenjit et al. (2008) for *A. brassicicola* and *Phythium aphanidermatum*, respectively. Taken together, volatile metabolites from *T. asperelleum* IsmT5 (6PP) are involved in direct and indirect interactions between fungi and plants thereby have the potential to influence biocontrol processes (Cottier and Mühlschlegel, 2012). Deciphering the signaling cascades in plants that are induced by *Trichoderma* volatiles is a future challenge. So far, to the best of our knowledge, only one example is known which showed that a WRKY transcription factor is part of the signaling cascade in bacterial volatile plant interactions (Wenke et al., 2012). In the future, further investigations at the molecular level are required to shed light on the role of *Trichoderma* volatiles, especially 6PP, and bacterial volatiles in activation of defense mechanisms in plants to improve plant resistance and to design plant protection systems.

Acknowledgments

Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00995

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Gaseous 3-pentanol primes plant immunity against a bacterial speck pathogen, *Pseudomonas syringae* pv. tomato via salicylic acid and jasmonic acid-dependent signaling pathways in *Arabidopsis*

Geun C. Song [1], Hye K. Choi [1] and Choong-Min Ryu [1,2]*

[1] Molecular Phytobacteriology Laboratory, Korea Research Institute of Bioscience and Biotechnology, Daejeon, South Korea,
[2] Biosystems and Bioengineering Program, University of Science and Technology, Daejeon, South Korea

3-Pentanol is an active organic compound produced by plants and is a component of emitted insect sex pheromones. A previous study reported that drench application of 3-pentanol elicited plant immunity against microbial pathogens and an insect pest in crop plants. Here, we evaluated whether 3-pentanol and the derivatives 1-pentanol and 2-pentanol induced plant systemic resistance using the *in vitro* I-plate system. Exposure of *Arabidopsis* seedlings to 10 μM and 100 nM 3-pentanol evaporate elicited an immune response to *Pseudomonas syringae* pv. tomato DC3000. We performed quantitative real-time PCR to investigate the 3-pentanol-mediated *Arabidopsis* immune responses by determining *Pathogenesis-Related* (*PR*) gene expression levels associated with defense signaling through salicylic acid (SA), jasmonic acid (JA), and ethylene signaling pathways. The results show that exposure to 3-pentanol and subsequent pathogen challenge upregulated *PDF1.2* and *PR1* expression. Selected *Arabidopsis* mutants confirmed that the 3-pentanol-mediated immune response involved SA and JA signaling pathways and the *NPR1* gene. Taken together, this study indicates that gaseous 3-pentanol triggers induced resistance in *Arabidopsis* by priming SA and JA signaling pathways. To our knowledge, this is the first report that a volatile compound of an insect sex pheromone triggers plant systemic resistance against a bacterial pathogen.

Keywords: 3-pentanol, induced resistance, volatile organic compound, *Arabidopsis*, bacterial speck pathogen

Introduction

Plants protect themselves against diverse microbial pathogens and insects using a variety of defense mechanisms (Agrios, 2004). Among these mechanisms, induced resistance represents a unique machinery against a broad spectrum of plant pathogens (Mysore and Ryu, 2004; Eyles et al., 2010). Induced resistance is classified into two major groups: systemic acquired resistance (SAR) elicited

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Abbreviations: Col-0, *Arabidopsis thaliana* ecotype Columbia; ET, ethylene; JA, jasmonic acid; *PR*, *Pathogenesis-Related* genes; *Pto*, *Pseudomonas syringae* pv. tomato DC3000; qRT-PCR, quantitative RT-PCR; SA, salicylic acid; VOC, volatile organic compound.

by avirulent pathogens and induced systemic resistance (ISR) by root-associated bacteria (Ryals et al., 1996; Kloepper et al., 2004; Pieterse et al., 2009; Balmer et al., 2013). Many of the microbial determinants that elicit plant immunity have been reported (Lyon, 2007). Microbial (fungus and bacteria) cell-wall components and secreted metabolites are major groups. Among secreted metabolites, volatile organic compounds (VOCs) have been reported to induce plant immunity when applied to plants (Farag et al., 2013; Chung et al., 2015; Kanchiswamy et al., 2015a). To apply VOC under field condition, rapid evaporation of VOCs in the open field is a major challenge (Farag et al., 2013). However, recent report shows that plants can be successfully protected against plant pathogens and insect herbivores using 3-pentanol and 2-butanone (Song and Ryu, 2013; Kanchiswamy et al., 2015b). In addition, the underlying mechanism of VOC-mediated enhancement of plant immunity remains elusive (Chung et al., 2015). Particularly, the signaling pathways involved in major plant defense mechanisms, such as those that mediate the effects of salicylic acid (SA), jasmonic acid (JA), and ethylene (ET), have been intensively studied using only 2,3-butanediol and tridecane VOC within the *Arabidopsis thaliana-Pectobacterium carotovorum/Pseudomonas syringae* pathosystem (Ryu et al., 2004; Han et al., 2006; Kwon et al., 2010; Rudrappa et al., 2010; Lee et al., 2012;). Additionally, there have been no reports to our knowledge of the defense signaling induced by insect-produced VOCs.

Among insect's produced VOCs, 3-pentanol is well-characterized with respect to its ability to induce plant immunity on pepper and cucumber plants (Song and Ryu, 2013; Choi et al., 2014). In many cases, 3-pentanol is also an important insect sex pheromone, particularly for the ambrosia beetle *M. mutatus* (Coleoptera, Curculionidae, Platypodinae), promotes the aggregation of males (Gatti Liguori et al., 2008; Funes et al., 2009, 2011), and facilitating mating behavior in several other species (Rossiter and Staddon, 1983; Bukovinszky et al., 2005; Manrique et al., 2006; Vitta and Lorenzo, 2009; Gols et al., 2011). A crucial role for 3-pentanol as an insect sex pheromone and attractant has been reported, but its function in eliciting plant defense responses against pathogens has only recently been studied (Zhuge et al., 2010; Song and Ryu, 2013).

Drench application of 3-pentanol induces an immune response in cucumber plants against angular leaf spot caused by *Pseudomonas syringae* pv. lachrymans and the sucking insect aphid (Song and Ryu, 2013). However, the molecular mechanism of 3-pentanol-mediated plant immunity is unknown. Here, we used the model plant *Arabidopsis thaliana* to investigate 3-pentanol-mediated immunity *in vitro*. We utilized the I-plate system, which is a Petri dish divided into two physically separated compartments that share the same headspace, to investigate the effects of 3-pentanol and its isoforms 1-pentanol and 2-pentanol on plant tissues and pathogen challenge. We focused on 3-pentanol activation of defense priming in plant immunity, which primes major plant defense signaling pathways involved in the plant immune response (Conrath, 2011). Defense priming is indicated by faster or stronger expression of defense-related genes by secondary biotic and abiotic stresses (Pare et al., 2005; Yang et al., 2009). We evaluated 3-pentanol-mediated defense priming in selected *Arabidopsis* mutants by performing qRT-PCR

FIGURE 1 | Effects of gaseous 1-pentanol, 2-pentanol, and 3-pentanol on *Arabidopsis* systemic resistance. (A,B) *Arabidopsis* seedlings were planted in an I-plate, which contains two physically separated compartments that share the same headspace. Plants are exposed to volatile 3-pentanol and its isoforms (or sterile distilled water control), which evaporates from solutions in the other compartment. All plants were drop-inoculated with *P. syringae* pv. tomato DC3000 (*Pto*) 7 days after exposure to volatiles from 1 nM, 100 nM, 10 μM, and 1 mM 1-pentanol, 2-pentanol, and 3-pentanol. Disease severity **(B)** was measured 7 days after pathogen inoculation. The asterisk in **(B)** indicates significant differences ($P = 0.05$ by LSD) between treatment and control. Error bars represent means $\pm$ SEM; $N = 12$ plants per treatment.

analysis of *Pathogenesis-Related* (*PR*) gene expression in SA, JA, and ET signaling pathways. Our results indicate that a volatile emission of the insect pheromone 3-pentanol elicits an induced resistance response that protects *Arabidopsis* plants against pathogen infection.

Materials and Methods

Disease Assay and Effect of 3-pentanol on *Arabidopsis*

Plant and bacterial preparations were conducted as described previously (Ryu et al., 2003a, 2004; Lee et al., 2012). Briefly, *Arabidopsis thaliana* ecotype Columbia (Col-0) seedlings that had been lowed to germinate and grow for at least 2 days were transferred to one compartment of an I-plate (SPL Lifesciences Co., Pocheon, Gyeonggi-do, South Korea) containing 1/2 Murashige and Skoog medium supplemented with 0.6% (w/v) agar and 1.5% (w/v) sucrose. Plants were cultivated in the I-plates in a growth chamber for 14 days at 21°C under a 16 h light/8 h

dark cycle before collecting samples for gene expression analysis. Bacterial pathogens were cultured overnight at 30°C in LB medium supplemented with 100 μg/ml rifampicin. *Arabidopsis thaliana* ecotype Columbia (Col-0) plants were prepared as described previously (Lee et al., 2012). The I-plate system was employed to assess induced resistance mediated by 1-pentanol, 2-pentanol, and 3-pentanol; 30 μl of 1 nM, 100 nM, 10 μM, and 1 mM of each C5 amyl alcohol (or sterile distilled water control) was added to one compartment of an I-plate (the a-compartment in **Figure 1A**) containing *Arabidopsis* plants in the other compartment, and the plate was tightly sealed with Parafilm. For the induced resistance assay, 2 μL of freshly prepared suspension of *P. syringae* pv. tomato DC3000 (*Pto*) in sterile distilled water [10^7 colony-forming units (CFUs) per mL] was drop-inoculated on leaves 7 days after exposure to each C5 amyl alcohol. Sterile distilled water was mock-inoculated as a negative control. Inoculated plants were placed in a dew chamber (100% humidity) under darkness for 1 d at 25°C. Disease severity was measured 5−7 days after pathogen challenge. The disease rate (0−5) of each plant was measured by recording the percentage of total plant leaf surface showing symptoms as follows: 0 = no symptoms, 1 = mild chlorosis at the inoculated site, 2 = chlorosis covering half of the leaf, 3 = chlorosis covering the whole leaf, 4 = severe chlorosis and mild necrosis, and 5 = most severe symptoms with necrosis (Lee et al., 2012). This was designed as a completely randomized experiment with 12 replications and one plant per replication. The entire experiment was repeated three times. For long-term storage, bacterial cultures were maintained at −80°C in King's B medium containing 20% glycerol.

RT-PCR and qRT-PCR

For qRT-PCR analysis to investigate defense priming of signaling marker genes, Col-0 plants were exposed to 3-pentanol, subsequently challenged with pathogen, and leaf tissues were collected at 0.2 (15 min), 6, and 12 h after pathogen challenge and used for further experiments (**Figure 3A**). Total RNA was isolated from *Arabidopsis* leaf tissues using the TRI reagent (Molecular Research Center, USA) according to the manufacturer's instructions. First-strand cDNA synthesis was performed with 2 μg of DNase-treated total RNA, oligo-dT primers, and Moloney murine leukemia virus reverse transcriptase (MMLV- RT, Enzynomics, Korea). PCR reactions were performed according to the manufacturer's instructions. Expression of the candidate defense priming genes *Chinase B* (*CHIB*) for ET response, *plant defense 1.2* (*PDF1.2*) for JA response, and *Pathogenesis-related gene 1* (*PR1*) for SA response was assessed using the following primers: 5′-GCTTCAGACTACTGTGAACC-3′ (*CHIB*-F), 5′-TCCACCGTTAATGATGTTCG-3′ (*CHIB* -R); 5′-AATGAGCTCTCATGGCTAAGTTTGCTTCC-3′ (*PDF1.2*-F), 5′-AATCCATGGAATACACACGATTTAGCACC-3′ (*PDF1.2*-R); and 5′-TTCCACAACCAGGCACGAGGAG-3′ (*PR1*-F), and 5′-CCAGACAAGTCACCGCTACCC-3′ (*PR1*-R). A Chromo4 Real-Time PCR system (Bio-Rad, USA) was used for qRT-PCR. Reaction mixtures consisted of cDNA, iQTM SYBR® Green Supermix (Bio-Rad), and 10 pM of each primer. Thermocycler parameters were as follows: initial polymerase activation, 10 min at 95°C; then 40 cycles of 30 s at 95°C, 60 s at 55°C, and 30 s
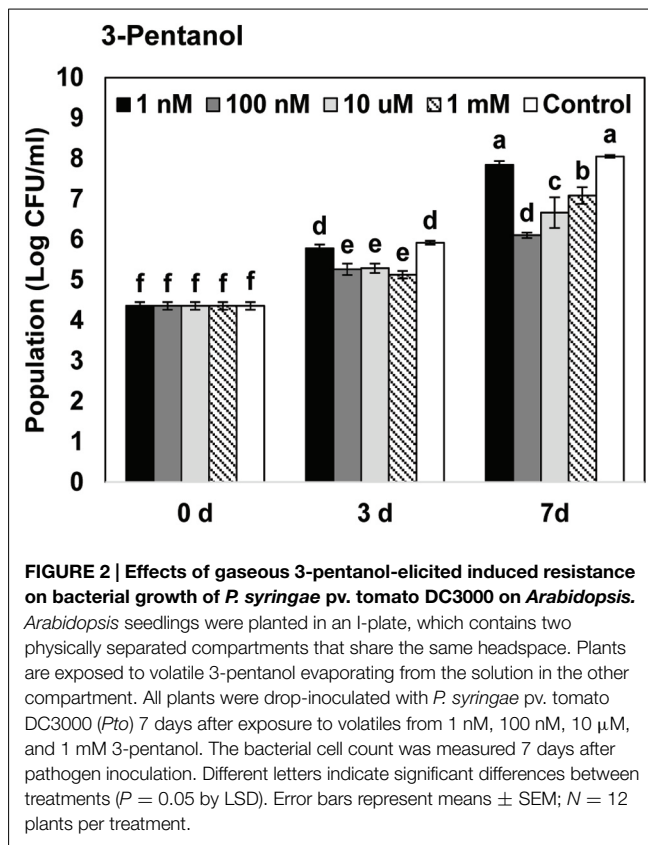
FIGURE 2 | Effects of gaseous 3-pentanol-elicited induced resistance on bacterial growth of *P. syringae* pv. tomato DC3000 on *Arabidopsis.* *Arabidopsis* seedlings were planted in an I-plate, which contains two physically separated compartments that share the same headspace. Plants are exposed to volatile 3-pentanol evaporating from the solution in the other compartment. All plants were drop-inoculated with *P. syringae* pv. tomato DC3000 (*Pto*) 7 days after exposure to volatiles from 1 nM, 100 nM, 10 μM, and 1 mM 3-pentanol. The bacterial cell count was measured 7 days after pathogen inoculation. Different letters indicate significant differences between treatments (*P* = 0.05 by LSD). Error bars represent means ± SEM; *N* = 12 plants per treatment.

at 72°C. Conditions were determined by comparing threshold values in a series of dilutions of the RT product, followed by a non-RT template control and a non-template control for each primer pair. Relative RNA levels were calibrated and normalized to the level of *AtAct2* mRNA.

Induced Resistance in *Arabidopsis* Mutants *npr1*, *sid2*, *jar1-1*, and *etr1-3* and Transgenic NahG Plants

To test whether 3-pentanol elicits induced resistance via the JA, SA, or ET pathway, *Pto*-induced disease development was assessed in wild-type Col-0 seedlings and the following mutants: *jar1-1* for JA signaling; *npr1*, NahG, and *sid2* for SA signaling; and *etr1-3* for ET signaling. The experimental protocols were essentially the same as those described previously (Ryu et al., 2003b). Briefly, all mutant and transgenic lines were derived from the parental *A. thaliana* ecotype Columbia (Col-0), which was obtained from the Ohio State University Stock Center, Columbus, OH, USA. The *Arabidopsis* seeds were surface-sterilized with 6% sodium hypochlorite, washed four times with SDW, and maintained at 4°C for 2 d to enhance germination. Seedlings preparation and growth condition were same as describe previously (Ryu et al., 2004). Bacterial pathogens were cultured overnight at 30°C in King's B medium supplemented with 100 μg/ml rifampicin (Ryu et al., 2004; Lee et al., 2012). The disease symptoms were photographed 7 days after pathogen challenge. The disease rate (0−5) of each plant was measured by recording the percentage of total plant leaf surface showing

symptoms as described above (Lee et al., 2012).The experimental protocol was designed to ensure complete randomization with 12 replications and one plant per replication. The entire experiment was repeated twice.

Statistical Analysis

Analysis of variance for experimental datasets was performed using JMP software version 5.0 (SAS Institute Inc., Cary, NC, USA). Significant treatment effects were determined by the magnitude of the F value ($P = 0.05$). When a significant F test was obtained, separation of means was accomplished by Fisher's protected LSD at $P = 0.05$.

Results

Gaseous 3-pentanol Triggers Induced Systemic Resistance in *Arabidopsis*

We first evaluated the effect of gaseous 3-pentanol on induced resistance against *Pto* DC3000 (**Figure 1A**). Disease severity after exposure to volatile emission from 1 nM, 100 nM, 10 μM, and 1 mM 3-pentanol was 3.1(error range: 0.6549), 1.9 (0.3758), 1.5 (0.3371), and 1.4 (0.2875), respectively, whereas it was 4.1 (0.3093) for the mock-inoculated water control (**Figure 1B**). Exposure to volatile emission from 1 mM 1-pentanol and 10 μM 2-pentanol significantly reduced disease severity compared to control. These experiments show that 3-pentanol is more effective than other isoforms to induce resistance against *Pto* DC3000 and a concentration of 100 nM was sufficient to significantly reduce disease severity (**Figure 1B**). The number of bacterial cells in leaf collected 3 and 7 days after inoculation was reduced significantly in plants exposed to 100 nM, 10 μM, and 1 mM 3-pentanol, whereas bacterial growth was not significantly different in plants exposed to 1 nM 3-pentanol and control plants (**Figure 2**. The results show that 10 and 100 nM 3-pentanol reduced bacterial cell counts by 100- and 25-fold, respectively, compared with mock-inoculated control. Therefore, we chose 10 μM and 100 nM 3-pentanol for further experiments. No direct inhibition was detected between different concentrations of 3-pentanol and *Pto* DC3000 indicating that the population reduction was caused by elicitation of induced resistance (data not shown).

3-pentanol-Mediated Induced Resistance Involves SA and JA Signaling Pathways

We evaluated the expression of defense-related genes in response to 3-pentanol and subsequent challenge with *Pto* DC3000 using Quantitative RT-PCR (qRT-PCR; **Figure 3A**). We first investigated the direct effect of 3-pentanol on the defense-related genes of *Arabidopsis*, including *PR1* (SA response), *PDF1.2* (JA response), and *CHIB* (ET response) that evaluated previously (Lee et al., 2012). The transcriptional level of the three signaling marker genes did not differ greatly from the control at day 0 and 7 after exposure to 3-pentanol before pathogen challenge (**Figure 3B**). Exposure to gaseous 3-pentanol and subsequent pathogen challenge caused a 32- and 16-fold upregulation of *PDF1.2* (JA signaling) and a 3.7- and 4.1-fold upregulation of

FIGURE 3 | Expression marker genes for defense priming of SA, JA, and ET signaling pathways in *Arabidopsis* Col-0 plants treated with volatile 3-pentanol (100 nM) and subsequently challenged with *P. syringae* pv. tomato DC3000 (*Pto*) (A). Expression of *PR1*, *PDF1.2*, and *CHIB* was analyzed with qRT-PCR at 0 and 7 days after volatile 3-pentanol treatment **(B)** and then at 0, 6, and 12 h after *Pto* inoculation **(C)**. The housekeeping gene, *AtActin*, was used to indicate equal loading. Asterisks indicate significant differences among treatments ($P = 0.05$ by LSD). Error bars represent means ± SEM; $N = 12$ plants per treatment.

PR1 (SA signaling) transcriptional level, respectively, compared with that of sterile distilled water control at 6 and 12 h post inoculation (hpi). By contrast, there was no significant difference in *CHIB* (ET signaling) transcriptional level at 0.2, 6, and 12 hpi in plants exposed to gaseous 3-pentanol or sterile distilled water control and subsequently challenged with pathogen. These results suggest that 3-pentanol treatment primes the JA and SA signaling pathways (**Figure 3C**). Interestingly, significant upregulation of *PR1* at 0.2 hpi was detected in control plant when compared to that of gaseous 3-pentanol treated plants (**Figure 3C**).

Next, we used the I-plate system to test the effects of 100 nM and 10 μM 3-pentanol evaporate on plant defense pathway signaling in five *Arabidopsis* mutants [*npr1* (SA signaling), NahG
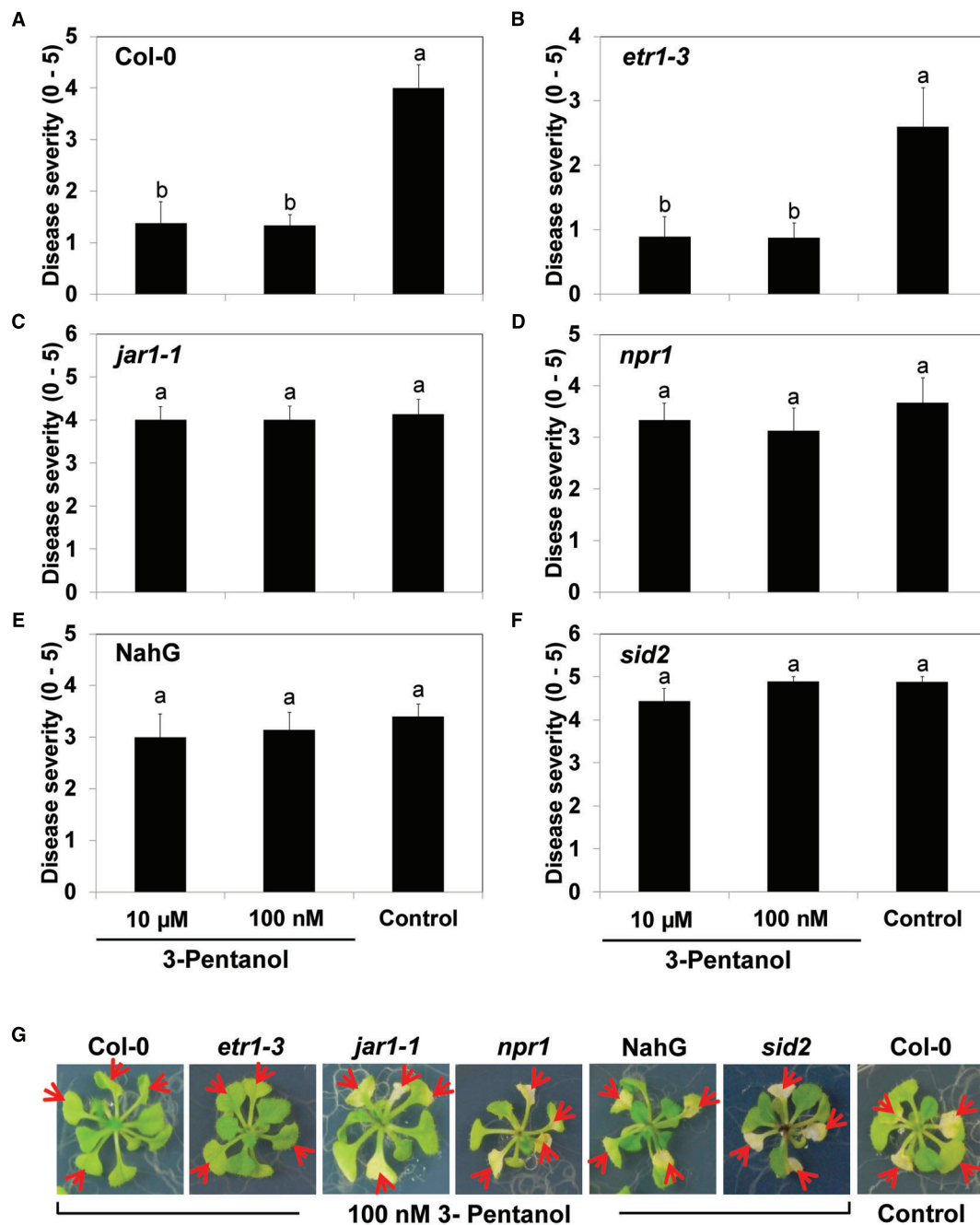
FIGURE 4 | Volatile 3-pentanol-mediated induced resistance in *Arabidopsis* mutants against *Pto*. *Arabidopsis* Col-0 seedlings **(A)**, *etr1-3* **(B)**, *jar1-1* **(C)**, *npr1* **(D)**, NahG **(E)**, and *sid2* **(F)** were exposed to 100 nM and 10 μM 3-pentanol evaporate or sterile distilled water control. After 5 days, all plants were drop-inoculated with *Pto*. Disease severity was measured 7 days after pathogen inoculation. Numbers represent means of 12 replications per treatment, with one seedling per replication. Different letters in **(A)** to **(F)** indicate significant differences between treatments ($P = 0.05$ by LSD). Error bars represent means ± SEM. **(G)** Plants were photographed 7 days after pathogen challenge.

(SA degradation), *sid2* (SA synthesis), *jar1-1* (JA-resistant), and *etr1-3* (ET receptor mutant)] challenged with *Pto* DC3000 (Lee et al., 2012). The SA signaling-related mutants *npr1*, NahG, and *sid2*, and the JA-resistant mutant *jar1-1* displayed severe disease symptoms (**Figures 4C–G**), whereas Col-0 and *etr1-3* consistently displayed induced resistance (**Figures 4A,B, G**). We obtained similar results from three independent experiments.

Discussion

Our previous results showed that drench application of 3-pentanol protected cucumber plants against bacterial pathogens and insects in an open-field experiment (Song and Ryu, 2013). In the current study, we took a step further and evaluated the molecular mechanisms behind 1-pentanol, 2-pentanol, and 3-pentanol-mediated plant systemic resistance. We also investigated which

plant defense signaling pathways may be primed by 3-pentanol-mediated induced resistance. This study shows that exposure to gaseous 3-pentanol upregulates the expression of marker genes *PDF1.2* and *PR1*, thereby indicating that 3-pentanol primes JA and SA defense signaling pathways.

Plant-associated bacteria including mainly *Bacillus* spp. and *Pseudomonas* spp. release 10–40 VOCs into the headspace above colonies grown on complex medium (Farag et al., 2013). *In planta*, 2,3-butanediol or acetoin were identified as bacterial VOCs responsible for reducing soft-rot symptoms caused by *Pectobacterium carotovorum* subsp. carotovorum in *Arabidopsis*, anthracnose caused by *Colletotrichum orbiculare* in *Nicotiana benthamiana*, and the fungal pathogens, *Microdochium nivale*, *Rhizoctonia solani*, or *Sclerotinia homoeocarpa* in *Agrostis stolonifera*, suggesting the possible use of bacterial VOCs as environmentally sound biochemical agents for agricultural applications (Ryu et al., 2004; Chung et al., 2015). However, there are several disadvantages to the use of bacterial VOCs in agricultural fields: (1) rapid evaporation rate, (2) inconsistencies between *in vitro* effects (e.g., I-plate) of VOCs and effects observed in open-field experiments, and (3) unstable effectiveness of target VOCs. To overcome these problems, we attempted to identify more effective bacterial volatiles and their derivatives that increased plant resistance. Previous studies on bacterial volatiles as chemical triggers of systemic resistance were carried out in cucumber (Song and Ryu, 2013). We initially identified 1-pentanol from a headspace analysis of bacterial colonies, and then assessed the derivative 3-pentanol as a trigger for systemic resistance in pepper plants cultivated in a greenhouse (data not shown). Unexpectedly, 3-pentanol had greater effect on induced resistance in pepper than 1-pentanol. We then evaluated the effectiveness of 3-pentanol in an open-field experiment. Drench application of 1 mM 3-pentanol into the soil conferred plant protection against bacterial spot caused by *Xanthomonas axonopodis* pv. vesicatoria (Choi et al., 2014). In the greenhouse and open-field experiments, we cannot rule out an effect of 3-pentanol as a VOC that triggers the induction of systemic resistance due to volatilization (evaporation) of 3-pentanol after drench application.

We tested the effect of gaseous 3-pentanol on induced resistance in *Arabidopsis* seedlings grown in I-plates; the results indicate that symptom development was significantly suppressed (**Figures 1** and **2**). Drench application of 3-pentanol to *Arabidopsis* seedlings grown under *in vitro* conditions triggered induced resistance (data not shown). Disease development was not significantly different between the two application protocols (exposure to gaseous 3-pentanol or drench application to the roots), indicating that volatile 3-pentanol may be the main

agent triggering ISR in drench application experiments. The previous study showed a significant behavioral response of the ambrosia beetle, *M. mutatus*, to 10 µg of 3-pentanol (= 100 nM 3-pentanol) (Manrique et al., 2006). The current study evaluated different 3-pentanol doses and clearly demonstrated biological relevance for triggering plant defense responses (**Figures 1B,C**). 3-pentanol did not show direct growth inhibition of *Pto* (data not shown). Furthermore, 3-pentanol volatile applications displayed no significant increase of *CHIB*, *PDF1.2*, and *PR1* expression levels (**Figure 3B**). Alternatively, 3-pentanol considerably primed the elicitation of transcriptional levels of *PDF1.2* and *PR1* when compared to controls after the pathogen challenge (**Figure 3C**). Thus the results clearly indicated that gaseous 3-pentanol mediate defense priming of SA (*PR1* marker gene upregulation) and JA (*PDF1.2* marker gene upregulation) signaling pathways (**Figures 3B,C**).

An unexpected increase in the level of *PR1* gene transcription at 0.2 h in the control plant (**Figure 3C**) can be speculated as wound-mediated defense priming, that is suppressed by *Pto* at 6 and 12 h (**Figure 3C**). Nevertheless, the detailed underlying mechanism remains to be understood.

This result is in agreement with a previous report. Drench application of 100 nM 3-pentanol on pepper seedlings primed the transcriptional level of pepper defense genes including *CaPR1* and *CaPR2* for SA signaling, *CaPIN2* for JA signaling, and *CaPR4* and *CaGLP1* for ET signaling at 3 and 6 h after pathogen challenge (Choi et al., 2014). In cucumber, drench application of 1 mM 3-pentanol upregulated *CsLOX1* at 6 h after pathogen challenge, indicating that the oxylipin pathway was triggered to recruit the natural enemy of aphids (Song and Ryu, 2013).

Conclusion

In conclusion, this study shows that a volatile 3-pentanol can trigger plant systemic resistance against *Pto* DC3000. Our results suggest that insect pheromones may be a rich source of chemical triggers that protect plants by enhancing plant immunity.

Funding

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Are Bacterial Volatile Compounds Poisonous Odors to a Fungal Pathogen *Botrytis cinerea*, Alarm Signals to *Arabidopsis* Seedlings for Eliciting Induced Resistance, or Both?

Rouhallah Sharifi[1,2] and Choong-Min Ryu[1,3]*

[1] Molecular Phytobacteriology Laboratory, Super-Bacteria Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon, South Korea, [2] Department of Plant Protection, College of Agriculture and Natural Resources, Razi University, Kermanshah, Iran, [3] Biosystems and Bioengineering Program, University of Science and Technology, Daejeon, South Korea

Biological control (biocontrol) agents act on plants via numerous mechanisms, and can be used to protect plants from pathogens. Biocontrol agents can act directly as pathogen antagonists or competitors or indirectly to promote plant induced systemic resistance (ISR). Whether a biocontrol agent acts directly or indirectly depends on the specific strain and the pathosystem type. We reported previously that bacterial volatile organic compounds (VOCs) are determinants for eliciting plant ISR. Emerging data suggest that bacterial VOCs also can directly inhibit fungal and plant growth. The aim of the current study was to differentiate direct and indirect mechanisms of bacterial VOC effects against *Botrytis cinerea* infection of *Arabidopsis*. Volatile emissions from *Bacillus subtilis* GB03 successfully protected *Arabidopsis* seedlings against *B. cinerea*. First, we investigated the direct effects of bacterial VOCs on symptom development and different phenological stages of *B. cinerea* including spore germination, mycelial attachment to the leaf surface, mycelial growth, and sporulation *in vitro* and *in planta*. Volatile emissions inhibited hyphal growth in a dose-dependent manner *in vitro*, and interfered with fungal attachment on the hydrophobic leaf surface. Second, the optimized bacterial concentration that did not directly inhibit fungal growth successfully protected *Arabidopsis* from fungal infection, which indicates that bacterial VOC-elicited plant ISR has a more important role in biocontrol than direct inhibition of fungal growth on *Arabidopsis*. We performed qRT-PCR to investigate the priming of the defense-related genes *PR1*, *PDF1.2*, and *ChiB* at 0, 12, 24, and 36 h post-infection and 14 days after the start of plant exposure to bacterial VOCs. The results indicate that bacterial VOCs potentiate expression of *PR1* and *PDF1.2* but not *ChiB*, which stimulates SA- and JA-dependent signaling pathways in plant ISR and protects plants against pathogen colonization. This study provides new evidence for bacterial VOC-elicited plant ISR that protects *Arabidopsis* plants from infection by the necrotrophic fungus *B. cinerea*. Our work reveals that bacterial VOCs primarily act via an indirect mechanism to elicit plant ISR, and have a major role in biocontrol against fungal pathogens.

Keywords: bacterial volatile organic compounds, phytohormones, induced systemic resistance, plant growth-promoting rhizobacteria, biofilm formation, leaf surface attachment

INTRODUCTION

Plants have evolved complex and efficient surveillance systems that respond to abiotic and biotic stresses, including pests and pathogens (Agrios, 2004). Small signaling molecules elicit plant cell defense responses throughout the plant; these include the phytohormones salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) (Pieterse et al., 2009). Each hormone has a particular function in eliciting plant immunity. For example, SA-dependent signaling is triggered by necrotizing avirulent pathogens, whereas JA signaling is triggered by necrotrophic pathogens and insect pests (Mysore and Ryu, 2004). Plants with activated immune systems produce diverse classes of pathogenesis-related (PR) proteins and toxic phenol compounds such as phytoalexins, which can function to induce subsequent defense mechanisms (Ryals et al., 1996; Pieterse et al., 2009; Balmer et al., 2013). Plant immune responses can be induced by pathogens, insects, and beneficial root-associated bacteria designated as plant growth-promoting rhizobacteria/fungi (PGPR/PGPF). PGPR/PGPF systems elicit similar plant immune responses as those elicited by pathogens and insects (Kloepper et al., 2004). Plant resistance to pathogens and insects is largely mediated by the two plant hormones, JA and ET, which elicit induced systemic resistance (ISR) responses (Pieterse et al., 2009; Balmer et al., 2013). Previous studies reported that ISR were effectively elicited by necrotrophic pathogens such as *Pectobacterium carotovorum* (Han et al., 2006; Farag et al., 2013). To identify microbial determinants that elicit ISR, microbial secreted products have been tested on plants under greenhouse and field conditions, including siderophores, phytohormone mimetics, *N*-acyl homoserine lactone, vitamins, and cell wall components such as chitin, glucan, and lipopolysaccharides (Lyon, 2007; Hartmann and Schikora, 2012; Kanchiswamy et al., 2015a,b; Lee et al., 2015).

New investigations clearly demonstrate that PGPR/PGPF emit volatile compounds that trigger robust plant systemic defense responses against pathogenic bacteria (Ryu et al., 2004; Kishimoto et al., 2007; Rudrappa et al., 2010; Song and Ryu, 2013). Ryu et al. (2004) and Rudrappa et al. (2010) shown that bacterial volatile organic compounds (VOCs) activate plant defenses in a strain-specific manner. Ryu et al. (2004) reported that activation of systemic defense in *Arabidopsis* against *Erwinia carotovora* subsp. *carotovora* elicited by *Bacillus subtilis* strain GB03 VOCs depends on ET pathways but was independent of JA and SA pathways. Rudrappa et al. (2010) showed that SA and ET are required for *B. subtilis* FB17 VOC-elicited ISR in *Arabidopsis* against the hemibiotrophic pathogen *Pseudomonas syringae* pv. tomato DC3000, whereas JA was not required. Not only bacteria strains but also a single volatiles can employ different signaling pathways to boost plant defense. Some bacteria emit 2,3-butanediol and its precursor acetoin as VOCs; these volatiles have roles in plant protection against pathogens (Farag et al., 2006). 2,3-butanediol activated *PR-4* expression and SA-dependent signaling in *Agrostis stolonifera* (Cortes-Barco et al., 2010a),

application of 100 µM 2,3-butanediol on *Nicotiana benthamiana* seedlings elicited resistance against the hemibiotrophic fungus *Colletotrichum orbiculare* (Cortes-Barco et al., 2010b) by increasing basic PR proteins expression, which are markers of JA-dependent signaling, whereas there was no change in acidic PR proteins expression, which are markers of SA-dependent signaling. 2,3-butanediol activated both SA- and JA-dependent signaling pathways in *Arabidopsis thaliana* in response to abiotic stress (Cho et al., 2008). Currently, there is debate in the literature regarding the function and activity of *Bacillus* VOCs.

Many studies report antagonistic effects of VOCs on plant pathogenic fungi (McCain, 1966; Kai et al., 2007). VOCs can act as antibiotics and directly inhibit mycelial growth and spore germination of pathogenic fungi (Kai et al., 2007; Vespermann et al., 2007). Fiddaman and Rossall (1993) and Chaurasia et al. (2005) show that *B. subtilis* VOCs deform mycelia and inhibit growth of some pathogenic and biocontrol fungi. *B. subtilis* VOCs induce protoplasm retraction in *Botrytis cinerea* hyphae (Chen et al., 2008). However, most of these reports utilize a dual culture method that exposes pathogenic fungi to extremely high VOC levels, which do not occur under natural conditions. Therefore, it is necessary to determine whether *Bacillus* VOCs protect *Arabidopsis* against *B. cinerea* via ISR or by inhibiting fungal growth or infection.

The objective of this work was to investigate direct and indirect mechanisms of *Arabidopsis* protection conferred by *B. subtilis* strain GB03 VOCs against the necrotrophic fungal pathogen *B. cinerea*. First, we evaluated the effects of different VOC concentrations on stages of the *B. cinerea* life cycle and during *Arabidopsis* infection. Secondly, we examined direct and indirect VOC effects on *Arabidopsis* protection against pathogenic fungal infection. Third, we investigated the priming of defense gene expression conferred by bacterial VOCs as a means of eliciting plant ISR. Our results indicate that low concentrations of *B. subtilis* GB03 VOCs induce plant systemic resistance and defense responses, and protect *Arabidopsis* against infection by the necrotrophic pathogen *B. cinerea*. This study broadens the understanding of plant defense mediated by bacterial volatile compounds.

MATERIALS AND METHODS

Evaluating Bacterial Volatile Effects on *Botrytis cinerea* Growth and Plant Protection from Infection

To study the effects of *B. subtilis* GB03 VOCs on *B. cinerea* spore germination, we placed a small Petri dish measuring 40 mm diameter and 10 mm depth inside a larger Petri dish measuring 90 mm diameter and 15 mm depth. Tryptic soy broth (Difco Co, MD, USA) agar (TSA) medium was poured into the larger plate and allowed to solidify. Then, 0–3 sterile filter paper disks (5 mm diameter) were placed around the periphery of the TSA

plate. The disks were saturated with a 30 μl suspension of 10^8 colony forming units (CFU)/ml of *B. subtilis* GB03. The inoculated plates were sealed with lids and incubated at 30°C for 24 h. Then, 4 ml of 1×10^5 CFU/ml of *B. cinerea* spores in half-strength potato dextrose broth (Difco Co, MD, USA) were added to the small 4 cm plate in the center of the inoculated 9 cm plate. The lid of the larger Petri dish was sealed with Parafilm and incubated at 25°C (**Figure 2B**). Spore germination was assessed after 6 h by observing 50 spores per replicate plate at 200× magnification (Nikon Eclipse E600, Osaka, Japan), and percentage germination was recorded. Spores with germinated mycelia longer than half of the spore diameter were considered as germinated.

The effect of bacterial VOCs on *B. cinerea* mycelial growth was investigated using the double-plate assay of Ting et al. (2011). A 9 cm dish containing TSA medium was prepared, 1–3 sterile filter paper disks (5 mm) were placed on the medium, and 30 μl of 10^8 CFU/ml of *B. subtilis* GB03 suspension was pipetted onto the filter disks. The plates were sealed with lids and incubated at 30°C for 24 h. Then, agar plugs of *B. cinerea* mycelia were taken from the periphery of a plate containing a young growing culture, and the plugs were inserted into the center of a 9 cm Petri dish containing 20 ml of PDA. This plate was inverted and securely fitted over the *B. subtilis* plate. The two plates were sealed together with Parafilm and incubated at 25°C (**Figure 2A**). Radial growth of the fungus was evaluated after 7 days.

The effect of bacterial VOCs on *B. cinerea* sporulation was examined using the same double-plate assay used for the mycelial growth test with slight modification. To evaluate sporulation, a plate was prepared with agar plugs of *B. cinerea* mycelia and incubated at 25°C until vegetative mycelial growth filled the plate. Then, this plate was inverted over the top of a TSA plate containing 1–3 sterile filter paper disks saturated with 30 μl of *B. subtilis* GB03 suspension, the plates were sealed together using Parafilm, and incubated at 25°C for 7 days. Fungal spores were harvested with 10 mM $MgCl_2$, and the spore suspensions were passed through gauze cloth to remove mycelial fragments. The numbers of spores produced under each bacterial VOC dosage were counted using a hemocytometer and a compound light microscope.

Analysis of Mycelial Attachment to Polystyrene Surface

The mycelial surface attachment evaluated in broth medium same as the method used to evaluate fungal spore germination. The layer of mycelium in top of broth medium showing the air-medium interface was photographed after 7 days (**Figure 4**). The surface attachment which reflects hydrophobic surface tendency, was evaluated using the crystal violet (CV) staining method according to Mowat et al. (2007). Briefly, the 4 cm Petri dishes (SPL, Pocheon-si, Gyeonggi-do, South Korea) containing fungi were washed four times with tap water to remove non-attached mycelia and spores. Petri dishes were air dried and attached mycelia were stained with 4 ml of 0.5% CV for 15 min at room temperature. Excess CV stain was removed

by washing several times and gently pipetting off the rinsate until excess stain was removed. The remaining CV stain was solubilized by the addition of 4 ml of 95% ethanol. Solubilized CV was gently pipetted off, and the absorbance at OD_{600} was determined using a spectrophotometer (Ultraspec 7000, Biochrom Co, Cambridge, England). Mycelia attachment to leaf surface have been assessed by microscopic inspection of *Arabidopsis* leaves after staining with Trypan blue according to (Koch and Slusarenko, 1990).

Assessment of ISR in Plants Treated with Bacterial Volatiles

Arabidopsis seeds were surface sterilized with 1% (v/v) sodium hypochlorite for 20 min, rinsed three times in sterile distilled water (SDW), and placed on Petri dishes containing half-strength Murashige and Skoog (MS) medium (Duchefa Biochemie, Haarlem, The Netherlands) containing 0.8% (w/v) plant agar and 1.5% (w/v) sucrose (pH 5.8). The plates were placed in a growth cabinet with a 16 h light/8 h dark cycle provided by fluorescent lights (8,000 lux), and temperature was maintained at 22°C with 50–60% relative humidity. Two days after vernalization, seedlings were transferred to specialized plastic Petri dishes that contained a center partition (designated as I-plates). One chamber of the I-plate contained half-strength MS agar medium and the other chamber contained TSA onto which 5 mm diameter sterile filter paper disks had been placed. The seedlings were transferred to the chamber containing MS agar medium. The chamber containing TSA was inoculated with 30 μl of a *B. subtilis* suspension or SDW applied dropwise onto the filter paper disk. Plates were sealed with Parafilm and transferred to the growth chamber using the same conditions. Fourteen days later, 5 μl of *B. cinerea* spore suspensions (10^5 CFU/ml) were drop-inoculated onto five leaves per *Arabidopsis* seedling. Leaves exhibiting symptoms were determined by visual inspection 3 days after inoculation.

Differentiating Direct and Indirect Mechanisms of Bacterial VOC-Mediated Plant Protection from *Botrytis cinerea* Infection

These experiments were performed using the I-plate method described by Ryu et al. (2004). Two-day-old *Arabidopsis* seedlings were transplanted into one chamber of an I-plate containing MS agar medium, and 30 μl of 10^8 CFU/ml *B. subtilis* GB03 suspension or distilled water control was pipetted onto a sterile filter paper disk on TSA medium in the other chamber. After 14 days of seedling exposure to VOCs, the plates were separated into two groups. In one group, the TSA medium and disks containing bacteria were removed from the I-plate (designated as the *B. subtilis*-removed group). In the other group, the TSA medium and disks containing bacteria was allowed to remain in the I-plate (designated as the *B. subtilis*-treated group). Subsequently, all plates were subjected to inoculation with 5 μl of *B. cinerea* spore suspensions (10^5 CFU/ml) onto five leaves per *Arabidopsis* seedling. Disease severity was evaluated 3 days after pathogen challenge.

Percentage of direct and ISR effect of VOCs on disease suppression calculated based on below formula:

$$\text{Direct effect} =$$

$$\frac{\text{DS in } B. \text{ subtilis removed} - \text{DS in } B. \text{ subtilis treated}}{\text{DS in control} - \text{DS in } B. \text{ subtilis treated}} \times 100$$

$$\text{ISR} = \frac{\text{DS in control} - \text{DS in } B. \text{ subtilis removed}}{\text{DS in control} - \text{DS in } B. \text{ subtilis treated}} \times 100$$

Where DS is disease severity percent.

Evaluation of Plant Defense Priming using Quantitative RT-PCR

Total RNA was isolated from inoculated 14-day-old leaf tissues 0, 12, 24, and 36 h after *B. cinerea* inoculation according to the protocol of Yang et al. (2009). Total RNA was treated with 1 U of RNase-free DNase (Promega, USA) for 10 min at 37°C, and then subjected to a second round of purification using TRI reagent. First-strand cDNA synthesis was performed in 20 µl of AccuPower RT PreMix (Bioneer, Korea) containing 1 µg of DNase-treated total RNA, oligo(dT) primers, and Moloney murine leukemia virus reverse transcriptase (MMLV- RT; Invitrogen, USA). PCR reactions were performed according to the manufacturer's instructions. The candidate gene was analyzed using the following primers: 5′-GCTTCA GACTACTGTGAACC-3′ (*ChiB_F*) and 5′-TCCACCGTTAATG ATGTTCG-3′ (*ChiB_R*); 5′-AATGAGCTCTCATGGCTAAGTT TGCTTCC-3′ (*PDF1.2_F*) and 5′-AATCCATGGAATACACAC GATTTAGCACC-3′ (*PDF1.2_R*); 5′-TTCCACAACCAGGCAC GAGGAG-3′ (*PR1_F*) and 5′-CCAGACAAGTCACCGCTACC C-3′ (*PR1_R*). The AGI codes were as follows: *CHIB* (AT3G 12500), *PDF1.2* (AT5G44420), *PR1* (AT2G14610), and *AtACT2* (AT3G18780). The control for equal loading was *AtActin* using the primers 5′-GTTAGCAACTGGGATGATATGG-3′ and 5′-CAGCACCAATCGTGATGACTTGCCC-3′. Candidate priming genes were PCR-amplified from 100 ng of cDNA using an annealing temperature of 60°C. Amplified PCR products were separated by 2% agarose gel electrophoresis. The qRT-PCR assays were performed using a Chromo4 Real-Time PCR system (Bio-Rad). Reaction mixtures (10 µl) contained 5 µl of 2 × Brilliant SYBR Green qPCR master mix (Bio-Rad), cDNA, and 10 pM of each primer. Thermocycle parameters were as follows: initial polymerase activation for 10 min at 95°C, and then 40 cycles of 30 s at 95°C, 30 s at 60°C, and 42 s at 72°C. Conditions were determined by comparing the threshold values in a dilution series of the RT product, followed by a non-RT template control and a non-template control for each primer pair. Relative RNA levels were calibrated and normalized to the level of *AtAct2* mRNA.

Statistical Analysis

Data were subjected to analysis of variance (ANOVA) using JMP software (SAS Institute Inc., Cary, NC, USA). Significance of *B. subtilis* GB03 VOC treatment effects were determined by the magnitude of the F value at $P = 0.05$. When a significant F value was obtained for treatments, separation of means was accomplished using Fisher's protected least significant difference (LSD) at $P = 0.05$. Experiments done in four replicate and each experiment repeated for three time. The results of repeated trials of each experiment were similar. Therefore, one representative trial of each experiment is reported in the section "Results."

RESULTS

Assessment of Bacterial VOC-Mediated Plant Protection Against *B. cinerea* Infection

We tested the effects of bacterial VOCs on different stages of *B. cinerea* growth, including spore germination, mycelial growth, and spore production. First, we confirmed that pretreatment of plants with bacterial VOCs conferred protection against subsequent *B. cinerea* infection (**Figure 1**). A few leaves pretreated with *B. subtilis* GB03 VOCs developed very mild scars at the fungal inoculation site, whereas control leaves developed severe necrosis (**Figure 1**). Bacterial VOCs suppressed fungal growth and development at all phenological stages in a dose-dependent manner (**Figures 2A–C**). In all of these tests, inoculation of one filter paper disk with bacteria, which represented a low dose of VOCs, did not significantly affect mycelial growth, spore germination, and spore production (**Figures 2A–C**). Exposing *B. cinerea* to VOCs from two inoculated disks did not significantly affect mycelial growth and spore germination (**Figures 2A,C**), but reduced spore production by 0.271 log CFU/ml (**Figure 2B**). Exposing fungi to VOCs from three inoculated disks reduced mycelial growth, spore germination, and spore production by 61, 64, and 7%, respectively (**Figure 2**). When the bacteria colonized the entire Petri dish, fungal growth was completely inhibited (data not shown).

Differentiation of Direct versus Indirect Mechanisms of Plant Protection against Pathogen Infection by Bacterial VOCs

We designed experiments to differentiate between direct inhibition of fungal growth by VOCs and indirect inhibition by VOC induction of plant systemic resistance. For these experiments, we used one filter disk inoculated with *B. subtilis* GB03. *Arabidopsis* seedlings were transplanted into one chamber of an I-plate and the other chamber contained TSA medium with one disk inoculated with *B. subtilis* GB03 or control disk with 30 µl of distilled water. Plates were incubated in a growth chamber for 14 days. The plates containing GB03 were separated into two groups. In the first group, the TSA medium and disk containing bacteria were removed immediately before pathogen challenge. These plates no longer contained bacterial VOCs, and were designated as the "*B. subtilis*-removed" group (**Figure 3A**). In this group, only plant ISR could function against pathogen infection because there were no longer any VOCs to confer direct plant protection against *B. cinerea* colonization. In the second group, the bacteria were not removed (designated as "*B. subtilis*-treated"); therefore, there was continuous production

FIGURE 1 | Plant protection against *Botrytis cinerea* by volatile compounds emitted from *Bacillus subtilis* GB03. Bacterial volatile compounds (BVCs) elicit induced systemic resistance (ISR) against *B. cinerea* infection. **(A)** Representative photo of ISR by BVCs using by the I-plate system. The right panel = BVC treatment; left panel = water control **(B)** Disease severity (0 = no symptom, 10 = severe necrosis) in the presence of bacterial VOCs and water-treated controls was recorded and plates were photographed 3 days after pathogen challenge. Different letters indicate significant differences between treatments according to Fisher's LSD test at $P = 0.05$.

FIGURE 2 | Dose-dependent inhibition of *B. cinerea* mycelial growth, spore germination, and spore development by *B. subtilis* GB03 volatile compounds. (A) Spore germination, **(B)** spore formation, and **(C)** mycelial growth of *B. cinerea* were measured after 7 days in the presence of one, two, or three spot-inoculated filter disks (5 mm diameter) of *B. subtilis* GB03 bacterial suspension (10^8 CFU/ml) that were allowed to grow for 24 h before addition of fungi. Different letters indicate significant differences between treatments according to Fisher's LSD test at $P = 0.05$. Error bars indicate standard error of the mean (SEM).

of volatiles, which could induce indirect fungal inhibition by eliciting plant ISR and direct antagonism (**Figure 3A**). Based on M&M formula, ISR proportion was 90.63% and direct inhibition of fungi was 9.36% which mean that ISR have main role in suppression of *B. cinerea* on *Arabidopsis* in low concentration of VOCs (**Figure 3B**). Monitoring *in vivo* growth of *B. cinerea* on *Arabidopsis* by quantitative measurement of β-*tubulin* expression revealed that fungal growth inhibition was primarily due to stimulation of plant defense responses. There were differences between β-*tubulin* expressions in the "*B. subtilis*-removed" and "*B. subtilis*-treated" groups within 10 h post-infection (hpi). However, these effects were temporally unstable and were not significantly different at 20 and 30 hpi. There were no differences in β-*tubulin* expression levels between the "*B. subtilis*-removed" and "*B. subtilis*-treated" groups in 20 and 30 hpi.

Effects of Bacterial VOCs on artificial Surface and Leaf Attachment of *B. cinerea*

We performed microscopic investigation of *B. cinerea* colonization of *Arabidopsis* leaves. The results revealed that

the "*B. subtilis*-treated" group displayed more aggressive epiphytic growth on leaf surfaces than that of the "*B. subtilis*-removed" group (**Figure 3B**). These experiments were performed using one filter disk inoculated with *B. subtilis* GB03; however, one inoculated disk did not significantly affect spore germination and mycelial growth. We concluded that VOCs might interfere in mycelial attachment to the leaf surface, and this could cause
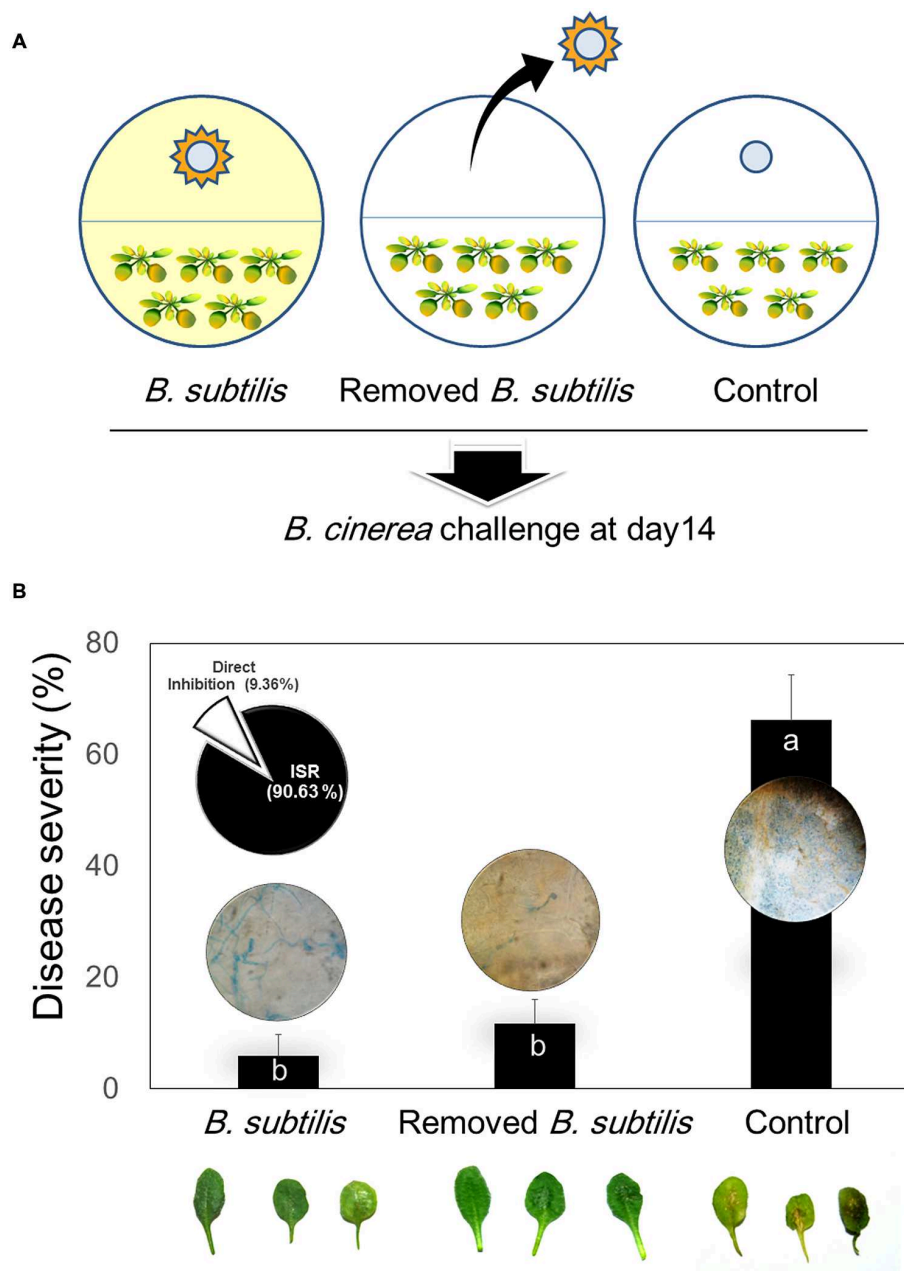
FIGURE 3 | Direct and indirect mechanisms of *B. subtilis* GB03 volatile compound effects on *Botrytis cinerea* and ISR of *Arabidopsis*. (A) Schematic of experimental design to test bacterial VOC-elicited ISR using the two-chamber I-plate system. *Arabidopsis* seedlings were grown in one chamber and *B. subtilis* GB03 were grown in the other chamber. The two chambers share the same head space, which allows bacterial volatiles to be transmitted to seedlings and inoculated fungal pathogens. In one group of plates, *B. subtilis* GB03 were removed before fungal inoculation at 14 days (middle image); in the other group, bacteria and volatile emission were retained (left image). Water-treated filter disks were used as control. **(B)** Disease severity caused by *B. cinerea* inoculation on *Arabidopsis* seedlings pretreated with *B. subtilis* GB03 VOCs, and in the presence and absence of continuing *B. subtilis* VOC emission. The inset pie graph indicates percentage plant protection conferred by VOC-elicited ISR (indirect mechanism) and by VOC-induced inhibition of fungal growth (direct mechanism). The inset photos above or inside bar graph indicate that *B. subtilis* GB03 volatile compounds inhibit leaf attachment of *B. cinerea.* Fungi growth were checked after staining with Trypan blue. Different letters indicate significant differences between treatments according to Fisher's LSD test at $P = 0.05$.

epiphytic mycelial growth and inability to penetrate and colonize host tissue. We investigated mycelial surface attachment in the presence of bacterial VOCs using a universal biofilm formation method (Mowat et al., 2007). The results showed that VOCs emitted from one disk inoculated with GB03 were sufficient to reduce mycelial attachment to the hydrophobic Petri dish surface by 51%. In the presence of VOCs emitted from three inoculated disks, mycelial surface attachment was reduced

FIGURE 4 | *Bacillus subtilis* GB03 volatile compounds inhibit surface biofilm formation of *Botrytis cinerea*. Fungal biofilm formation in the presence of different concentrations of bacterial VOCs was measured using the CV staining method. The absorbance of CV retained in the fungal biofilm was measured spectrophotometrically at 600 nm, and represents the biofilm quantity. Fungal cultures were photographed 7 days after the start of VOC exposure. Different letters indicate significant differences between treatments according to Fisher's LSD test at $P = 0.05$.

by sevenfold compared with that of the control (**Figure 4**). However, Higher VOC levels increased the air-liquid interface biofilm which means that fungi colonized surface of liquid media (**Figure 4**). A possible explanation for this aerotactic response could be an increase in mycelial hydrophilicity. Talbot et al. (1996) reported that production of cutinase and SC3 hydrophobin increased mycelial hydrophilicity. Therefore, it was possible that VOCs reduced mycelial attachment to the hydrophobic cuticular surface of the leaf. Further work is needed to confirm VOC effects on surface attachment regulation in *B. cinerea*. Our observations support the hypothesis that VOCs increase epiphytic growth of *B. cinerea* on *Arabidopsis* leaves. Although low VOC concentrations do not significantly affect fungal growth, they can affect some virulence factors such as host penetration (**Figure 3**).

Bacterial VOC-Mediated Priming of Plant Defense Against *B. cinerea* Infection

Volatile organic compounds-mediated plant defense priming was investigated using qRT-PCR analysis. The results indicate that JA signaling has a key role in VOC-elicited plant defense responses, because *PDF1.2* expression was much higher in VOC-treated plants than in control plants. Maximum increases in *PDF1.2* expression levels in VOC-treated and

control plants were 13 and 4.6-fold, respectively, at 36 hpi (**Figure 5B**). *PR-1* expression, which is a marker of SA signaling, had a maximum 2.8-fold increase in VOC-treated plants at 12 hpi (**Figure 5A**). The activation of *PDF1.2* and *PR-1* at 36 and 12 hpi, respectively, represents early stages of disease establishment and plant defense priming. *CHIB* expression, which is a marker ET signaling, did not display statistically significant differences in VOC-treated and control plants (**Figure 5C**). This result is not in agreement with our previous results (Ryu et al., 2004), and other ET-dependent marker genes should be analyzed. Previous studies report that a necrotrophic pathogen activated the JA signaling pathway and suppressed SA-dependent pathways (Pieterse and Van Loon, 2007), and activation of one pathway (JA or SA) had antagonistic effects on the other (Pieterse et al., 2009). However, the present study indicates that GB03 VOCs activate both pathways.

DISCUSSION

The debate on direct and indirect effects of bacterial VOC-mediated plant protection against pathogens arises because bacterial volatile compounds can directly inhibit pathogen growth (Chaurasia et al., 2005; Vespermann et al., 2007; Chen et al., 2008; Yuan et al., 2012) and indirectly elicit plant immune responses against target pathogens (Lee et al., 2012; Song and Ryu, 2013; Choi et al., 2014). Studies on direct effects of volatile compounds generally utilize very high volatile concentrations, which do not occur under natural conditions. Pedigo (1999) mentioned that high concentrations of any chemical can induce toxic effects. Studies on indirect effects of volatile compounds utilized low VOC concentrations by exposing plants and pathogens to one filter paper disks (5 mm diameter) with confluent PGPR growth (Ryu et al., 2004; Lee et al., 2012). In present study, one filter disk did not significantly affect mycelial growth, spore germination, and spore production, but reduced disease severity by 60.26% (**Figures 1** and **2**). This indicates that VOC-elicited plant ISR is involved in plant protection when VOC concentrations are low. Park et al. (2015) shown that one colony of bacteria was enough to increase plant growth up to 80% in natural condition. They revealed that 5 ng of 2-butanone is enough for promote plant growth. However, three filter paper disks significantly reduced mycelial growth, spore production, and spore germination (**Figure 2**). Exposing fungi to whole Petri dishes with confluently growing *B. subtilis* GB03 abolished mycelial growth. These results indicate that high VOC concentrations could be toxic for fungi. By contrast, treatment with low VOC concentrations (Ryu et al., 2004) or low levels of individual volatiles such as 0.2 pg of 2,3 butanediol (Ryu et al., 2004), 10 μM of MeJA (Jiang et al., 2015; Wang et al., 2015), 1 ppm of 2,4-Di-*tert*-butylphenol (Sang and Kim, 2012), 0.001 ppm of acetoin (Ann et al., 2013) or 100 μM hexadecane (Park et al., 2013) reveal the indirect mechanism of VOC-elicited plant ISR. In our previous work, we showed that 100 μM of pure volatiles acetoin, 2,3 butanediol, 3-pentanol and 1-pentanol had no negative effect on growth of

FIGURE 5 | *Bacillus subtilis* GB03 volatile compounds elicit *Arabidopsis* defense priming of the jasmonic acid and salicylic acid signaling pathways. Defense priming gene expression levels were measured by time-course qRT-PCR analysis of **(A)** *PR1* in the salicylic acid signaling pathway, **(B)** *PDF1.2* in the jasmonic acid signaling pathway, and **(C)** *ChiB* in the ethylene signaling pathway **(C)**. The ratio of gene expression in the *B. subtilis* GB03-treated plants versus that in the water-treated control relative to expression of the *Actin* gene is computed as the mean ± SEM. Different letters indicate significant differences between treatments **(A,B)** according to Fisher's LSD test at $P = 0.05$.

B. cinerea but suppressed disease on *Arabidopsis* (Sharifi et al., 2013).

The current study showed that VOC-elicited plant defense responses continued to be active after the bacteria and their emitted volatiles were removed, and plant immunity to infection was still observed at the time of pathogen challenge (**Figure 3B**). We suggests that direct VOC inhibition of fungal growth is only responsible for a small percentage (9.36%) of the total VOC effect in suppressing *B. cinerea*. Microscopic inspection showed that volatiles have effect on surface attachment of *B. cinerea*. In the presence of VOCs emitted from one inoculated filter disks, fungi growth more epiphytic (**Figure 3**). However, expression of β-tubulin showed that there were difference in colonization

between *B. subtilis* and removed *B. subtilis* just in 10 h. There was no difference in leaf colonization in the presence or absence of VOCs at 20 and 30 hpi. Bacterial VOCs emitted from one filter disk were sufficient to reduce *B. cinerea* attachment to the leaf surface (**Figure 4**). This could suppress fungal penetration and induce epiphytic growth on the leaf surface (Talbot et al., 1996; Tucker and Talbot, 2001). Biotrophic and necrotrophic fungi need to tightly attach to the host cuticular surface and then penetrate the host tissue. Attachment to the host surface during the initial stage of fungal pathogenesis is crucial for fungal establishment on the host (Talbot et al., 1996; Tucker and Talbot, 2001; Harding et al., 2009). Altering the surface attachment efficiency of *B. cinerea* reduced or inhibited its pathogenicity (Doss et al., 1995; Epstein and Nicholson, 1997). Quintana-Rodriguez et al. (2015) shown that volatiles emitted from diseased plant could increase resistance in neighbor susceptible plants. These volatiles also were able to directly inhibited conidia germination *in vivo* and *in vitro* but in dose dependent manner.

We evaluated VOC-elicited plant defense priming by performing a time-course qRT-PCR analysis of defense marker gene expression (**Figure 5**). We analyzed the expression of *PDF1.2*, *PR-1*, and *ChiB*, which are involved in JA, SA, and ET signaling pathways, respectively. The highest gene expression achieved in *PDF1.2* after *B. subtilis* GB03 treatment. *PDF1.2* expression reached maximum at 36 hpi, which is within the typical plant defense priming temporal window. The JA signaling pathway is the primary pathway induced by necrotrophic fungi such as *B. cinerea* (Glazebrook, 2005). Our previous work revealed that Me-JA could suppress *B. cinerea* disease on *Arabidopsis* up to 40% (Sharifi et al., 2013). *PR-1* expression also displayed evidence of defense priming, with maximum expression occurring at 12 hpi. The present study observed that *B. subtilis* GB03 VOCs activated both JA and SA signaling pathways. Several studies report that *Bacillus* strains can simultaneously activate JA and SA pathways, and SA, JA, and ET pathways (Buensanteai et al., 2009; Rudrappa et al., 2010; Niu et al., 2011; Lee et al., 2012; Choi et al., 2014). The *B. subtilis* volatile compound acetoin activated expression of both *PR-1* and *PDF1.2* (Rudrappa et al., 2010). Tridecane from *Paenibacillus polymyxa* E681 induced *PR-1* and *VSP2* expression (Lee et al., 2012).

CONCLUSION

We intended to link information from two types of reports. Researcher who mentioned that, volatiles are responsible for direct fungi growth inhibition and researchers who report that, ISR is mechanism of volatiles action. The optimum VOCs concentration that did not inhibit the fungus *in vitro* still elicit plant defense strategies to prevent subsequent *Botrytis* infection (**Figures 2** and **4**). Low VOCs concentrations elicit plant ISR, which is an indirect mechanism that results in 90.63% of disease suppression (**Figure 3**). By contrast, direct inhibition of fungal growth and development by low VOC concentrations accounts for 9.36% of the total disease suppression (**Figure 3**). The direct mechanism may become

more prominent with higher VOC concentrations in a dose-dependent manner. JA was the main signaling pathway in activation of plant defense by means of *B. subtilis* GB03 volatiles (**Figure 5**). However, there were no cross-talk between SA and JA signaling pathway as expression of both genes increased.

AUTHOR CONTRIBUTIONS

RS conceived and performed experiments, interpreted data and contributed to the drafting of the manuscript. CMR gave experimental advice, interpreted data, and contributed to the

drafting of the manuscript. All authors contributed to the discussion and approved the final manuscript.

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The effects of bacterial volatile emissions on plant abiotic stress tolerance

*Xiao-Min Liu and Huiming Zhang**

Shanghai Center for Plant Stress Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China

Plant growth-promoting rhizobacteria (PGPR) are beneficial plant symbionts that have been successfully used in agriculture to increase seedling emergence, plant weight, crop yield, and disease resistance. Some PGPR strains release volatile organic compounds (VOCs) that can directly and/or indirectly mediate increases in plant biomass, disease resistance, and abiotic stress tolerance. This mini-review focuses on the enhancement of plant abiotic stress tolerance by bacterial VOCs. The review considers how PGPR VOCs induce tolerance to salinity and drought stress and also how they improve sulfur and iron nutrition in plants. The potential complexities in evaluating the effects of PGPR VOCs are also discussed.

Keywords: plant growth-promoting rhizobacteria, volatile organic compounds, abiotic stress, salinity, drought, sulfur nutrition, iron deficiency

Introduction

Plants live naturally with many microorganisms, and the nutrient-rich environment of the rhizosphere is especially conducive to interactions between microorganisms and plants. While many soil microorganisms have no observable effects on plants, others enhance or inhibit plant growth. Plant growth-promoting rhizobacteria (PGPR) are beneficial soil microorganisms that can stimulate plant growth or increase tolerance to stresses. Some PGPR have been applied in agriculture, resulting in increased seedling emergence, plant weight, crop yield, and disease resistance (Kloepper et al., 1980, 1991, 1999). PGPR promote plant growth by producing non-volatile substances, such as the hormones auxin and cytokinin, as well as 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which reduces plant ethylene levels, and siderophores, which facilitate root uptake of metal nutrients (Loper and Schroth, 1986; MacDonald et al., 1986; Glick, 1999; Timmusk et al., 1999). In addition, certain PGPR promote plant growth by emitting volatile organic compounds (VOCs). Microbial VOC emission, which was reported in 2003 by Ryu et al., is now recognized as an important aspect of plant-microorganism interactions (Ryu et al., 2003; Wenke et al., 2010; Blom et al., 2011; Bitas et al., 2013; Farag et al., 2013). VOC emission is indeed a common property of a wide variety of soil microorganisms, although the identity and quantity of volatile compounds emitted vary among species (Effmert et al., 2012; Kanchiswamy et al., 2015).

Although PGPR VOCs do not contain any known plant growth hormones or siderophores (Farag et al., 2013; Kanchiswamy et al., 2015), VOC-mediated regulation of plant endogenous auxin homeostasis and of iron uptake by roots has been documented (Zhang et al., 2007, 2009;

Abbreviations: FIT1, Fe deficiency-induced transcription factor 1; FRO2, ferric reductase oxidase 2; HKT1, high affinity potassium transporter1; IRT1, iron regulated transporter 1; NR1 and 2, nitrate reductase 1 and 2; PEAMT, phosphoethanolamine N-methyltransferase; SOS, salt overly sensitive; VOCs, Volatile Organic Compounds.
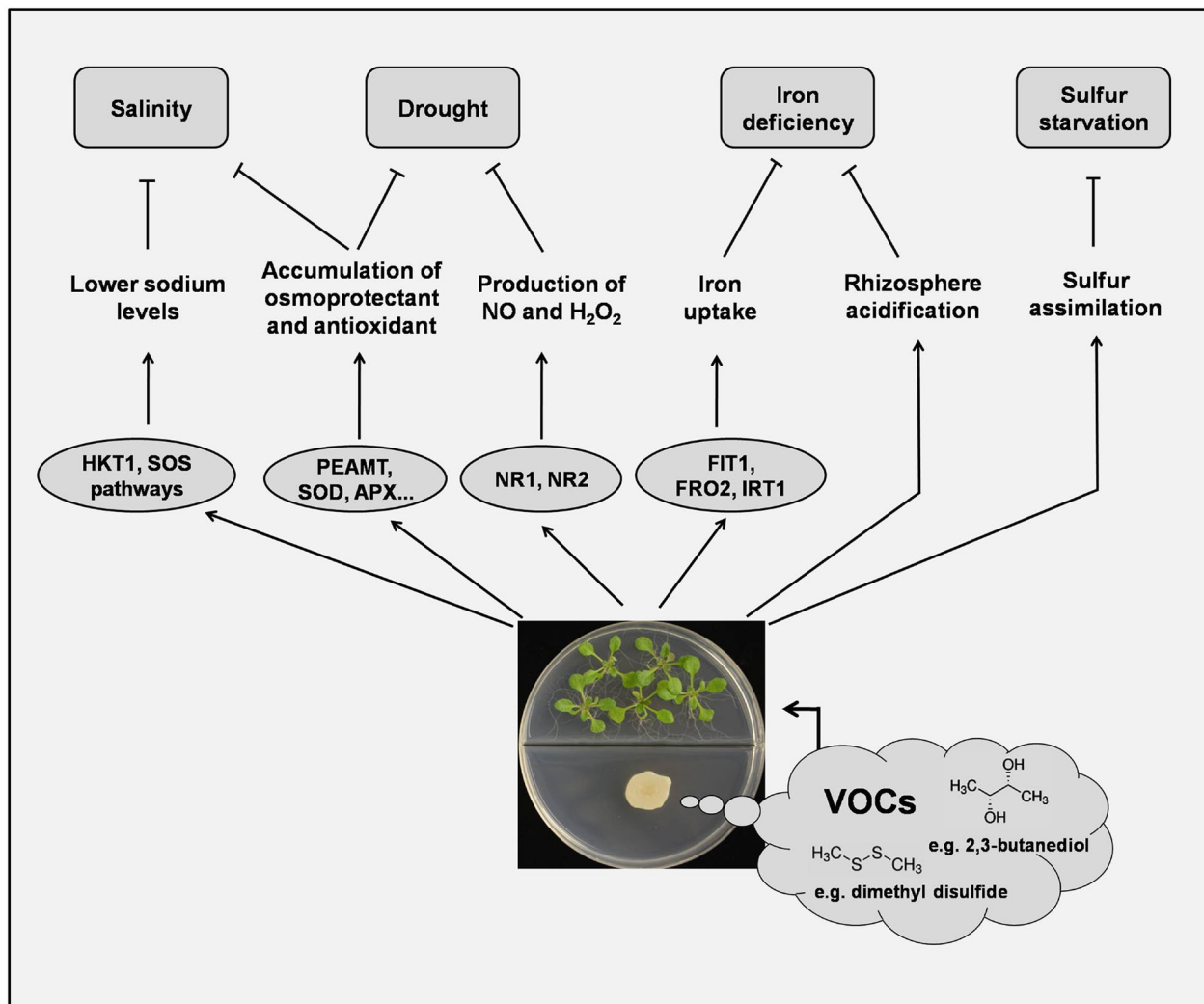
FIGURE 1 | The effects of microbial volatiles on plant abiotic stress tolerance. VOCs can modulate *Arabidopsis* sodium homeostasis *via* tissue-specific regulation of *HKT1* and possibly also *via* the SOS pathway. Accumulation of H_2O_2 and nitric oxide is involved in the VOC-induced drought tolerance in plants. Accumulation of choline synthesized by VOC-induced *PEAMT* and other osmo-protectants may be a common mechanism for increasing osmotic protection in salt- or drought-stressed plants. VOCs also trigger the expression of *FIT1*, *FRO2*, and *IRT1* to facilitate iron uptake and plant growth. Under conditions of sulfur starvation, plants directly take up and assimilate the S-containing compounds (e.g., dimethyl disulfide) emitted from some PGPR. See text for details.

Farag et al., 2013). In addition to promoting plant growth, microbial VOCs may also induce disease resistance and abiotic stress tolerance, although the latter phenomenon has been studied only a few times. As a result of the increasing interests in VOCs in mediating plant-microorganism interactions, recent reviews have summarized the chemical nature of microbial VOCs, as well as the effects of microbial VOCs on plant biomass production and disease resistance (Wenke et al., 2010; Bailly and Weisskopf, 2012; Bitas et al., 2013; Farag et al., 2013; Garbeva et al., 2014; Audrain et al., 2015; Kanchiswamy et al., 2015). This mini-review focuses on the enhancement of plant abiotic stress tolerance by bacterial VOCs. In addition to providing an overview of PGPR VOC-induced tolerance to salinity and drought stress, we consider how PGPR VOCs improve sulfur and iron nutrition in plants (**Figure 1**). Finally, we discuss

the potential complexities in evaluating the effects of PGPR VOCs.

Increased Salt Tolerance

Excessive sodium (Na^+) creates both ionic and osmotic stresses for plant cells, leading to suppression of plant growth and reduction in crop yields (Zhu, 2001). Bacteria-induced salt tolerance in plants has been observed for several PGPR strains, among which *Bacillus amyloliquefaciens* GB03 (originally described as *Bacillus subtilis* GB03; Choi et al., 2014) displays VOC-mediated effects (Mayak et al., 2004; Barriuso et al., 2008; Zhang et al., 2008a). Salt-stressed *Arabidopsis* plants treated with GB03 VOCs showed greater biomass production and less Na^+ accumulation compared to salt-stressed plants without

VOC treatment (Zhang et al., 2008a). Such VOC-induced stress tolerance was observed in wild-type (WT) plants but not in the *hkt1* null mutant, suggesting a key role of HKT1 in mediating the salt stress tolerance triggered by GB03 VOCs. *Arabidopsis* HKT1 is a xylem parenchyma-expressed Na^+ transporter that is responsible for Na^+ exclusion from leaves by removing Na^+ from the xylem sap (Sunarpi et al., 2005; Horie et al., 2009; Møller et al., 2009). Under salinity stress, GB03 VOCs reduce Na^+ accumulation in *Arabidopsis* shoots, presumably by enhancing HKT1-dependent shoot-to-root Na^+ recirculation, because VOCs transcriptionally up-regulate *HKT1* in shoots and concomitantly down-regulate *HKT1* in roots (Zhang et al., 2008a). While it remains unclear how GB03 VOCs regulate *HKT1* transcription, the organ-specific patterns appear to be critical for VOC-induced salt tolerance as well as for auxin-mediated growth promotion (Zhang et al., 2007, 2008a).

GB03 VOCs decreased the Na^+ level in entire WT *Arabidopsis* plants by approximately 50%, indicating either reduced Na^+ uptake, enhanced Na^+ exudation, or both. Intriguingly, GB03 reduced plant Na^+ levels by only 15% in the *Arabidopsis sos3* mutant (Zhang et al., 2008a). SOS3 is required for post-transcriptional activation of the H^+/Na^+ antiporter SOS1, which controls root Na^+ exudation and long-distance Na^+ transport in plants (Shi et al., 2000). Therefore, SOS3-dependent Na^+ exudation is likely required, as a part of the integrated regulation of Na^+ homeostasis, for the decreased accumulation of Na^+ in VOC-treated plants. In addition, GB03 VOCs also cause rhizosphere acidification (Zhang et al., 2009), thereby producing a proton gradient that could potentially facilitate the SOS1-mediated export of Na^+ from roots.

In response to salinity, plants adjust their endogenous metabolism to cope with osmotic stress caused by the excessive accumulation of Na^+. PGPR-induced salt tolerance was recently reported in soybean plants exposed to volatile emissions from *Pseudomonas simiae* strain AU; the emissions not only decreased root Na^+ levels but also increased the accumulation of proline, which protect cells from osmotic stress (Vaishnav et al., 2015). Consistent with induced systemic tolerance under salinity, plants treated with AU VOCs showed higher levels of the vegetative storage protein (VSP) and several other proteins that are known to help sustain plant growth under stress conditions (Vaishnav et al., 2015).

Protection from Water Loss

Dehydration is a common threat to plants experiencing osmotic stress caused by salinity, drought, or cold conditions. Elevated accumulation of osmo-protectants in plants under dehydration stress can increase cellular osmotic pressure to lower the free water potential of cells and thereby prevent water loss, and can also stabilize proteins and membrane structures (Yancey, 1994). Under osmotic stress, *Arabidopsis* exposed to GB03 VOCs accumulated higher levels of choline and glycine betaine than plants without VOC treatment (Zhang et al., 2010). Choline and glycine betaine are important osmo-protectants that confer dehydration tolerance in plants (Rhodes and Hanson, 1993). Consistent with the elevated osmo-protectant levels, plants treated

with GB03 VOCs or directly inoculated with GB03 displayed enhanced tolerance to dehydration stress. PEAMT, an essential enzyme in the biosynthesis pathway of choline and glycine betaine (Nuccio et al., 1998; Mou et al., 2002), was suggested to play a key role in mediating VOC-induced plant tolerance to dehydration, because VOC treatment increased the level of *PEAMT* transcripts and because genetic dysfunction of *PEAMT* abolished VOC-induction of dehydration tolerance (Zhang et al., 2010).

GB03 VOCs contain 2,3-butanediol, which promotes plant growth and induces disease resistance (Ryu et al., 2003, 2004). In addition to being found in GB03 VOCs, 2,3-butanediol is also found in the VOCs of some other PGPR strains including *Pseudomonas chlororaphis* strain O6, a bacterium that can trigger induced systemic resistance in plants. Under drought conditions, *Arabidopsis* plants inoculated with *P. chlororaphis* O6 or exposed to 2,3-butanediol exhibited increased stress tolerance, which evidently resulted from increased stomatal closure and reduced water loss (Cho et al., 2008). The application of *P. chlororaphis* O6 or 2,3-butanediol to mutants defective in various hormone signaling pathways indicated that the induced drought tolerance is regulated by multiple classic hormones including salicylic acid (SA), jasmonic acid (JA), and ethylene. In addition, SA appears to play a primary role in the induced drought tolerance, because free SA levels significantly increased in plants treated with *P. chlororaphis* O6 or 2,3-butanediol (Cho et al., 2008). In a subsequent study, 2,3-butanediol was found to induce plant production of nitric oxide (NO) and hydrogen peroxide, while chemical perturbation of NO accumulation impaired 2,3-butanediol-stimulated plant survival under drought stress; these results indicated an important role for NO signaling in the drought tolerance induced by 2,3-butanediol (Cho et al., 2013).

The phytohormone abscisic acid (ABA) is known to control plant stress responses under dehydration conditions. However, the enhanced osmo-protection of plants treated with GB03 VOCs appears to be unrelated to ABA, or at least to ABA production, because osmotic stress caused ABA to increase to similar levels in plants with and without exposure to GB03 VOCs (Zhang et al., 2010). That ABA is not the reason for PGPR-induced plant drought tolerance is further supported by observations that PGPR-treated *Arabidopsis* and cucumber plants accumulated less ABA than control plants (Cho et al., 2008; Kang et al., 2014). An indirect involvement of ABA in such PGPR-triggered abiotic-stress tolerance cannot be completely ruled out, however, given the complex cross-talk among ABA, NO, SA, and hydrogen peroxide signaling pathways in plants (Denancé et al., 2013; León et al., 2014; Song et al., 2014). PGPR-induced drought tolerance can also be mediated through elevated antioxidant responses at the levels of enzyme activity and metabolite accumulation, as was observed in wheat inoculated with *Bacillus safensis* strain W10 and *Ochrobactrum pseudogregnonense* strain IP8 (Chakraborty et al., 2013). Enhanced proline accumulation and gene expression of ROS-scavenging enzymes were observed in PGPR-treated potato plants, which displayed increased tolerance to various abiotic stresses including salinity, drought, and heavy-metal toxicity (Gururani et al., 2013). It would be useful to determine whether volatile emissions enhance antioxidative processes in plants that are stressed by dehydration. Some PGPR strains, such as

Pseudomonas aeruginosa strain Pa2, produce exopolysaccharides that enhance the ability of the bacteria to maintain soil moisture content and increase drought tolerance in plants (Naseem and Bano, 2014). Certain bacterial VOCs such as acetic acid can induce the formation of biofilms, which contain exopolysaccharides as major constituents (Chen et al., 2015). Thus, it is possible that certain PGPR VOCs may indirectly increase plant drought tolerance by mediating exopolysaccharide production.

Enhancement of Sulfur Acquisition

As an essential element in many primary metabolites such as the amino acids cysteine and methionine, the macronutrient sulfur (S) is critical for plant survival. Under S-deficient conditions, plants suffer from repression of photosynthesis and disruption of primary metabolism(Burke et al., 1986; Gilbert et al., 1997). While plants mainly acquire S through root uptake of SO_4^{2-} from soil, plants can also assimilate S from S-containing compounds in the air, including some volatile compounds that are emitted by soil microorganisms (Meldau et al., 2013). Dimethyl disulfide (DMDS) is an S-containing volatile compound commonly produced by many soil bacteria and fungi (Kanchiswamy et al., 2015). Emission of DMDS from *Bacillus* sp. strain B55, a natural symbiont of *Nicotiana attenuata* plants, rescued plant growth retardation caused by S deprivation (Meldau et al., 2013). The incorporation of bacteria-emitted S into plant proteins was demonstrated by adding radio-labeled ^{35}S to the bacterial growth medium. In addition to detecting DMDS, Meldau et al. (2013) also detected the S-containing compound S-methyl pentanethioate in *Bacillus* sp. B55 VOCs. The authors attributed most of the S nutrition provided by *Bacillus* sp. B55 VOCs to DMDS rather than to S-methyl pentanethioate for two reasons. First, DMDS was detected as a major component of the volatile emissions while S-methyl pentanethioate was present in only trace amounts. Second, synthetic DMDS was superior to the natural VOC blends in rescuing S-starvation phenotypes of *N. attenuata* plants (Meldau et al., 2013).

Sulfur in SO_4^{2-} is in an oxidative state and thus requires an energy-consuming reduction process for biological assimilation (Takahashi et al., 2011). In contrast, sulfur in DMDS is in a chemically reduced state. Therefore, it appears that DMDS may not only provide S to plants but may also help plants avoid expending energy on sulfate reduction. Consistent with this hypothesis, DMDS supplementation significantly decreased the expression of S assimilation genes as well as methionine biosynthesis and recycling (Meldau et al., 2013). Like DMDS in *Bacillus* sp. B55 VOCs, other S-containing volatile compounds such as dimethyl sulfide and dimethyl trisulfide have been detected in high concentrations in other microbial VOC blends (Kanchiswamy et al., 2015). Whether these microbial VOCs may also enhance S assimilation by plants remains to be determined.

Optimization of Iron Homeostasis

The transition between ferrous iron (Fe^{2+}) and ferric iron (Fe^{3+}) generates a redox potential that is important for electron transfer reactions including photosynthesis. Deprivation of Fe severely impairs the photochemical capacity and is accompanied by leaf chlorosis. Graminaceous monocots produce siderophores that increase Fe^{3+} mobility in soil and directly uptake Fe^{3+} without reduction, while non-graminaceous monocots and dicots not only acidify the rhizosphere to increase Fe^{3+} mobility but also use plasma membrane ferric reductase to reduce Fe^{3+} and subsequently transport Fe^{2+} into roots (Curie and Briat, 2003). Augmented Fe uptake was observed in *Arabidopsis* exposed to GB03 VOCs, which do not contain any known siderophores (Farag et al., 2006; Zhang et al., 2009). Under Fe-sufficient growth conditions, plants treated with GB03 VOCs displayed typical Fe-deficiency responses, including transcriptional up-regulation of the root Fe^{3+} reductase gene *FRO2* and of the Fe^{2+} transporter gene *IRT1*, increases in FRO2 enzyme activity, and rhizosphere acidification (Zhang et al., 2009). As a result, Fe levels were elevated in VOC-treated plants, consistent with greater amounts of Fe-rich photosynthetic apparatus (Zhang et al., 2008b).

GB03 VOC-triggered gene induction of *IRT1* and *FRO2* requires the transcription factor FIT1, because VOC failed to induce *IRT1* or *FRO2* in the *fit1* knockout mutant (Zhang et al., 2009). VOC treatment also failed to increase iron uptake or photosynthesis in the *fit1* mutant. Still, it remains unknown how VOC-treated plants initiate the inducible iron-deficiency responses. One possibility is that a demand for more iron may result from VOC-induced leaf cell expansion (Zhang et al., 2007) and/or photosynthesis augmentation (Zhang et al., 2008b). Also unclear is the identity of the component(s) in GB03 VOCs that induces plant iron-deficiency responses. On the other hand, acid components such as diethyl acetic acid possibly account for the rhizosphere acidification that is directly caused by VOC exposure (Farag et al., 2006; Zhang et al., 2009).

Potential Complexities of VOC Effects on Plants

Although PGPR VOCs have been shown to benefit plants *via* direct growth promotion, induced resistance to biotic stress, and increased tolerance to abiotic stress (Bailly and Weisskopf, 2012; Bitas et al., 2013; Farag et al., 2013), most of the data concerning these beneficial effects have been obtained in artificial environments. Current studies with PGPR VOCs typically use I-plates (**Figure 1**), in which a central partition separates plants from bacteria but allows bacterial VOCs to diffuse throughout the plate. This experimental setup appears to favor perception of volatile compounds by leaves, but in natural environments, PGPR VOCs that diffuse through rhizosphere soil pores would mainly be perceived by roots. Therefore, information obtained using I-plates may not apply to natural situations. Another concern with the use of I-plates is that, in addition to releasing volatiles, soil microorganisms also secrete non-volatile compounds (Glick, 1999), which may be taken up by roots and interfere with plant responses to VOCs.

Another complexity in studying the effects of PGPR VOCs on plants is that VOCs from the same PGPR strain may have different effects on plant growth and stress tolerance depending on the nature of the growth medium and the population density

of the bacterium (Blom et al., 2011). An increase in the bacterial population in the same space may alter VOC profiles and result in the production of new, toxic components or elevated proportions or quantities of pre-existing toxic components. Alternatively, the VOC component that is responsible for plant growth promotion may accumulate to such high levels that it adversely affects plant growth. Indole, for example, promoted plant growth when applied at low levels but killed plants when applied at high levels (Blom et al., 2011). Similarly, both growth promotion and growth inhibition have been observed for plants treated with DMDS (Kai et al., 2010; Meldau et al., 2013). A change in plant growth conditions may also cause beneficial PGPR VOCs to become inhibitory. GB03 VOCs induce expression of *FIT1* and *IRT1* genes, which enhance plant iron uptake and photosynthesis (Zhang et al., 2009). In addition to transporting iron, IRT1 transports other metal ions such as cadmium into roots (Nishida et al., 2011). It therefore seems possible that GB03 VOCs may aggravate cadmium toxicity in cadmium-stressed plants.

Conclusion

To date, research on abiotic stress tolerance induced by PGPR VOCs in plants has revealed some interesting phenotypes and initial insights into underlying mechanisms. Nonetheless, further insights into *in planta* molecular mechanisms are needed, especially regarding how VOC signals are perceived by plants and how plants assimilate certain VOC components as metabolites. Future research should also consider the possibility that PGPR VOCs have developed as a consequence of co-evolution. The survival of soil microorganisms is largely dependent on the growth and productivity of the plant community. In addition to supplying leaf litter for decomposers, plants also release up to 30% of their photosynthetic output in the form of root exudates that attract and maintain fungal and bacterial colonies in the soil (Smith et al., 1993; Jones et al., 2003). Therefore, mutually beneficial effects including enhancement of plant abiotic stress tolerance by PGPR VOCs could have resulted from the co-evolution of PGPR with their plant symbionts. Researchers have increasingly recognized that microbial VOCs play important roles in mediating inter- and intra-species interactions. Continued research on PGPR VOCs should lead to improved protection of plants from abiotic stress and to a better understanding of the underlying molecular mechanisms.

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Microbial Small Talk: Volatiles in Fungal–Bacterial Interactions

Ruth Schmidt[1]*, Desalegn W. Etalo[1], Victor de Jager[1], Saskia Gerards[1], Hans Zweers[1], Wietse de Boer[1,2] and Paolina Garbeva[1]

[1] Department of Microbial Ecology, Netherlands Institute of Ecology, Wageningen, Netherlands, [2] Department of Soil Quality, Wageningen University, Wageningen, Netherlands

There is increasing evidence that volatile organic compounds (VOCs) play an important role in the interactions between fungi and bacteria, two major groups of soil inhabiting microorganisms. Yet, most of the research has been focused on effects of bacterial volatiles on suppression of plant pathogenic fungi whereas little is known about the responses of bacteria to fungal volatiles. In the current study we performed a metabolomics analysis of volatiles emitted by several fungal and oomycetal soil strains under different nutrient conditions and growth stages. The metabolomics analysis of the tested fungal and oomycetal strains revealed different volatile profiles dependent on the age of the strains and nutrient conditions. Furthermore, we screened the phenotypic responses of soil bacterial strains to volatiles emitted by fungi. Two bacteria, *Collimonas pratensis* Ter291 and *Serratia plymuthica* PRI-2C, showed significant changes in their motility, in particular to volatiles emitted by *Fusarium culmorum.* This fungus produced a unique volatile blend, including several terpenes. Four of these terpenes were selected for further tests to investigate if they influence bacterial motility. Indeed, these terpenes induced or reduced swimming and swarming motility of *S. plymuthica* PRI-2C and swarming motility of *C. pratensis* Ter291, partly in a concentration-dependent manner. Overall the results of this work revealed that bacteria are able to sense and respond to fungal volatiles giving further evidence to the suggested importance of volatiles as signaling molecules in fungal–bacterial interactions.

Keywords: soil microorganisms, fungal–bacterial interactions, volatiles, terpenes, signaling, motility

INTRODUCTION

In terrestrial ecosystems fungi and bacteria live in complex multi-species networks (Frey-Klett et al., 2011; Hung et al., 2015). Within those networks, both fungi and bacteria produce a plethora of secondary metabolites of diverse chemical classes (Schulz and Dickschat, 2007; Morath et al., 2012; Muller et al., 2013). Several of these secondary metabolites are diffusible molecules, such as antibiotics and antibiotic-like substances or signaling molecules, which are important in interactions between fungi and bacteria (Ryan and Dow, 2008; Frey-Klett et al., 2011; Haq et al., 2014).

A group of metabolites that is increasingly recognized to play important roles in microbial interactions and communications are volatile organic compounds (VOCs). Those compounds are low molecular weight carbon-containing compounds (<400 Da) that evaporate easily at normal temperatures and air pressures (Schulz and Dickschat, 2007; Bitas et al., 2013). Their physico-chemical properties facilitate evaporation and diffusion through both water- and gas-filled

pores in soil and rhizosphere environments (Schmidt et al., 2015). Hence, they possess important functions for long distance fungal–bacterial interactions in the porous soil matrix.

Although it is known that many soil microorganisms produce a wide range of volatile compounds (Wheatley, 2002; Effmert et al., 2012) relatively little attention has been paid to fungal volatiles and to their ecological role. In these studies, over 300 distinct volatiles have been identified from fungi, belonging to different chemical classes including alcohols, benzenoids, aldehydes, alkenes, acids, esters, and ketones (Morath et al., 2012; Piechulla and Degenhardt, 2014). However, most research focused on volatiles produced by single species growing under nutrient rich conditions which is far from the nutrient-limited growth that most microbes experience in soil (Garbeva and de Boer, 2009; Kai et al., 2009; Weise et al., 2012). Furthermore, it has been shown that the composition of volatiles can vary depending on several factors, such as the fungal growth stage, moisture, temperature and pH (Wheatley, 2002; Insam and Seewald, 2010; Romoli et al., 2014).

Within the past years, it has become evident that microbial volatiles can play two major roles in long-distance interactions within soil microbial communities: (i) as infochemical molecules affecting the behavior, population dynamics and gene expression in responding microorganisms and (ii) as interference competition tools suppressing or eliminating potential enemies (Effmert et al., 2012; Garbeva et al., 2014a,b; Schmidt et al., 2015). Currently, most research on microbial volatiles is focused on the effect of bacterial volatiles on other bacteria and/or fungi whereas the effect of fungal volatiles on bacteria remains largely unknown.

In this study, we aimed to profile volatiles emitted by a range of fungal and oomycetal soil strains and to test the effect of these volatiles on the behavior of phylogenetically different soil bacteria which are known from previous studies to interact with fungi (Leveau et al., 2010; Mela et al., 2011; Garbeva et al., 2014b). The main research questions we addressed were (1) Can bacteria sense the presence of fungal and oomycetal volatiles and react with specific phenotypical responses and (2) Is the response dependent on the nutrient conditions and growth stage of the fungal and oomycetal strains?

Materials and Methods
Bacterial, Fungal, and Oomycetal Strains and Growth Conditions

All bacterial, fungal and oomycetal strains used in this study (**Table 1**) have been isolated from bulk or rhizosphere soil. The bacterial strains *Collimonas fungivorans* Ter331 and Ter6, *C. pratensis* Ter91, Ter291, and *C. arenae* Ter10 and Ter282, *Burkholderia* sp. AD24, *Pedobacter* sp. V48 and *Paenibacillus* sp. AD87 are strains from sandy dune soils in The Netherlands (De Boer et al., 1998; de Boer, 2004). *Serratia plymuthica* PRI-2C strain was isolated from maize rhizosphere, The Netherlands (Garbeva et al., 2004). Bacterial strains were pre-cultured from frozen glycerol stocks on 0.1 Tryptic Soya Broth plates (0.1 TSB; 5 g L^{-1} NaCl, 1 g L^{-1} KH$_2$PO$_4$,

3 g L^{-1} TSB, 20 g L^{-1} Merck Agar; pH = 6.5; Garbeva and de Boer, 2009) and grown for 3 days at 20°C prior usage.

The fungal strains *Trichoderma harzianum* PVDG2, *Mucor hiemalis* PVDG1 and *Fusarium culmorum* PV were also isolated from a sandy dune soil in The Netherlands (De Boer et al., 1998). *Verticillium dahliae* JR2 was isolated from tomato, Canada and *Rhizoctonia solani* AG2.2IIIB was isolated from sugar beet, the Netherlands (Garbeva et al., 2014b). The fungal oomycete *Pythium ultimum* P17 was isolated from tulip bulb rhizosphere, The Netherlands (Garbeva et al., 2014b). All fungal and oomycetal strains were pre-cultured on 0.5 Potato Dextrose Agar plates (19.5 g L^{-1} PDA, 7.5 g L^{-1} Merck Agar; pH = 5.5–6; Fiddaman and Rossall, 1993) and incubated for 6 days at 20°C prior usage.

Screening for Volatile-Mediated Phenotypes

To investigate the effect of fungal volatiles on bacterial phenotypes, variations of assays in double plate-within-a-plate system (Lee et al., 2015) were performed (schematically described in **Supplementary Figure S1**). A 3.5-cm Petri dish containing the fungal and oomycetal strains was placed into a partitioned 9-cm Petri dish containing the bacterial strains. Plates containing only sterile medium was used as a control. The bacterial response to fungal and oomycetal volatiles was studied by comparing the phenotypic responses of the bacteria under the two nutrient conditions.

Test of Bacterial Growth and Antimicrobial Activity

The 3.5-cm Petri dish contained either 3 mL 0.5 PDA medium or 1.5% water-agar (5 g L^{-1} NaCl, 1 g L^{-1} KH$_2$PO$_4$, 15 g L^{-1} Merck Agar; pH 6.5) supplied with artificial root exudates (WA + ARE). The artificial root exudates stock solution contained 18.4 mM glucose; 18.4 mM fructose; 9.2 mM saccharose; 4.6 mM citric acid; 9.2 mM lactic acid; 6.9 mM succinic acid; 18.4 mM L-serine; 11 mM L-glutamic acid and 18.4 mM L-alanine (C/N 10.4). Per liter of water-agar, 70.4 mL of ARE stock solution was added. A small plate (3.5 cm) containing the fungal and oomycetal plugs (6 mm in diameter) was placed into the partitioned Petri dish (9 cm) and grown for 3 days at 20°C (**Supplementary Figure S1A**). Bacterial strains were grown in 10 mL 0.1 TSB broth overnight at 20°C. The cells were washed twice with sterile 10 mm sodium phosphate buffer (1.361 g KH$_2$PO$_4$ in 1 L milliQ, pH 6.5), adjusted to a range of 1 × 10^6–10^2 cells/mL, 5 µl of cell suspension was spotted on 1.5% WA + ARE of the partitioned Petri dish containing the fungal and oomycetal strains in the other compartment. The Petri dish was then closed and incubated for 3 days at 20°C. On day 6, bacterial growth was determined by comparing the cfu/mL of bacteria exposed to fungal and oomycetal volatiles to that of bacteria exposed only to sterile growth media only.

To test the triggering of antimicrobial activity by bacterial strains in response to fungal and oomycetal volatiles, an agar

TABLE 1 | Bacterial, fungal, and oomycetal strains used in this study.

	Phylum/Class	Source	Accession number	Reference
Bacterial strains				
Collimonas fungivorans Ter331	Proteobacteria, β-Proteobacteria	Inner coastal dune soil in Terschelling, the Netherlands	NR_074756	De Boer et al., 1998; de Boer, 2004
Collimonas fungivorans Ter6	Proteobacteria, β-Proteobacteria	Inner coastal dune soil in Terschelling, the Netherlands	CP013232	De Boer et al., 1998; de Boer, 2004
Collimonas pratensis Ter91	Proteobacteria, β-Proteobacteria	Inner coastal dune soil in Terschelling, the Netherlands	CP013234	De Boer et al., 1998; de Boer, 2004
Collimonas pratensis Ter291	Proteobacteria, β-Proteobacteria	Inner coastal dune soil in Terschelling, the Netherlands	CP013236	De Boer et al., 1998; de Boer, 2004
Collimonas arenae Ter10	Proteobacteria, β-Proteobacteria	Inner coastal dune soil in Terschelling, the Netherlands	CP013233	De Boer et al., 1998; de Boer, 2004
Collimonas arenae Ter282	Proteobacteria, β-Proteobacteria	Inner coastal dune soil in Terschelling, the Netherlands	CP013235	De Boer et al., 1998; de Boer, 2004
Burkholderia sp. AD24	Proteobacteria, β-Proteobacteria	Rhizosphere and bulk soil of *C. arenaria*	KJ685239	De Ridder-Duine et al., 2005
Paenibacillus sp. AD87	Firmicutes, Bacilli	Rhizosphere and bulk soil of *C. arenaria*	KJ685299	De Ridder-Duine et al., 2005
Pedobacter sp. V48	Bacteroidetes, Sphingobacteriia	Coastal dune soil, the Netherlands	DQ778037	de Boer et al., 2007
Serratia plymuthica PRI-2C	Proteobacteria, γ-Proteobacteria	Maize rhizosphere soil, the Netherlands	AJTB00000000	Garbeva et al., 2004
Fungal and Oomycetal strains				
Trichoderma harzianum PVDG2	Ascomycota	Coastal dune soil, the Netherlands	KC888990	De Boer et al., 1998
Fusarium culmorum PV	Ascomycota	Coastal dune soil, the Netherlands	KT992460	De Boer et al., 1998
Verticillium dahliae JR2	Ascomycota	Tomato soil, Canada	PRJNA175765	Huang, 2014
Mucor hiemalis PVDG1	Zygomycota	Coastal dune soil, the Netherlands	KC888987	De Boer et al., 1998
Rhizoctonia solani AG2.2IIIB	Basidiomycota	Sugar beet rhizosphere soil, the Netherlands	KT124637	Garbeva et al., 2014b
Pythium ultimum P17	Oomycota	Rhizosphere of bulb, the Netherlands	KT124638	Garbeva et al., 2014b

overlay assay was performed on day 6 (Tyc et al., 2014). The two indicator organisms *Escherichia coli WA321* and *Staphylococcus aureus 533R4* were grown in liquid LB broth overnight at 37°C, 220 rpm. Fresh LB-agar (1.5% Merck Agar) was prepared, cooled down to ∼45°C and the target organisms were added to 6×10^5 cells/mL (*E. coli* WA321) or 4×10^5 cells/mL (*S. aureus* 533R4). A volume of 5 mL liquid LB-agar containing the target organisms was poured over the compartment in which bacteria were growing. After solidification of the overlay agar, plates were incubated overnight at 37°C. The next day, plates with bacteria exposed to fungal and oomycetal volatiles were examined for visible zones of inhibition (ZOI) compared to the bacteria exposed only to sterile media.

Test of Bacterial Motility

The effect of fungal and oomycetal volatiles on bacterial swarming and swimming motility was assessed on soft WA + ARE [0.6% wt/vol and 0.3% wt/vol, adapted from de Bruijn and Raaijmakers (2009)]. After autoclaving, the medium was cooled down in a water bath to 60°C. Next, 10 mL of the medium was pipetted into the partitioned Petri dish and the plates were kept for 24 h at room temperature (20°C) prior to the swarming and swimming assay. For all swarming and swimming assays, the same conditions (agar temperature and

volume, time period of storage of the poured plates) were kept constant to maximize reproducibility. A plate containing the fungal and oomycetal plugs (6 mm in diameter) that were grown on either 0.5 PDA or 1.5% water-agar supplied with artificial root exudates (WA + ARE) was placed into the partitioned Petri dish and grown for 3 days at 20°C (**Supplementary Figure S1B**). Recipient bacteria were grown in 10 mL 0.1 TSB broth overnight at 20°C. The cells were washed twice with sterile 10 mm sodium phosphate buffer (1.361 g KH_2PO_4 in 1 L milliQ, pH 6.5), adjusted to 1×10^7 cells/mL and 5 μl of cell suspension was spotted in the center of the soft WA + ARE of the partitioned Petri dish containing the fungal and oomycetal strains. The Petri dish was then closed and incubated for 3 days at 20°C. On day 6, volatile effect was determined by comparing the motility diameter of bacteria exposed to fungal and oomycetal volatiles to that exposed only to media. Motility diameter was calculated by measuring the radial swimming and swarming zones of the bacteria in two directions and calculating the mean for each of the three replicates.

Test of Bacterial Biofilm Formation

The test for biofilm formation was adapted and modified from O'Toole et al. (1999). A small plate containing the fungal and

oomycetal plugs (6 mm in diameter) that were grown on either 0.5 PDA or 1.5% water-agar supplied with artificial root exudates (WA + ARE) was placed into the partitioned Petri dish and grown for 3 days at 20°C (**Supplementary Figure S1C**). Recipient bacteria were grown in 10 mL 0.1 TSB broth overnight at 20°C. The cells were washed twice with sterile 10 mm sodium phosphate buffer, adjusted to 1×10^7 cells/mL and 20 µl of cell suspension was added into six-wells strip of a flat-bottom 96-well plates made of transparent polystyrene (Greiner) with 180 µl 0.1 TSB broth per well. Part of the 96-well plates was placed into the partitioned Petri dish containing the fungal and oomycetal strains in the other compartment. The Petri dish was then closed and incubated for 2 days at 20°C. On day 6, the six-well strip was removed from the large Petri-dish and 10 µl of 1% crystal violet solution was added to each well. These were incubated for 15 min at room temperature and rinsed three times with demi water. Biofilm formation was estimated by solubilization of crystal violet by adding 200 µl of 96% ethanol and determining the OD600. Volatile activity was determined by comparing biofilm formation from bacteria exposed to fungal and oomycetal volatiles to that of bacteria exposed to media.

Fungal and Oomycetal Volatile Trapping and GC-Q-TOF Analysis

For the collection of fungal and oomycetal volatiles, glass Petri dishes with leads to which a steel trap containing 150 mg Tenax TA and 150 mg Carbopack B (Markes International Ltd., Llantrisant, UK) could be fixed were used (Garbeva et al., 2014a). Fungi/oomyctes were grown on either 0.5 PDA or 1.5% water-agar supplied with artificial root exudates (WA + ARE) for 3 and 6 days at 20°C. Petri dishes containing medium only served as controls. All treatments were inoculated in triplicates. The Tenax steel traps were collected at two time points for two fungal and oomycetal growth stages (days 3 and 6) and under two nutrient conditions. Traps were removed, capped and stored at 4°C until analysis using GC-Q-TOF. Volatiles were desorbed from the traps using an automated thermal desorption unit (model UnityTD-100, Markes International Ltd., Llantrisant, UK) at 210°C for 12 min (He flow 50 mL/min) and trapped on cold trap at −10°C. The trapped volatiles were introduced into the GC-QTOF (model Agilent 7890B GC and the Agilent 7200A QTOF, Santa Clara, CA, USA) by heating the cold trap for 3 min to 280°C with split ratio set to 1:20. The column used was a 30 mm × 0.25 mm ID RXI-5MS, film thickness 0.25 µm (Restek 13424-6850, Bellefonte, PA, USA). Temperature program used was as follows: 39°C for 2 min, from °C to 95°C at 3.5°C/min, then to 165°C at 6°C/min, to 250°C at 15°C/min and finally to 300°C at 40°C/min, hold 20 min. The Volatiles were detected by the MS operating at 70 eV in EI mode. Mass spectra were acquired in full scan mode (30–400 amu, 4 scans/s). MassHunter Qualitative Analysis Software V B.06.00 Build 6.0.633.0 (Agilent Technologies, Santa Clara, CA, USA) was used to control the instrument and for data acquisition and analysis. The mass chromatogram that were generated exported

as mzData files and were processed (peak picking, baseline correction and peak alignment) in untargeted manner using the MetAlign software package (Lommen, 2009). Extraction and reconstitution of compound mass spectra were performed according to the method described previously by Tikunov et al. (2012). Identification of metabolites was performed using NIST-MS Search and accurate mass, retention indices, and spectra match factor using NIST 2014 V2.20 (National Institute of Standards and Technology, USA, http://www.nist.gov) and Wiley ninth edition spectral libraries and by their linear retention indexes (lri). The lri values were compared with those found in the NIST and in the in-house NIOO lri database.

Test of Terpene Compounds on Bacterial Motility

Four fungal volatiles, α-Terpinene, β-Phellandrene, 3-Carene, and Camphene were confirmed though injection of authentic standards obtained from Sigma–Aldrich and the Natural Products Laboratory, Leiden. Volatile chemicals were dissolved in ethanol with concentrations of 10 nM, 100 nM, 10 µM, 100 µM, 10 mM, and 100 mM. The effect of individual terpene volatiles on the motility of *C. pratensis* Ter291 and *S. plymuthica* PRI-2C was investigated using the double plate-within-a-plate system as described previously. The pure compounds were applied as a 10-µl droplet on a sterile filter paper (1 cm × 1 cm) on the bottom of a 3.5 cm Petri dish which was then transferred into the partitioned Petri dish. Plates were sealed immediately and incubated for 3 days at 20°C. The activity of the pure compounds was determined by comparing the motility diameter of bacteria exposed to pure volatile compounds as single compounds to that of bacteria exposed to control (only ethanol). Motility diameter was calculated by measuring the radial swimming and swarming zones of the bacteria in two directions and calculating the mean for each of the three replicates.

Statistical Analysis

In all experiments, both for the treatments and the controls three independent replicates were considered. For the metabolomics analyses Genemaths XT software (Applied Maths, Belgium) was used for ANOVA (with Bonferroni correction), Principal Component Analysis (PCA) and Hierarchical Cluster Analysis. Pearson's correlation coefficients were used to calculate the distance or similarity between two entries and the resulting clusters were summarized using a complete linkage algorithm. The raw values of each sample were log-transformed and auto-scaled by the use of the average as an offset and the standard deviation as scale [raw value-average (offset)/SD (scale)].

Data obtained from the phenotypical assays were expressed as standard error of the mean and analyzed using OriginPro 2015 (OriginLab Corporation, MA) and SPSS (Science Inc., IL). Student's *t*-test ($p < 0.05$) and one-way analysis of variance (ANOVA) between groups (treatments and control) were performed for all data.

RESULTS

Volatile Profiles of Fungal and Oomycetal Strains

Based on metabolomics analysis a total of 306 putative volatiles were detected when the fungal and oomycetal strains were grown on the nutrient poor WA + ARE medium. 106 of these volatiles differed significantly in abundance between at least two of the fungal/oomycetal strains. A total of 578 putative volatiles were detected from the head space of the fungal and oomycetal strains when grown on the nutrient rich PDA with 173 volatiles significantly different in their abundance between at least two fungal/oomycetal strains. Volatiles that differed significantly were further used to compute PCA and Hierarchical Cluster Analysis (HCA). In the PCA, the first three principal components (PC) explained 63% of the total variation observed between the fungal/oomycetal strains that were grown on nutrient poor medium (**Figure 1**). The first principal component PC1 explained 29% of the total variation and is primarily attributed to volatiles that were altered depending on the growth stage of the fungal/oomycetal strains (**Figure 1**). The emission profile of these volatiles is indicated in cluster 5 of the HCA and is characterized by volatiles with higher abundance at the early stage of growth in all strains (**Supplementary Figure S2A**). Volatiles in cluster 4 of the HCA also showed higher emission in four out of the six strains (namely *M. hiemalis*, *R. solani*, *T. harzianum*, *F. culmorum*) at the early stage of growth while their emission pattern and abundance was fairly similar for the remaining two strains (*P. ultimum* and *V. dahliae*) at both growth stages. The second PC explained 21% of the total variation and is attributed to the volatiles indicated in clusters 6 and 7 of the HCA (**Figure 1** and **Supplementary Figure S2A**, clusters 6 and 7). Volatiles in cluster 6 are primarily consisting of terpenes and were largely produced by *F. culmorum* at both growth stages with increased emission at the later growth stage (**Supplementary Figure S2A**). Volatiles in cluster 7 were abundantly detected in *F. culmorum* and *T. harzianum* at both growth stages with a noticeable increase in emission at the later growth stage. Some volatiles within this cluster were also detected in *V. dahliae* and were increasingly abundant at the later growth stage (**Supplementary Figure S2A**, cluster 7). The third PC explained 13% of the total variation. The volatiles that explained this variation are indicated in cluster 2 of the HCA and are mainly emitted by *P. ultimum* at the later stage of growth (**Supplementary Figure S2A**, cluster 2).

Principal Component Analysis based on volatiles from the fungi and oomycete grown on nutrient rich PDA showed that the first three PCs explained 60% of the total observed variation between the strains (**Figure 2**). The first PC that explained 37% of the total variation is related to compounds that are found in cluster 3 of the HCA (**Figure 2** and **Supplementary Figure S2B**, cluster 3). This cluster is characterized by higher emission of the volatiles at the early growth stage by all strains except *P. ultimum* and *V. dahlia*. The second PC explained 14% of the variation and is primarily associated to volatiles grouped

in the cluster 6 of the HCA that predominantly explains the growth stage dependent emission of volatiles by the strains (**Figure 2** and **Supplementary Figure S2B**, cluster 6). Similar to the observation under the nutrient poor conditions, this group of volatiles showed higher emission at the early growth stage in all the strains. The third PC explained 8% of the total variation and the volatiles associated to this variation are indicated in cluster 2 of the HCA (**Figure 2** and **Supplementary Figure S2B**, cluster 2). This cluster consisted of terpenes and they are emitted predominantly by *F. culmorum* at the later growth stage. Although these terpenes and other volatiles in this cluster were emitted by *F. culmorum* on both media, they were emitted to higher extent at the early stages of growth on WA + ARE (**Supplementary Figure S2A**, cluster 6 and **Supplementary Figure S2B**, cluster 2).

The volatiles belonging to the unique clusters of *F. culmorum* were investigated in more detail for both nutrient poor and nutrient rich conditions. Cluster 6 consisted of 19 volatiles belonging to the classes of terpenes (monoterpenes and sesquiterpenes), alkylbenzenes, cycloalkenes and furans and Cluster 2 consisted of 17 volatiles belonging to the classes of terpenes (monoterpenes and sesquiterpenes), alkaloids, benzenoids and furans (**Table 2**). Some volatiles could not be identified and are thus indicated as unknown compounds with their respective retention times and accurate masses. In both clusters terpenes represented the most abundant class with unique volatiles for both nutrient conditions. The identity of the terpenes α-Terpinene, β-Phellandrene, 3-Carene, and Camphene were confirmed with commercially available authentic standards.

Screening for Bacterial Phenotypes in Response to Fungal and Oomycetal Volatiles

A screening using variations of assays of a double-plate-within-a-plate system was performed to test phenotypical responses of 10 bacterial strains to volatiles emitted by six fungal and oomycetal strains. The two different media, WA + ARE and PDA, for which fungal and oomycetal volatile production was analyzed, were also used for screening the bacterial response to volatiles. Out of all phenotypes tested (growth, antimicrobial activity, biofilm formation, and motility), motility was the only phenotype affected by the fungal volatiles.

From all bacterial strains screened, *C. pratensis* Ter291 and *S. plymuthica* PRI-2C revealed the strongest and highly reproducible responses in motility upon exposure to the fungal and oomycetal volatiles (**Figure 3** and **Supplementary Table S1**). For the other bacterial strains high variability was observed between replicates (**Supplementary Table S1**). Consequently, we focused on the description of the response in motility of *C. pratensis* Ter291 and *S. plymuthica* PRI-2C.

Overall, swimming motility was more strongly affected than swarming motility and the effect on motility was much more pronounced by fungal and oomycetal volatiles emitted from PDA than from WA + ARE (**Figure 3**). Only *C. pratensis* Ter291 swarming motility was significantly reduced when exposed
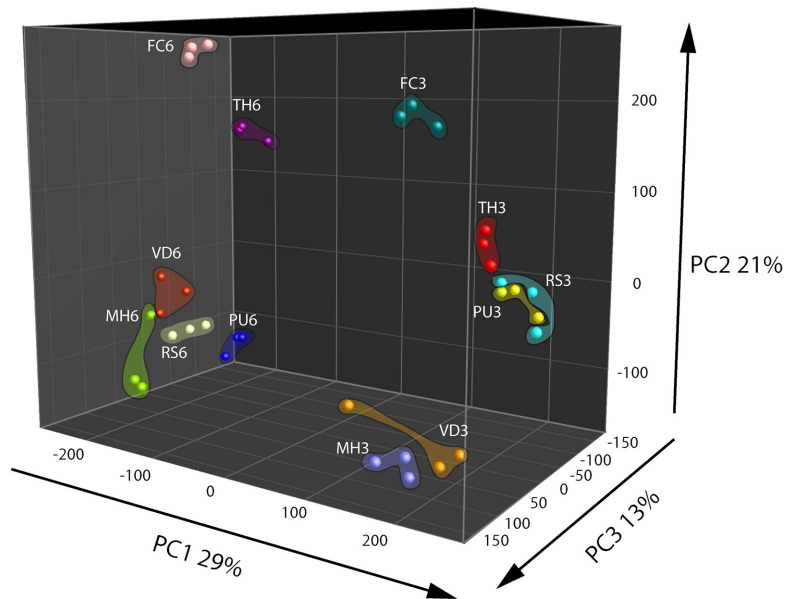
FIGURE 1 | Principal Component Analysis (PCA) of fungal and oomycetal strains based on 106 volatiles that were significantly different (*P* < 0.05 with Bonferroni correction) in abundance between at least two strains when grown on water agar supplied with artificial root exudates at day 3 (early growth stage) and day 6 (late growth stage). MH, *Mucor hiemalis*; RS, *Rhizoctonia solani*; PU, *Pythium ultimum*; VD, *Verticillium dahliae*; FC, *Fusarium culmorum*; TH, *Trichoderma harzianum*.
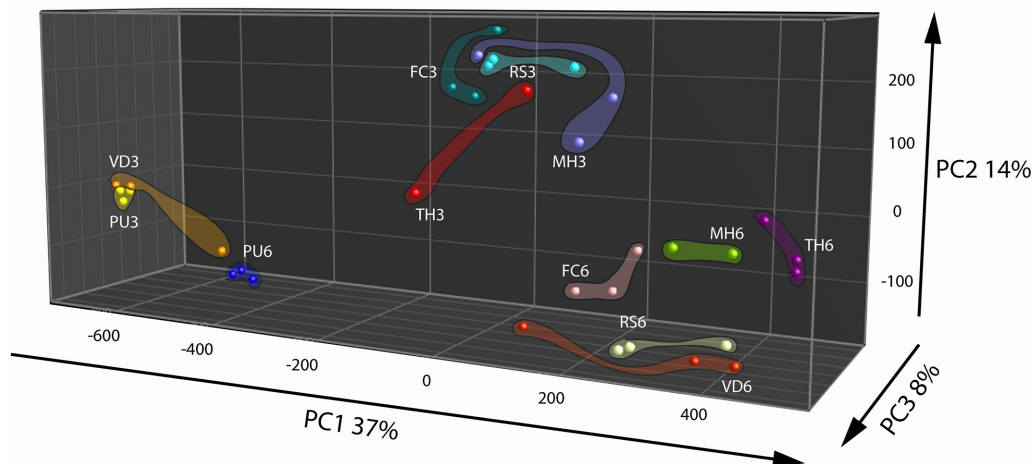
FIGURE 2 | Principal Component Analysis of fungal and oomycetal strains based on 173 volatiles that were significantly different (*P* < 0.05 with Bonferroni correction) in their abundance at least between two strains when grown on potato dextrose agar at day 3 (early growth stage) and day 6 (late growth stage). MH, *Mucor hiemalis*; RS, *Rhizoctonia solani*; PU, *Pythium ultimum*; VD, *Verticillium dahliae*; FC, *Fusarium culmorum*; TH, *Trichoderma harzianum*.

to volatiles emitted by *M. hiemalis* and *R. solani* on PDA (**Figure 3A**). No significant effect was observed in swarming motility of *C. pratensis* Ter291 and *S. plymuthica* PRI-2C by fungal and oomycetal volatiles emitted from WA + ARE and for *S. plymuthica* PRI-2C by volatiles emitted from PDA (**Figure 3A**).

The swimming motility of *C. pratensis* Ter291 was significantly reduced by volatiles emitted by *T. harzianum* and *P. ultimum*

growing on WA + ARE (**Figure 3B**). No such effect was observed for *S. plymuthica* PRI-2C. Volatiles emitted by all fungal and oomycetal strains growing on PDA revealed a significant reduction of the swimming motility of *C. pratensis* Ter291 with a very strong effect observed by *R. solani* (**Figures 3B,C**). For *S. plymuthica* PRI-2C swimming motility was significantly induced when exposed to volatiles produced by *F. culmorum* on PDA whereas the swimming motility was significantly reduced

TABLE 2 | Characteristics of volatile compounds of cluster 6 (water agar supplied with artificial root exudates) and 2 (potato dextrose agar) emitted by _F. culmorum_.

#	Compound	MSI*	Average RT** (min)	Accurate mass	RI***	Class
Cluster 6 WA + ARE						
(1)	2-Furancarboxaldehyde	2	4.73		771	Furans
(2)	Unknown		12.41	77.038		
(3)	α-Phellandrene	2	13.61		1005	Monoterpenes
(4)	Pentamethylcyclopentadiene	2	13.88		1006	
(5)	3-Carene	1	14.33		1017	Monoterpenes
(6)	o-Cymene	2	14.42		1026	Alkylbenzenes
(7)	Unknown	2	21.48	93.067	1197	
(8)	Unknown		27.60	93.068		
(9)	Unknown		29.22	229.001		
(10)	α-Copaene	2	29.52		1433	Sequiterpenes
(11)	1,3-Cyclopentadiene-1-butanenitrile, α-ethyl-	2	29.73		1433	Cycloalkenes
(12)	Unknown		29.84	161.128		
(13)	(-)-Isoledene	2	30.38		1472	Sequiterpenes
(14)	Unknown		30.51	93.067		
(15)	Unknown		31.09	67.053		
(16)	Unknown		31.83	80.059		
(17)	cis-Farnesol	2	32.59		1503	Sequiterpenes
Cluster 2 PDA						
(1)	2,4-Dimethylfuran	2	4.08		714	Furans
(2)	Unknown		4.88	95.047		
(3)	Camphene	1	12.79		970	Monoterpenes
(4)	α-Terpinene	1	13.85		1004	Monoterpenes
(5)	β-Phellandrene	1	14.55		1032	Monoterpenes
(6)	1,3,8-p-Menthatriene	2	19.03		1136	Sesquiterpenes
(7)	2,6-Dichloroanisol	2	20.65		1157	Benzenoids
(8)	Unknown		25.42	189.164		
(9)	Unknown		27.76	121.097		
(10)	Longifolene	2	27.91		1347	Sesquiterpenes
(11)	Ledene	2	27.98		1348	Sesquiterpenes
(12)	Y-Muurolene	2	28.28		1356	Sesquiterpenes
(13)	Streptazone C	2	29.47		1411	Alkaloids
(14)	Germacrene-D	2	29.77		1433	Sesquiterpenes
(15)	δ-Guaiene	2	30.04		1412	Sesquiterpenes
(16)	Unknown		30.31	105.068		
(17)	Unknown		30.67	67.054		
(18)	Unknown	2	31.77	109.100	1471	
(19)	α-Bisabolene	2	32.55		1500	Sesquiterpenes

*1: Identified metabolites based on authentic standards; 2: Putatively annotated compounds (e.g., without chemicals reference standards, based upon physicochemical properties and/or spectral similarity with public/commercial NIST 2014 V2.20 and Wiley ninth edition spectral libraries). The reporting grades (1 and 2) are assigned according to the proposed minimum reporting standards for chemical analysis [metabolomics standards initiative (MSI)] (Sumner et al., 2007).
**Retention time.
***Retention index.

when exposed to volatiles produced by _R. solani_ on PDA (**Figures 3B,C**).

Volatiles emitted by _R. solani_ growing on PDA reduced swarming as well swimming motility (**Figures 3B,C**). The HCA (**Supplementary Figure S2B**, cluster 1) resulted in a unique cluster for _R. solani_, however, the compounds could not be identified with certainty and

thus no correlation between the reduction in motility and the potentially involved compounds could be drawn.

Interestingly, volatiles emitted by one fungus lead to different responses in the two strains. For example _F. culmorum_ grown on PDA revealed a reduction of swimming motility of _C. pratensis_ Ter291 and an induction in _S. plymuthica_ PRI-2C (**Figures 3B,C**).

FIGURE 3 | Effect of fungal and oomycetal volatiles on *Collimonas pratensis* Ter291 and *Serratia plymuthica* PRI-2C swarming motility (0.6% wt/vol agar) and swimming motility (0.3% wt/vol agar) on water agar supplied with artificial root exudates (A) and potato dextrose agar (B). Setup of experiment and effect of FC and RS volatiles on swimming motility of *C. pratensis* Ter291 and *S. plymuthica* PRI-2C **(C)**. MH, *M. hiemalis*; RS, *R. solani*; PU, *P. ultimum*; VD, *V. dahliae*; FC, *F. culmorum*; TH, *T. harzianum*, Control: media. Five microliters of washed overnight cultures of *C. pratensis* Ter291 and *S. plymuthica* PRI-2C was spotted in the center of a soft agar partitioned Petri dish containing the fungal and oomycetal strains and incubated for 3 days at 20°C. As an indicator of motility the average swimming and swarming diameter (cm) was measured. The error bars represent standard errors of the mean of three independent biological replicates. The asterisks indicate statistically significant ($P < 0.05$) differences relative to the control.

Effect of Individual Terpenes on *S. plymuthica* PRI-2C and *C. pratensis* Ter291 Motility

To test whether terpenes may play a role in the observed motility response of *S. plymuthica* PRI-2C and *C. pratensis* Ter291, four terpenes (α-Terpinene, β-Phellandrene, 3-Carene, and Camphene) were selected from the unique *F. culmorum* cluster to be tested individually. These compounds showed a reliable annotation based on their retention indices and mass spectral similarity, with a match score >800, and were commercially (synthetically) available as authentic standards. The identity of the four pure compounds was confirmed by GC-MS by comparing their mass spectra and RI with those found in the *F. culmorum* volatile profile. Their respective mass spectra are given in **Supplementary**

Figure S3. A range of different concentrations, previously reported in experiments with microorganisms (Blom et al., 2011; Kim et al., 2013), was used to test their effect on motility of *S. plymuthica* PRI-2C and *C. pratensis* Ter291.

The screening showed that *S. plymuthica* PRI-2C was affected in both swarming and swimming motility, while *C. pratensis* Ter291 was only affected in swarming motility (**Figure 4**). For some of the pure compounds, a concentration dependent effect was observed. For example, α-Terpinene affected *S. plymuthica* PRI-2C swimming motility in concentrations of 10 nm, 100 nM, and 100 μM, but no effect was observed with high concentrations (10 mM and 100 mM; **Figure 4D**). ß-Phellandrene induced swimming motility in *S. plymuthica* PRI-2C in concentrations from 10 μM to 100 mM (**Figure 4D**). Interestingly, depending on the concentration, 3-Carene affected the swarming motility in *C. pratensis* Ter291 in different ways. At concentrations of 10 and 100 nM, *C. pratensis* Ter291 swarming motility was significantly increased while being significantly decreased at concentration of 10 μM (**Figure 4A**). Independently of the concentrations

applied, ß-Phellandrene significantly reduced swarming motility of *C. pratensis* Ter291 (**Figure 4A**). Likewise, 3-Carene and Camphene lead to a significant decrease in *S. plymuthica* PRI-2C swarming and swimming motility, respectively (**Figures 4B,D**).

DISCUSSION

Volatile compounds form an important part in the interactions between different soil inhabiting microorganisms (Insam and Seewald, 2010; Wenke et al., 2010; Effmert et al., 2012; Garbeva et al., 2014a). They can have different ecological functions, including inhibition or promotion of other (micro)-organisms (Kai et al., 2007, 2009; Vespermann et al., 2007; Bailly and Weisskopf, 2012; Bitas et al., 2013). However, one important role that has been long overlooked is the ability of volatiles to act as signaling molecules in the communication between different soil microorganisms despite their physico-chemical properties that facilitate diffusion through soil. To date, very little is known about the role of fungal volatiles in fungal–bacterial interactions. Thus, the aim of this study was to investigate the effect of fungal volatiles on bacteria.

Our results added to fill this gap in knowledge by showing that fungal and oomycetal volatiles can play an important role in long distance fungal–bacterial interactions, and can lead to specific phenotypical responses in the interacting partners. Out of all the phenotypical responses considered namely growth, antimicrobial activity, biofilm formation and motility, motility of bacteria, both swimming (individual cells moving in more liquid environments) and swarming (direct, signal-dependent movement powered by rotating flagella), were significantly affected. Fungal and oomycetal volatiles either triggered or suppressed bacterial motility depending on the interacting partner. This finding could, therefore, reflect a potential strategy employed by the fungus to attract mutualistic bacteria toward itself and to repel competitors from common niches by manipulating their motility through volatiles. The composition and abundance of volatiles is affected by the growth stage of the fungal/oomycetal strains and the nutrient conditions. Several independent studies have reported that the volatile profiles of bacteria are also dependent on growth condition and nutrient availability (Korpi et al., 2009; Insam and Seewald, 2010; Blom et al., 2011; Bitas et al., 2013; Garbeva et al., 2014b).

Besides the growth stage- and nutrient condition-dependent changes in the global volatile profile, certain groups of volatiles are emitted in higher amounts by specific individual strains. Terpenes emitted by *F. culmorum* are the most salient example from our study: a nutrient-poor growing condition triggers higher levels of terpene emission at an early growth stage and an even higher emission at a later growth stage; on the contrary, under nutrient-rich conditions, the emission of this volatile cluster was induced only at a later growth stage. This suggests that fungi and oomycetes can invest their carbon resources toward formation of specific blends of volatiles depending on their growth stages and the nutrient availability in their environment. Our findings are in line with those by Korpi et al. (2009), who demonstrated that a

lack of certain nutrients leads to terpene emission, suggesting that some volatiles are produced only under nutrient-limited conditions, which is often the case in natural environments. Terpenes represent the biggest and most diverse family of primary and secondary metabolites found in a variety of organisms, among which several fungi (Keller et al., 2005; Gioacchini et al., 2008; Strobel et al., 2011; Muller et al., 2013; Busko et al., 2014). Most studies, however, focused mainly on the detection and chemical characterization of these molecules, while only few addressed the biological function of terpenes. The latter studies indicate that fungal terpenes may be used in defense against competitors (e.g., caryophyllene) or as a signaling molecule (e.g., farnesol; Martins et al., 2007). Our work, based on the screening of fungal and oomycetal strains and using pure terpene compounds, proved that individual terpenes affect the motility of the exposed bacteria.

Several studies showed that fungi have a high sensitivity to volatiles emitted by bacteria leading to reduction and inhibition in spore germination and growth (fungistasis; Garbeva et al., 2011, 2014b; Effmert et al., 2012; Schmidt et al., 2015). The difference in susceptibility between fungi and oomycetes may be due to the structure of their cell wall (Schmidt et al., 2015). It was recently proposed that bacteriostasis (inability of bacteria to multiply in soil; Ho and Ko, 1982; Effmert et al., 2012) might also involve volatile compounds. However, within this study we did not observed effect on bacterial growth by fungal volatiles. In contrast to fungi, bacteria seem to be more resistant to volatiles. It has been speculated that variations in sensitivity of bacteria to volatiles may possibly be mediated by an ATP-dependent efflux mechanism, which has been investigated for several terpene compounds against *Pseudomonas aeruginosa* (Cox and Markham, 2007) as well as the ability of volatiles to disintegrate the outer membrane (Longbottom et al., 2004). These findings may indicate that bacteria are more resistant to volatiles emitted by fungi and oomycetes.

The identity of volatile molecules is an important basis for understanding their ecological roles. However, it is a challenging task to unambiguously identify the high number of compounds detected, just as it is to set the right ranges of concentrations that are representative of the natural condition during screenings with pure compounds. The technology used in this study does not allow measuring the actual concentration of the volatile compounds produced by the strains. Thus, when testing the effect of pure compounds we adopted a range of concentrations known to be relevant in such assays (Blom et al., 2011; Kim et al., 2013). Interestingly, some of the pure compounds showed a dose-dependent effect on the motility. This suggests that by regulating the emission of volatiles, fungi might be able to influence bacterial responses in different ways. For instance, the emission of volatiles in lower concentrations might attract the bacterium to move toward the fungus, while volatiles emitted in higher concentrations might be toxic and thus repel the bacteria away from the fungus. For example, bacteria from the genus *Collimonas*, used in this study, were previously shown to colonize and grow on living fungal hyphae (a phenomenon called mycophagy; de Boer, 2004), implying that volatiles might play

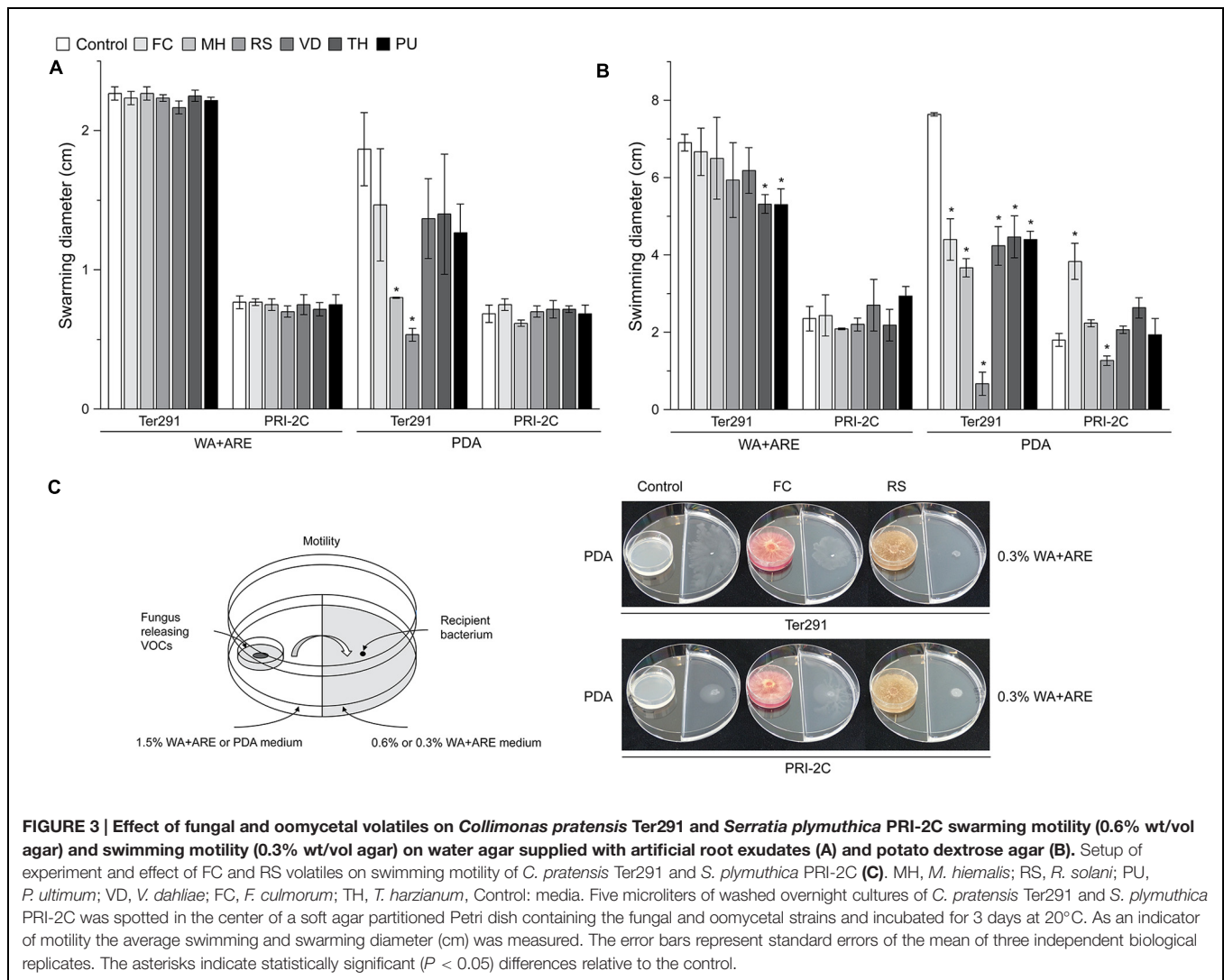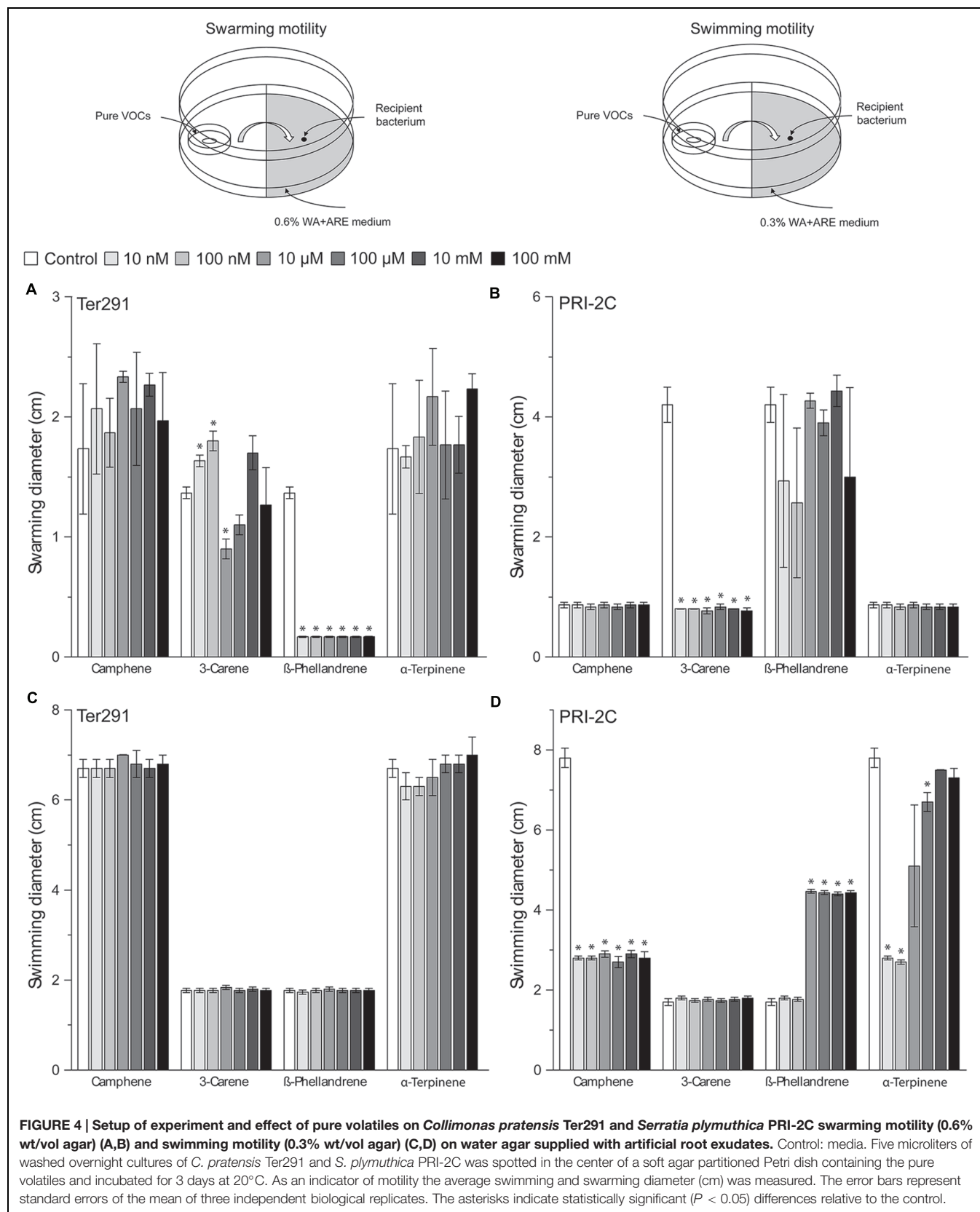**FIGURE 4 | Setup of experiment and effect of pure volatiles on** *Collimonas pratensis* **Ter291 and** *Serratia plymuthica* **PRI-2C swarming motility (0.6% wt/vol agar) (A,B) and swimming motility (0.3% wt/vol agar) (C,D) on water agar supplied with artificial root exudates.** Control: media. Five microliters of washed overnight cultures of *C. pratensis* Ter291 and *S. plymuthica* PRI-2C was spotted in the center of a soft agar partitioned Petri dish containing the pure volatiles and incubated for 3 days at 20°C. As an indicator of motility the average swimming and swarming diameter (cm) was measured. The error bars represent standard errors of the mean of three independent biological replicates. The asterisks indicate statistically significant ($P < 0.05$) differences relative to the control.

a role as long-distance signals for attracting such mycophagous bacteria.

CONCLUSION

Bacteria can sense fungal and oomycetal volatiles and respond with changes in motility. This response was dependent on the volatile blend emitted by the organisms, which was influenced by the nutrient conditions and, for some strains, by their growth stage. Several identified volatile terpenes were shown to affect motility. To better understand how bacteria perceive fungal volatiles on a cellular level, a valuable insight could stem from future studies involving transcriptomics and proteomics tools.

ACKNOWLEDGMENTS

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.01495

TABLE S1 | Effect of fungal and oomycetal volatiles on bacterial phenotypes (growth, antimicrobial activity, biofilm formation and motility) on WA+ARE and PDA.

FIGURE S1 | Variations of assays in double plate-within-a-plate system used to test the effect of fungal volatile compounds on bacterial growth and antimicrobial activity (A), motility (B) and biofilm formation (C) as described in section "Materials and Methods."

FIGURE S2 | Hierarchical cluster analysis (HCA) of fungal and oomycetal strains based on volatiles that were significantly different (P < 0.05 with Bonferroni correction) in abundance between at least two strains when grown on water agar supplied with artificial root exudates (A) and on potato dextrose agar (B) at day 3 (early growth stage) and day 6 (late growth stage). MH, *Mucor hiemalis*; RS, *Rhizoctonia solani*; PU, *Pythium ultimum*; VD, *Verticillium dahliae*; FC, *Fusarium culmorum*; TH, *Trichoderma harzianum*; C, Control (media). The color code below the figure indicates the abundance of the volatiles, which is log2-transformed and scaled by Mean/SD. The boxes indicated in different colors discern the eight distinct clusters that determine the spatial separation of the samples in the PCA.

FIGURE S3 | Comparison of mass spectra of the pure volatile compounds with those found in *F. culmorum*.

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A novel assay for the detection of bioactive volatiles evaluated by screening of lichen-associated bacteria

Tomislav Cernava[1], Ines A. Aschenbrenner[1,2], Martin Grube[2], Stefan Liebminger[3] and Gabriele Berg[1]*

[1] Institute of Environmental Biotechnology, Graz University of Technology, Graz, Austria, [2] Institute of Plant Sciences, University of Graz, Graz, Austria, [3] Roombiotics GmbH, Graz, Austria

Volatile organic compounds (VOCs) produced by microorganisms are known both for their effect on pathogens and their role as mediators in various interactions and communications. Previous studies have demonstrated the importance of VOCs for ecosystem functioning as well as their biotechnological potential, but screening for bioactive volatiles remained difficult. We have developed an efficient testing assay that is based on two multi-well plates, separated by a sealing silicone membrane, two tightening clamps, and variable growth media, or indicators. The experiment design as presented here is a novel and robust technique to identify positive as well as negative VOC effects on the growth of a target organism and to test for specific substances e.g., hydrogen cyanide which can be detected with a suitable indicator. While the first pre-screening assay is primarily based on indicator color change and visible growth diameter reduction, we also introduce an advanced and quantitatively precise experiment design. This adaptation involves qPCR-based quantification of viable target cells after concluding the treatment with VOCs. Therefore, we chose preselected active isolates and compared the partial 16S rRNA gene copy number of headspace-exposed *E. coli* with non-treated controls. Separately obtained headspace SPME and GC/MS-based profiles of selected bacterial isolates revealed the presence of specific and unique signatures which suggests divergent modes of action. The assay was evaluated by screening 100 isolates of lung lichen-associated bacteria. Approximately one quarter of the isolates showed VOC-based antibacterial and/or antifungal activity; mainly *Pseudomonas* and *Stenotrophomonas* species were identified as producers of bioactive volatiles.

Keywords: volatiles, VOCs, antifungal, antibacterial, lichen symbiosis

Introduction

Volatile organic compounds (VOCs) are organic compounds that have a high vapor pressure at ordinary room temperature. VOCs are produced by the majority of organisms and they often function as communication molecules (Effmert et al., 2012). The most notable characteristic of all VOCs is the extent of their range of influence as compared to non-volatile substances. While other secreted metabolites rely on close contact between interacting organisms or diffusion through

separating matter, VOCs can overcome much greater distances. Bacterial as well as fungal strains produce a broad spectrum of bioactive VOCs with multi-functional effects, which are not restricted to the same species. Their action across organismal kingdoms was shown by Ryu et al. (2003) who demonstrated that bacterial volatiles promoted growth in *Arabidopsis thaliana*. In contrast, some bacterial isolates were also shown to reduce the growth of *A. thaliana* through emission of bioactive volatiles (Vespermann et al., 2007; Blom et al., 2011; Weise et al., 2013). Moreover, bacterial VOCs were shown to be able to suppress the growth of soil-borne pathogenic fungi, e.g., *Rhizoctonia solani* (Kai et al., 2007). Bacteria are able to communicate over long distances within the root system, both among bacteria and with plant hosts, where they elicit induced systemic resistance (ISR) and growth promotion (Farag et al., 2013). VOCs emitted by different soil bacteria can affect the growth, antibiotic production, and gene expression of soil bacteria (Garbeva et al., 2014). Owing to these multi-functional roles of VOCs, they have an enormous potential for biotechnological applications (Strobel, 2006; Schalchli et al., 2014). Currently, there is no efficient testing assay that allows for rapid screening of bioactive volatile metabolites in interrelation between two different microorganisms within the same headspace.

Host-associated microbiomes are important reservoirs for VOC-producing organisms because communication and pathogen defense are essential functions of the microbiome, and recently shown to be integral for healthy plant and human life (Blaser et al., 2013; Philippot et al., 2013). For the rhizosphere microbiome located on/in plant roots, the proportion of VOC producers is often high because in plants the root-associated microbiome acts as a primary protection shield against soil-borne pathogens (Cook, 1990; Weller et al., 2002). A similar role was also attributed to bacteria in the self-sustaining lichen symbiosis (Grube et al., 2015). In each microbiome a certain proportion of microorganisms with antagonistic activity against pathogens is involved in this function. Using a combination of metagenomic, -proteomic, and cultivation approaches, a proportion of 7% antagonists was identified for the lung lichen (Grube et al., 2015). Identification of antagonistic microorganisms is still a challenge (Berg et al., 2013), but nevertheless important for a more profound understanding of ecosystem functioning and also a necessary tool for bioprospecting in biotechnology (Strobel, 2006). The discovery of novel bioactive compounds facilitates improvement in disinfection strategies and drug discovery, both of which are in high demand due to the increasing rates of resistance to antibiotics (Woolhouse and Farrar, 2014). Antagonistic microorganisms harbor a vast potential to produce active biomolecules for direct activity against pathogens but also for mediators in various interactions, e.g., pathogen defense, quorum sensing, microorganism-host-interaction. Some of these biomolecules are highly active modifications of known antimicrobial substances and are therefore less susceptible to existing resistance mechanisms. In the past, most efforts focused on antibiotics for which high-throughput screening strategies were already developed (Conery et al., 2014; Seyedsayamdost, 2014). Although previous studies have demonstrated promising effects of bacterial and fungal volatile compounds, they are difficult to

detect as well as to identify. Due to their inspiring odors, lichen extracts are used as raw materials in perfumery (Joulain and Tabacchi, 2009). As the specificity of bacterial communities in this phylogenetically old symbiosis was only recently detected (Grube et al., 2009), nothing is yet known about VOCs produced by the abundant lichen-associated bacteria.

The objective of our study was to develop a well plate-based and cost-effective testing assay for the emission of bioactive VOCs. We chose lichen-associated bacteria for evaluation purposes. One hundred lichen-associated bacterial isolates were tested for volatile antagonistic activity in order to evaluate our assay. A noteworthy screening assay for biological hydrogen production developed by Schrader et al. (2008) was used as the basis for developing our testing system. The assay is based on two micro-well plates, separated by a sealing silicone membrane, two tightening clamps, and variable growth media or indicators. The suggested experiment design can be used to differentiate between target organism inhibition or growth promotion by a pure substance and also for the same effects caused by volatile mixtures emitted by living microorganisms. This, as well as an increased throughput compared to classic single plate-tests illustrates the novelty of the presented assay in comparison to already described experiment setups. In addition, it can be employed to test for specific substances which can be detected with a suitable indicator (**Figure 1**). Using this design we identified 30 out of 100 lichen-associated bacterial isolates, which produced bioactive volatiles and induced growth inhibition in two distinct target organisms. Since many lichenicolous organisms are characterized by slow growth rates and difficult or impossible to grow on media, two classic model targets were employed for evaluation purposes. *E. coli* was used in this experimental approach as a model for a typical human pathogen and *B. cinerea* as a model for a plant pathogen. Additional GC/MS-based headspace analysis with different lichen-associated isolates was applied to demonstrate the occurrence of isolate-specific VOC profiles.

Material and Methods

Isolation of Lichen-Associated Bacteria

Lobaria pulmonaria lichen thalli were sampled from three different locations in Austria (Tamischbachgraben, N 47°32′40″, E 14°37′35″, Johnsbach, N 47°38′07″, E 14°44′45″, and St. Oswald ob Eibiswald, N 46°44′50″, E 15°04′26″). The lichen samples were ground with mortar and pestle, and subsequently combined with a ratio of 1:10 0.85% sterile NaCl in a lab stomacher to form a homogenate (BagMixer; Interscience, St Nom, France). The diluted fractions were then plated onto agars R2A (Carl Roth, Karlsruhe, Germany), R2A with 25 μg ml^{-1} cycloheximide, starch casein agar (SCA; Küster and Williams, 1964), and ISP2 (Shirling and Gottlieb, 1966). Distinctive bacterial colonies were transferred onto R2A plates for sub-cultivation within 5 days of incubation at room temperature. After subsequent testing for antagonism against different pathogens among other physiological tests, 100 lichen-associated bacterial isolates were selected out of 388 available isolates from the in-house culture collection. All of these isolates met at least one of the following criteria: (i) antagonistic activity against *E. coli* K12, (ii) antagonistic
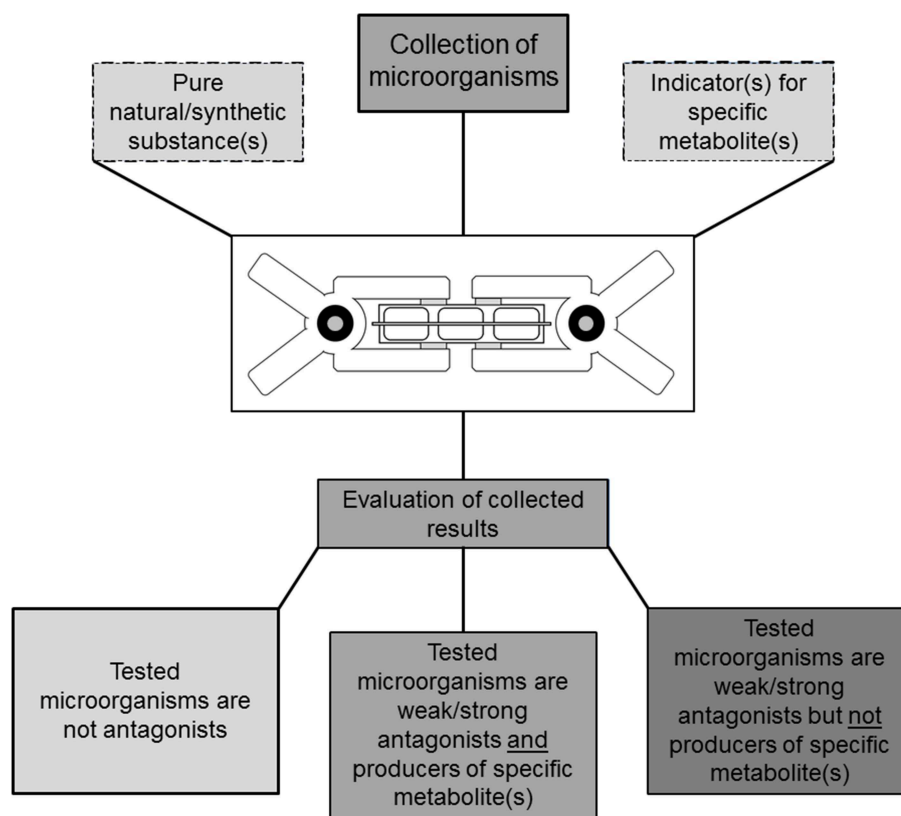
FIGURE 1 | Illustration of a TCVA-based screening approach for bioactive volatile compounds. The experiments are based on microorganism cultures suitable for cultivation in well-plates. Parallel setup of different experiments facilitates selectivity and can be used to eliminate producers of predefined compounds e.g., HCN if indicator substances are available. Utilization of additional reference substances enables gradual evaluation of visually inspected experiments.

activity against *Staphylococcus aureus* ATCC 25923, (iii) antagonistic activity against *Botrytis cinerea* (SCAM, culture collection of the institute of Environmental Biotechnology, Austria), (iv) antagonistic activity against *Rhinocladiella* sp. (culture collection of the Institute of Plant Sciences, University of Graz) in dual-culture experiments, (v) chitinase activity on chitin agar and in chitin-RBV assay, (vi) β-glucanase activity with chromogenic AZCL-Barley β-glucan.

Two Clamp VOCs Assays (TCVAs)

Depending on the experiment type, 6-, 12-, and 24-well plates (Greiner Bio-One, Frickenhausen, Germany) were used together with a perforated (0.5 cm ø) 1 mm silicone foil (detailed specifications are presented in Table S1) for tightening connected wells and usual clamps for fixation. Sterile plates were acquired and the silicone foils used were washed and autoclaved at 121°C (holding time of 20 min). 6-, 12-, and 24-well plates were filled with respectively 5, 3.5, and 1.5 mL sterile media per well. The preparation steps and the final setup are pictured sequentially in Figure S1.

TCVA with *B. cinerea* and Lichen-Associated Bacteria

The bacterial isolates were streaked onto Nutrient Agar (NA; Sifin, Berlin, Germany) in 6-well plates and pre-incubated for

24 h at 30°C. Next, 5 mm diameter plugs were cut from a donor plate evenly covered with *B. cinerea*. These plugs were placed in the center of each well in the 6-well plates containing Synthetic Nutrient-Poor Agar (SNA). After the inoculated plates were checked for sufficient growth, silicone foils were placed between plate pairs containing lichen isolates and *B. cinerea*, respectively. The plates were then clamped together; the lichen-associated bacteria plate was placed upside-down over the *B. cinerea* plates on the bottom. The plates were incubated in the dark at 21°C for 4 days and subsequently visually inspected for mycelium growth and compared to untreated controls (**Figure 2A**). Two types of controls were implemented; one containing NA wells without any bacteria and one inoculated with *E. coli* K12 instead of lichen-associated isolates.

TCVA with *E. coli* and Lichen-Associated Bacteria

The bacterial isolates were streaked onto NA in 12-well plates and pre-incubated for 24 h at 30°C. Following the incubation time, a fluid Nutrient Broth (NB; Sifin, Berlin, Germany) culture of *E. coli* K12 was grown to an $OD_{600} = 0.4–0.6$. 6 mL aliquots were then sequentially transferred to 200 mL NA (20%) supplemented with 0.2 mg/mL 2–(4–iodophenyl)–3–(4–nitrophenyl)–5–phenyltetrazolium chloride (INT; Sigma-Aldrich, St. Louis, MO, USA) and immediately pipetted into sterile 12-well plates.
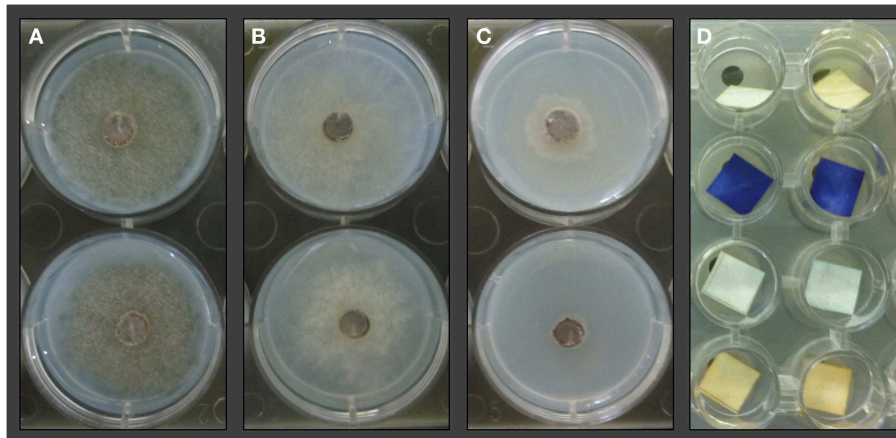
FIGURE 2 | TCVA with lichen-associated bacterial isolates and *Botrytis cinerea*. Mycelium growth and sporulation was compared to untreated controls **(A)** after 4 days of co-incubation. Inhibition of sporulation was recorded for wells where discoloring of the mycelium was not observable **(B)**. Inhibition of mycelial growth was recorded for wells with 50% or less mycelium proliferation **(C)** compared to negative controls. TCVA with HCN indicator **(D)** based on copper(II) ethyloacetoacetate and 4,4'-methylenebis(N,N-dimethylaniline). The second row shows positive reactions where bacterial isolates from counterpart wells secreted HCN into the headspace which led to the color change of indicator strips.

INT can be utilized to detect dehydrogenase activity due to visible color changes. Hence, approximate differences in bacterial abundance can be correlated to the grade of visible discoloring of INT-supplemented growth medium. After solidification of the *E. coli* K12 containing plates, silicone foils were placed between plate pairs containing lichen isolates and *E. coli* K12, respectively. The plates were then clamped together; the lichen-associated bacteria plate was placed upside-down over the *E. coli* K12 plates on the bottom. After 24 h incubation at 21°C, the plates were checked for differences in indicator color change and compared to positive and negative controls. Positive controls were obtained using different commercial disinfectants to determine sufficient OD$_{600}$ values (0.4–0.6) for *E. coli* K12 and an adequate concentration of INT (Figure S2). Two types of negative controls were implemented: one containing NA wells without any bacteria, and one inoculated with *E. coli* K12 instead of lichen-associated isolates.

qPCR Validation of TCVA Results

This experiment is an adaption of the aforementioned TCVA with *E. coli* K12, the only modification being that semi-solid 0.3% NA was used instead of solid 1.5% NA in the initial steps of the experiment. After the incubation time, 500 µL of the semi-solid medium with *E. coli* K12 and INT was transferred into 2 mL reaction tubes with 1 mL 0.85% NaCl and subsequently dissolved via vortex. Each tube was supplemented with 10 µL (1:100 solution) propidium monoazide (PMA; GenIUL, Barcelona, Spain) and incubated on ice in the dark while shaking at 100 rpm for 50 min. The tube lids were then opened after incubation and placed under a LED light source for activation of PMA with an emission maximum of 520 nm for 10 min. PMA forms covalent bonds with available DNA but cannot pass through undisrupted cell membranes. This step was performed to exclusively detect gene fragments from living *E. coli* K12 in the qPCR-based quantification. The suspension was then transferred to glass bead containing tubes and mechanically disrupted for 2 × 45 s at 6 m/s with a FastPrep®-24 Instrument (MP Biomedicals Europe, Illkirch, France) and centrifuged at 3000 × g for 2 min to sediment beads and residual Agar. DNA was subsequently extracted from 500 µL of supernatant using the GeneJET Genomic DNA Purification Kit (Thermo Scientific, Waltham, MA, USA). Quantification of 16S rDNA fragments from the DNA extract was conducted with primer pair Unibac-II-515f/Unibac-II-927r as described by Lieber et al. (2003), and standards containing the Unibac-II fragments were prepared according to Köberl et al. (2011). For standard preparation, the gene fragments from *Bacillus subtilis* subsp. *subtilis* Sd3-12 were cloned into the pGEM®-T Easy Vector (Promega, Madison, WI, USA) and later re-amplified with vector specific primers. Total DNA extract treated with amplification-grade DNase I (Sigma-Aldrich, St. Louis, MO, USA) was used to determine the inhibitory effects of co-extracted substances. Based on these results, the extracted DNA was then diluted 1:10 and the target regions were amplified using KAPA SYBR FAST qPCR Kit (Kapa Biosystems, Woburn, MA, USA). Two independent runs with three replicates for each sample were performed on the Rotor Gene 6000 (Corbett Research, Mortlake, Australia) according to Bragina et al. (2013). The specificity of the amplicons and qPCR products was confirmed using melting-curve analysis and gel-electrophoresis, respectively.

TCVA with a Hydrogen Cyanide (HCN) Indicator and Lichen-Associated Bacteria

The bacterial isolates were streaked onto NA in 24-well plates and pre-incubated for 24 h at 30°C. Indicator strips were prepared using blotting paper submerged in 10 mL chloroform (Carl Roth, Karlsruhe, Germany) solution with 50 mg copper(II) ethylacetoacetate (Sigma-Aldrich, St. Louis, MO, USA) and 50 mg 4,4-methylenebis(N,N-dimethylaniline) (Sigma-Aldrich, St. Louis, MO, USA) and left to air dry. After the pre-incubation time,

1 × 1 cm HCN indicator strips were placed in each well of a 24-well plate. Silicone foils were placed between the upside-down lichen-associated bacteria plates and those containing the HCN indicators. The plate pairs were then clamped together and incubated for 48 h at 30°C. Lastly, the indicator plates were checked for an intense blue color change in the corresponding upper wells. Negative controls were conducted with non-inoculated NA wells.

Identification of Active Isolates by 16S rDNA Sequencing

Isolated DNA from pure cultures was amplified with primer pair 27F/1492r according to Lane (1991). The PCR product was purified with Wizard® SV Gel and PCR Clean-Up System (Promega, Madison; WI, USA) followed by Sanger sequencing (LGC Genomics, Berlin, Germany). The sequences were aligned with BLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and 16S ribosomal RNA sequences database. Identification of the closest match was applied for the retrieved results.

Headspace SPME and GC/MS Analysis of Bacterial VOCs

The utilized GC/MS SPME headspace method was adapted with minor changes from Verginer et al. (2010). For sample preparation from bacterial isolates, single colonies were transferred with an inoculating loop on 10 mL NA slope agar (1.5%) in 20 mL headspace vials (75.5 × 22.5 mm; Chromtech, Idstein, Germany). The isolates were streaked out in 3 parallel lanes to ensure similar bacterial lawn density after incubation. Following 48 h of incubation at 30°C the vials were sealed with adequate crimp seals and incubated for additional 2 h. Solid phase micro extraction (SPME) was performed with an automated sampler and 50/30 μm Divinylbenzen/CarboxenTM/ Polydimethylsiloxane (PDMS) 2 cm Stableflex/SS fiber (Supelco, Bellefonte, PA, USA). Volatile compounds were enriched for 30 min at 30°C. Compound separation and detection was performed on a system combining a GC 7890A with a quadrupol MS 5975C (Agilent Technologies, Waldbronn, Germany). Samples were run through a (5%-phenyl)methylpolysiloxane column, 60 m × 0.25 mm i.d., 0.25 μm film thickness (DB-5MS; Agilent Technologies, Waldbronn, Germany), followed by electron ionization (EI; 70 eV) and detection (mass range 25–350). The inlet temperature was adjusted to 270°C. For the temperature gradient the GC column was kept at 40°C for 2 min, raised to 110°C at a rate of 5°C/min, then to 280°C at 10°C/min and finally maintained at 280°C for 3 min. The helium flow rate was set to 1.2 mL/min. Serial analysis was done with up to 12 samples per run. Obtained spectra were compared with NIST Mass Spectral Database 08 entries. Specific compounds were identified based on their retention indices and comparison to reference substances (Sigma-Aldrich, St. Louis, MO, USA). Origin 8.5 (OriginLab, Northampton, MA, USA) was applied for visualization of total ion chromatograms (TICs). Background-subtracted mass spectra were used for the depiction of unidentified substances.

Statistical Analysis

The statistical analysis was conducted with ANOVA within RStudio (version 0.97.551) and one-sided t-test ($P < 0.001$). Gene

copy numbers of the UniBac-II fragment from TCVA-exposed samples ($n = 36$) were compared to untreated controls ($n = 12$). The gene copy numbers were obtained from two biological samples and three qPCR repeats respectively.

Results

Testing Volatile Activity Against *E. coli* and *Botrytis Cinerea*

The here presented Two Clamp VOCs Assay (TCVA) made it possible to detect bioactive VOC producers within 100 lichen-associated bacterial isolates. Sporulation reduction (**Figure 2B**) was demonstrated for five isolates; four isolates reduced sporulation of *B. cinerea* in three out of four replicate experiments, while one isolate reduced sporulation in all four trials. *B. cinerea* growth was repeatedly reduced after exposure to 21 different lichen-associated bacterial isolates in the TCVA, and mycelium proliferation was visibly affected (**Figure 2C**) for these isolates when compared to negative controls. Moreover, 16 isolates reduced proliferation in three out of four replicate experiments, while five isolates reduced proliferation in all four trials. Only one of the identified growth-reducing isolates was later shown to release HCN into the headspace. TCVAs with *E. coli* allowed identification of 10 lichen-associated isolates that are associated with the exertion of antagonistic activity through headspace. Low INT-based growth media discoloring indicating a reduced number of metabolically active bacteria was observed in all three replicate experiments. Comparison to the corresponding HCN TCVAs showed that two of the growth-reducing isolates did not release HCN into headspace. Only one isolate inhibited the growth of both target organisms and was later identified as *Pseudomonas umsongensis* 313P5BS. From all identified antagonists we have selected the 15 most active isolates against one or both target organisms and one non-inhibiting isolate for Sanger sequencing (**Table 1**).

Screening for HCN Producers

All lichen isolates were tested for HCN production in a modified TCVA by imposing indicator strips to the headspace. Nine bacterial isolates induced dark blue discoloring of the indicator strips in all three replicate experiments. Eight of the identified HCN-producing isolates also reduced growth of *E. coli* in previous experiments. These isolates were later assigned to *Pseudomonas* spp., while the non-antagonistic HCN producer had the highest sequence similarity to a *Bacillus pumilus* isolate.

Validation of TCVA Results by Quantitative PCR

DNA extracts from wells containing *E. coli* that had shown low discoloring of INT after exposition to lichen-associated bacteria were used to determine the gene copy number of the Unibac-II fragment. DNA from dead or disrupted cells was blocked by PMA which enabled a correlation between gene copy number and living cells. A significantly lower gene copy number compared to controls was shown for all samples exposed to the headspace of highly active antagonists that were pre-screened based on INT discoloring. An approximately 4-fold decrease of the gene copy

TABLE 1 | Overview of identified isolates including corresponding activity in TCVAs.

Strain ID	Closest BLASTn match	GenBank accession #	Inhibition of *E. coli*	Inhibition of *B. cinerea*	HCN producer
43P2BR	*Bacillus pumilus*	KP739785		✓	
236P5S	*Pseudomonas umsongensis*	KP739786	✓		✓
268P3S	*Pseudomonas umsongensis*	KP739787	✓		✓
269P3R	*Burkholderia sordidicola*	KP739788	✓		
271P3S	*Pseudomonas umsongensis*	KP739789	✓		✓
279P5I	*Pseudomonas umsongensis*	KP739790	✓		✓
288P4R	*Burkholderia sordidicola*	KP739791	✓		
293P5BI	*Pseudomonas umsongensis*	KP739792	✓		✓
300P5BR	*Chryseobacterium piscium*	KP739793		✓	
301P5BS	*Pseudomonas umsongensis*	KP739794	✓		✓
313P5BS	*Pseudomonas umsongensis*	KP739795	✓	✓	✓
409P5	*Pseudomonas lini*	KP739796	✓		✓
418P4B	*Stenotrophomonas rhizophila*	KP739797		✓	
439P1B	*Stenotrophomonas rhizophila*	KP739798		✓	
460P5B	*Stenotrophomonas rhizophila*	KP739799		✓	
471P3B	*Bacillus pumilus*	KP739800			✓

Listed species represent the closest match of BLASTn searches within the 16S ribosomal RNA sequences database (NCBI). The 16S gene fragment sequences were deposited at GenBank (http://www.ncbi.nlm.nih.gov/genbank).

number was observed with the least inhibiting antagonist *Pseudomonas* sp. 279P5I, while the most effective antagonist *Pseudomonas* sp. 236P5S decreased the gene copy number of *E. coli* approx. 15-fold (**Figure 3**).

Taxonomic Assignment of Active Lichen-Associated Isolates

Sanger sequencing revealed multiple occurrence of some dominant genera. Furthermore, sequencing of 16S rRNA gene fragments from the 15 most active isolates and a non-antagonistic HCN producer revealed the majority belonging to the genus *Pseudomonas* (8 isolates), followed by *Stenotrophomonas* (3 isolates) and three other genera with lower occurrence: *Bacillus*, *Burkholderia,* and *Chryseobacterium*. Utilization of the TCVA demonstrated that *E. coli* inhibition was mostly observed after exposure to the headspace of *Pseudomonas* sp., while *B. cinerea* growth reduction was mostly observed after exposition to the headspace of *Stenotrophomonas rhizophilia*. Moreover the sequencing approach revealed that different *Pseudomonas* sp. inhibited *E. coli* growth accompanied by HCN release into

headspace. Identified isolates are presented together with corresponding TCVA results in **Table 1**.

GC/MS-Based Headspace Analysis with Selected Isolates

Three representative isolates which were shown to inhibit growth of headspace-exposed target microorganisms and which were taxonomically assigned to reoccurring genera were used for subsequent GC/MS headspace SPME profiling. Isolate-specific VOCs were identified by overlays of total ion chromatograms (TIC; **Figure 4**). A total of 21 compounds (Table S2) were found to be unique and only present in TICs of a specific isolate. *Bacillus pumilus* 43P2BR emitted nine distinctive volatile compounds (compound IDs: 2, 5, 8, 10, 13, 14, 15, 18, and 21), followed by *Pseudomonas umsogensis* 313P5BS with eight distinctive compounds (compound IDs: 1, 6, 7, 9, 16, 17, 19, and 20). *S. rhizophila* 418P4B was shown to emit only four distinctive compounds (compound IDs: 3, 4, 11, and 12). Compound identification indicated that *B. pumilus* 43P2BR emitted 1-butanol, 3-methyl-2-pentanone and seven unidentified substances (Figures S3-18). 2-butanol, 2-methyl-1-propanol and two unidentified substances were found within spectra of *S. rhizophila* 418P4B. Conversely, *P. umsogensis* 313P5BS emitted methyl thiocyanate as well as seven unidentified substances.

Discussion

The results of the screening for bacteria-derived bioactive VOCs demonstrated the applicability of a novel testing system, which is not restricted to bacteria associated with lichens, but can be widely applied with microorganisms sampled in other habitats. With the presented setup several 100 isolates can be tested simultaneously for VOCs-driven effects on target microorganisms and occurrence of specific metabolites. This facilitates screening programs for strain-specific biological effects. Thus, the method is also not limited to observations of growth inhibition such as demonstrated in the present study, but could also be used to identify growth promoting effects. The method, however, reveals the effect of the total "volatilome" of a bacterial strain and does not dissect the effect of individual substances. The composition of the mixture of volatile substances must still be assessed by chemical analysis, after which individual compounds might be tested separately.

We have selected 100 isolates for evaluation purposes that met predefined criteria such as antagonism in classic dual-culture experiments. Thus, we expected to identify a sufficient number of isolates which emit bioactive VOCs to validate the experimental design and the presented workflow. Utilization of different TCVA variations allowed the identification of 30 growth inhibiting bacterial isolates with a novel and reliable technique. Subsequent analysis of the headspace from taxonomically dissimilar bacterial isolates by employing headspace SPME GC/MS demonstrated the presence of isolate-specific TIC profiles and unique compounds in each sample. We have identified five out of 21 unique compounds, emitted by three distinct bacterial strains, to exemplify the presented workflow. While some compounds most likely originate from bacterial degradation of growth media
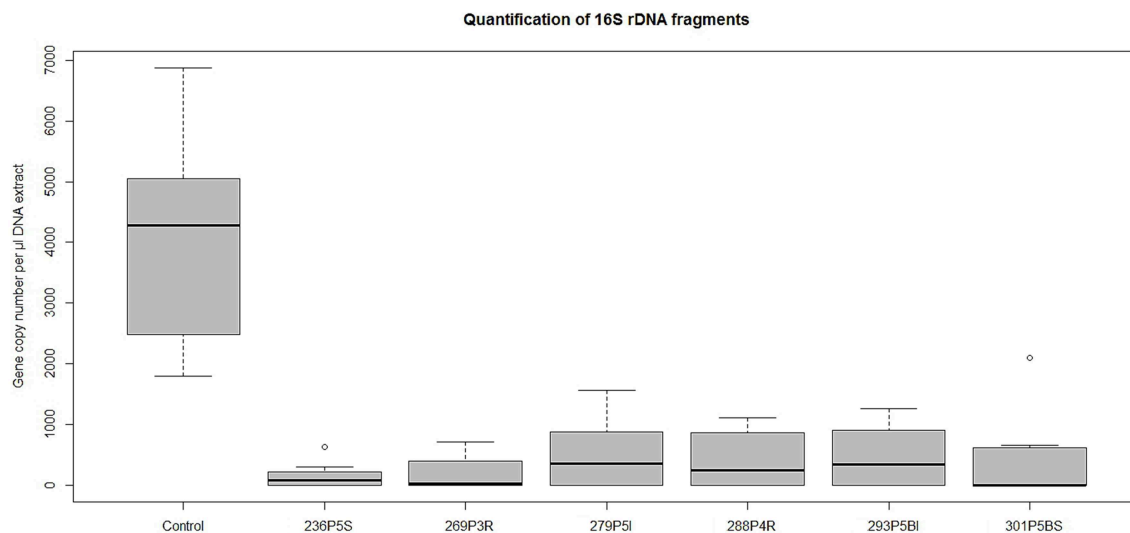
FIGURE 3 | A qPCR approach was used to determine gene copy numbers of *E. coli* K12 after exposition to strong VOCs antagonists. TCVA-exposed samples were treated with PMA and gene copy numbers of viable cells were quantified with Unibac-II primers. Values for the treated samples were obtained from respectively six qPCR runs and additional 12 qPCR runs for untreated controls. Statistical analysis with ANOVA and one-sided *t*-test confirmed a highly significant decrease of gene copy numbers of the treated samples compared to the control group ($P < 0.001$).

(waste products of bacterial metabolism) and therefore do not target pathogens specifically, other compounds might either be involved in molecular signaling or inhibition of competing organisms. These differences and their significance in natural systems merit further exploration. It might be hypothesized that bacterial bioconversion of the natural substrate may result in volatile compounds with signaling effect. Specifically, the odor of lichens, which may attract reindeer or is part of perfumes, could be influenced not only by the genuine compounds produced by the fungal or algal symbiont, but possibly also by VOCs produced by the bacteria themselves or by compounds released from the fungal matrix due to the metabolic activity of associated bacteria.

The presented workflow includes a pre-incubation of the tested isolates to minimize their inhibition by volatiles emitted by the target organisms. Due to intended growth advantage of the tested organisms, emitted volatiles from the target organisms might not play an important role during the co-incubation. Still, such effects cannot be completely avoided with the presented setup. An inverted approach where the "target" is pre-incubated and subsequently tested against the respective isolate collection could be implemented to obtain a more holistic view on occurring interactions.

Interestingly, *P. umsongensis,* a bacterial species isolated from soil as well as from fungal hyphae and described as "fungiphilic" (Warmink et al., 2009), was the only representative of *Pseudomonas* isolates that consistently inhibited *B. cinerea* growth. This may have resulted from better and faster growth on solid medium compared to the other utilized strains and therefore a higher accumulation of cyanide in the headspace. Various *Pseudomonas* species are known to be cyanogenic bacteria and therefore enhanced toxicity toward various prokaryotes and

eukaryotes can be expected even if they are not in close contact. While the employed headspace SPME GC/MS method was not suitable for detection of hydrogen cyanide, we were able to detect methyl thiocyanate above cultures of *P. umsongensis* 313P5BS. Conversion of cyanide to thiocyanate is accomplished by bacterial rhodanese and these co-occurring molecules can be extracted simultaneously from headspace above living cultures (Broderick et al., 2008). Weise et al. (2013) have highlighted the importance of bacterial ammonia production and demonstrated accompanied growth inhibition of *Arabidopsis thaliana*. Specific indicator stripes in the TCVA would allow to test for ammonia in the headspace, which would also imply an unspecific inhibition of target organisms.

Some bacteria are well known for pronounced antifungal effects against phytopathogenic fungi. This effect is typical for several strains belonging to *Stenotrophomonas* (Wolf et al., 2002), which was also observed with headspace experiments (Ryan et al., 2009). *Bacillus* species were shown in prior studies not only to produce antifungal VOCs (Fiddaman and Rossall, 1993), but also volatiles that promoted growth in *A. thaliana* (Ryu et al., 2003). Our study demonstrates such antagonistic effects with a robust well plate-based approach and provides various options for modifications to study further effects e.g., growth promotion of bacteria on plants in an adaptable testing system. Moreover, this approach could also be applied to study the prevalence of similar bioactive effects across entire bacterial genera and to correlate volatile effects with the occurrence of strains in particular ecological niches.

As we are convinced that bacterial volatiles might play an important role to modify the composition of host-associated communities, future research needs to focus on the, possibly context-dependent, effects of such small molecules. We anticipate
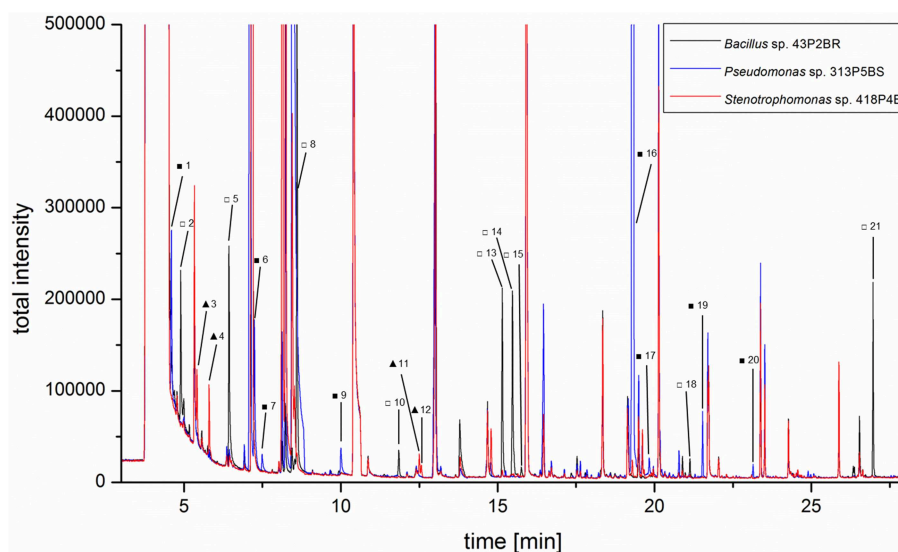
FIGURE 4 | Three lichen-associated bacterial isolates were subjected to headspace SPME GC/MS analysis to identify isolate-specific VOC profiles. An overlay of the respective TIC chromatograms illustrates the presence of specific metabolites in the headspace of *Bacillus pumilus* 43P2BR (empty squares), *Pseudomonas umsongensis* 313P5BS (filled squares), and *Stenotrophomonas rhizophila* 418P4B (filled triangles). Identified *Bacillus*-specific compounds were: 1-butanol (5□) and 3-methyl-2-pentanone (8□). Methyl thiocyanate (7■) was identified as *Pseudomonas*-specific compound. *Stenotrophomonas*-specific compounds were: 2-butanol (3▲) and 2-methyl-1-propanol (4▲). More details in Table S2.

that this newly developed testing approach will be a major step forward to facilitate such studies.

Acknowledgments

Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00398/abstract

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Diversity and functions of volatile organic compounds produced by *Streptomyces* from a disease-suppressive soil

Viviane Cordovez[1,2], Victor J. Carrion[1], Desalegn W. Etalo[1], Roland Mumm[3,4], Hua Zhu[5], Gilles P. van Wezel[1,5] and Jos M. Raaijmakers[1,5]*

[1] Department of Microbial Ecology, Netherlands Institute of Ecology (NIOO-KNAW), Wageningen, Netherlands, [2] Laboratory of Phytopathology, Wageningen University, Wageningen, Netherlands, [3] Plant Research International, Business Unit Bioscience, Wageningen University and Research Centre, Wageningen, Netherlands, [4] Centre for Biosystems Genomics, Wageningen, Netherlands, [5] Molecular Biotechnology, Institute of Biology, Leiden University, Leiden, Netherlands

In disease-suppressive soils, plants are protected from infections by specific root pathogens due to the antagonistic activities of soil and rhizosphere microorganisms. For most disease-suppressive soils, however, the microorganisms and mechanisms involved in pathogen control are largely unknown. Our recent studies identified Actinobacteria as the most dynamic phylum in a soil suppressive to the fungal root pathogen *Rhizoctonia solani*. Here we isolated and characterized 300 isolates of rhizospheric Actinobacteria from the *Rhizoctonia*-suppressive soil. *Streptomyces* species were the most abundant, representing approximately 70% of the isolates. *Streptomyces* are renowned for the production of an exceptionally large number of secondary metabolites, including volatile organic compounds (VOCs). VOC profiling of 12 representative *Streptomyces* isolates by SPME-GC-MS allowed a more refined phylogenetic delineation of the *Streptomyces* isolates than the sequencing of 16S rRNA and the house-keeping genes *atpD* and *recA* only. VOCs of several *Streptomyces* isolates inhibited hyphal growth of *R. solani* and significantly enhanced plant shoot and root biomass. Coupling of *Streptomyces* VOC profiles with their effects on fungal growth, pointed to VOCs potentially involved in antifungal activity. Subsequent assays with five synthetic analogs of the identified VOCs showed that methyl 2-methylpentanoate, 1,3,5-trichloro-2-methoxy benzene and the VOCs mixture have antifungal activity. In conclusion, our results point to a potential role of VOC-producing *Streptomyces* in disease suppressive soils and show that VOC profiling of rhizospheric *Streptomyces* can be used as a complementary identification tool to construct strain-specific metabolic signatures.

Keywords: Actinobacteria, SPME-GC-MS, antifungal activity, plant growth promotion, suppressive soil

Introduction

Disease-suppressive soils are soils in which plants are effectively protected from infections by specific root pathogens due to antagonistic activities of soil and rhizosphere (micro)organisms (Hornby, 1983; Weller et al., 2002). This phenomenon has been described worldwide, but the responsible (micro)organisms and underlying mechanisms are largely unknown for most

suppressive-soils (Weller et al., 2002; Mendes et al., 2011; Chapelle et al., 2015). In recent studies, we identified the microbiome of a soil suppressive to *Rhizoctonia solani*, an economically important soil-borne fungal pathogen of many crops including sugar beet, potato, and rice (Mendes et al., 2011; Chapelle et al., 2015). PhyloChip-based metagenomics detected more than 33000 bacterial and archaeal taxa in the rhizosphere of sugar beet seedlings grown in the *Rhizoctonia*-suppressive soil and revealed bacterial groups consistently associated with the disease suppressive state. Among the top 10% of most dynamic taxa (i.e., taxa relatively more abundant in suppressive than in non-suppressive soil), Actinobacteria were the most dynamic phylum found in the rhizosphere of sugar beet seedlings growing in the suppressive soil.

Actinobacteria are ubiquitously found in nature and the phylum comprises more than 500 formally described species (Goodfellow, 2012; Labeda et al., 2012). Many Actinobacteria are multicellular bacteria with a complex life cycle and are renowned for the production of an exceptionally large number of bioactive metabolites (Claessen et al., 2014). Members of the genus *Streptomyces* produce over 10000 secondary metabolites, including volatile organic compounds (VOCs) (Bérdy, 2005; Hopwood, 2007; van Wezel et al., 2009). Approximately 1000 microbial VOCs have been identified to date (Piechulla and Degenhardt, 2014). Although the production of VOCs by microorganisms is known for many years (Zoller and Clark, 1921; Stotzky and Schenck, 1976), it is only since the last decade that an increasing number of studies have reported on the diversity and potential functions of these compounds. The blend of VOCs released by microorganisms is diverse and complex. Microbial VOCs belong to different classes of compounds such as alkenes, alcohols, ketones, terpenes, benzenoids, aldehydes, pyrazines, acids, esters, and sulfur-containing compounds (Effmert et al., 2012). The same VOCs can be found for different, often unrelated, microorganisms but some VOCs are unique to specific microorganisms (Schulz and Dickschat, 2007; Garbeva et al., 2014). Microbial VOCs display versatile functions: they inhibit bacterial and fungal growth, promote or inhibit plant growth, trigger plant resistance and attract other micro- and macro-organisms (Ryu et al., 2003, 2004; Vespermann et al., 2007; Kai et al., 2009; Verhulst et al., 2009; Bailly and Weisskopf, 2012; Hagai et al., 2014; Schmidt et al., 2015). Furthermore, VOCs have been proposed to function as signaling molecules in inter- and intra-specific interactions and in cell-to-cell communication. To date, however, the natural functions of microbial VOCs and their modes of action remain largely unknown (Kai et al., 2009; Kim et al., 2012; Schmidt et al., 2015).

Here we studied the diversity and functions of VOCs produced by different *Streptomyces* from the rhizosphere of sugar beet seedlings grown in a *Rhizoctonia*-suppressive soil. We first isolated and characterized 300 Actinobacteria. As *Streptomyces* represented almost 70% of all isolates, subsequent VOC analyses, phylogeny, antifungal activity and plant growth assays were conducted with this group of Actinobacteria. By coupling SPME-GC-MS and hierarchical clustering of VOC profiles, we identified VOCs potentially involved in antifungal activity.

Materials and Methods

Selective Isolation of Actinobacteria

Actinobacteria were isolated from the rhizosphere (roots with adhering soil) of sugar beet plants grown in a soil suppressive to *R. solani*. The soil was previously collected in 2003 and 2004 from an agricultural sugar beet field close to the town of Hoeven, the Netherlands (51°35′10″N 4°34″44′E). For the collection of Actinobacteria from the rhizosphere, sugar beet seeds (cultivar Alligator) were sown in square PVC pots containing 250 g of field soil with an initial moisture content of 10% (v/w). Plants were grown in a growth chamber (24°C/24°C day/night temperatures; $180 \, \mu mol$ light $m^{-2} \, s^{-1}$ at plant level during 16 h/d; 70% relative humidity) and watered weekly with standard Hoagland solution (macronutrients only). After 3 weeks of plant growth, 1 g of sugar beet roots with adhering soil was suspended in 5 mL of potassium-phosphate buffer (pH 7.0). Samples were vortexed and sonicated for 1 min. To enrich for different genera of Actinobacteria, a number of treatments were applied to the soil suspension (Supplementary Table S1). Single colonies were picked based on the morphology and purified on fresh agar plates. Isolates were stored in glycerol (20%, v/v) at −20 and −80°C.

Characterization of Actinobacteria

All 300 Actinobacterial isolates were characterized by 16S rRNA gene sequencing. PCR amplifications were conducted using primers 8F (5′- AGAGTTTGATCCTGGCTCAG - 3′) and 1392R (5′- ACGGGCGGTGTGTACA - 3′) or 27F (5′- GAGTTTGAT CCTGGCTCAG - 3′) and 1492R (5′- ACCTTGTTACGACGAC TT - 3′) (Lane, 1991; Deangelis et al., 2009). For obtaining DNA, bacterial cells were disrupted by heating at 95°C for 10 min. For spore forming isolates, cells were disrupted in the microwave at 650 W for 30 s in TE buffer. Suspensions were centrifuged at 13000 rpm for 10 min. After centrifugation, 2 μl of the supernatants were used for the PCR reactions. PCR products were purified and sequenced at Macrogen Inc. Isolates were characterized based on sequence identity with 16S rRNA gene sequences in the Greengenes database (McDonald et al., 2012) (http://greengenes.lbl.gov/).

Coupling *Streptomyces* Isolates to OTUs Detected by PhyloChip

16S rRNA gene sequences of 173 *Streptomyces* isolates were compared with the 16S rRNA gene sequences of *Streptomyces* OTUs previously identified by PhyloChip-based metagenomic analysis as the top 10% of most abundant taxa associated with disease suppressiveness (Mendes et al., 2011). Phylogenetic analysis was performed with Muscle in MEGA6 (Tamura et al., 2013) and iTOL (Letunic and Bork, 2011) (http://itol.embl.de/). A Neighbor-joining consensus tree (Saitou and Nei, 1987) with 1000 bootstrap replicates (Felsenstein, 1985) was constructed using Tamura-Nei model (Tamura and Nei, 1993) with gamma distribution. A total of 11 isolates, which were closely related to the isolates detected by PhyloChip, was selected to study the composition of emitted VOCs and their *in vitro* effects on fungal and plant growth. *Streptomyces*

lividans 1326 (Cruz-Morales et al., 2013) was used as a reference strain.

Characterization of Selected *Streptomyces* Isolates

The 11 *Streptomyces* isolates were characterized based on colony morphology and by sequence analysis of the house-keeping genes *recA* (recombinase A) and *atpD* (ATP synthase subunit B). These genes were amplified and sequenced as previously described (Guo et al., 2008). Partial sequences of *recA* (500 bp), *atpD* (423 bp), and 16S rRNA (516 bp) genes of *Streptomyces* were concatenated to yield an alignment of 1439 sites. A concatenated phylogenetic tree supplemented with sequences of *Streptomyces* strains with a sequenced genome (NCBI database) was constructed using UPGMA with the Tamura-3 parameter calculation model with gamma distribution and 1.000 bootstrap replicates. All sequences were deposited to GenBak and have been assigned to accession numbers: KT60032-KT600042 (16S rRNA gene), KT600043-KT600053 (*recA* gene), and KT600054-KT600064 (*atpD* gene).

Collection and Analysis of *Streptomyces* VOCs

For trapping the VOCs, the *Streptomyces* isolates were inoculated individually in 10 ml sterile glass vials containing 2.5 ml of GA medium (Zhang, 1990) with three replicates each. Vials containing medium only served as controls. All vials were closed and incubated at 30°C. After 7 days, VOCs from the headspace of each vial were collected by solid phase microextraction (SPME) with a 65-mm polydimethylsiloxane-divinylbenzene fiber (Supelco, Bellefonte, USA).

Streptomyces VOCs were analyzed by GC-MS (Agilent GC7890A with a quadrupole MSD Agilent 5978C). VOCs were thermally desorbed at 250°C by inserting the fiber for 2 min into the hot GC injection port. The compounds released were transferred onto the analytical column (HP-5MS, 30 m × 0.25 mm ID, 0.25 μm—film thickness) in splitless mode. The temperature program of the GC oven started at 45°C (2-min hold) and rose with 10°C min^{-1} to 280°C (3-min hold). Mass scanning was done from 33 to 300 *m/z* with a scan time of 2.8 scans s^{-1}. GC-MS raw data were processed by an untargeted metabolomics approach. MetAlign software (Lommen and Kools, 2012) was used to extract and align the mass signals (s/n = 3). MSClust was used to remove signal redundancy per metabolite and to reconstruct compound mass spectra as previously described (Tikunov et al., 2012). VOCs were tentatively annotated by comparing their mass spectra with those of commercial (NIST08) and in-house mass spectral libraries. Linear retention indices (RI) of VOCs were calculated as previously described (Strehmel et al., 2008) and compared with those in the literature. VOCs selected for *in vitro* antifungal assays [methyl butanoate (≥98%), methyl 2-methylpentanoate (≥98%), methyl 3-methylpentanoate (≥97%), 1,3,5-trichloro-2-methoxy benzene (99%) and 3-octanone (≥98%)] were confirmed with authentic reference standards obtained at Sigma-Aldrich. Processed VOC data were log transformed and auto-scaled using the average as an offset and the standard deviation as scale [raw value-average (offset)/SD (scale)]. Log transformed

data were then subjected to multivariate statistical analysis. One-way ANOVA was performed with GeneMaths XT Version 2.11 (Applied Maths, Belgium) to identify VOCs significantly different from the control (medium only) [$p < 0.05$; with false discovery rate (FDR) correction]. After that, hierarchical cluster analysis (HCA) using Pearson's correlation coefficient with UPGMA algorithm was performed.

VOC-mediated Antifungal Activity

The effect of *Streptomyces* VOCs on the growth of the fungus *R. solani* was investigated using the bottoms of two 90-mm-diameter Petri dishes allowing physical separation between the bacteria and the fungus. One bottom contained a *Streptomyces* isolate on GA medium, previously incubated at 30°C for 4 days. The other bottom contained a plug of *R. solani* mycelium on 1/10th Tryptone Soy Agar (TSA, Oxoid). Both Petri dishes were sealed facing each other and incubated at 25°C with the Petri dish containing the *Streptomyces* on the bottom to avoid spores transferring to the plate with the fungus. As a control, the Petri dish containing *R. solani* was exposed to a Petri dish containing GA medium only. Fungal growth inhibition was calculated by measuring the radial growth of the fungal hyphae after 1, 2, and 3 days of incubation. Percentage of inhibition was calculated as [(diameter of fungus in control − diameter of fungus exposed to VOCs)*100/diameter of fungus in control] for each of the 3 replicates. Student's *t*-Test was performed to determine statistically significant differences compared to the control ($p < 0.05$, $n = 3$).

Antifungal Activity of Synthetic VOCs

Methyl butanoate (≥98%), methyl 2-methylpentanoate (≥98%), methyl 3-methylpentanoate (≥97%), 1,3,5-trichloro-2-methoxy benzene (99%), and 3-octanone (≥98%) were obtained at Sigma-Aldrich. All VOCs were dissolved in methanol with final concentrations ranging from 1 M to 1 nM (10-fold dilutions). Assays were performed using a standard 90 mm-diameter Petri dish with the fungal plug on 1/10th TSA medium on top and with a sterile paper filter (1.5 × 1.5 cm) on the bottom. Twenty microliters of each VOC dissolved in methanol were applied on the paper filter, plates were immediately sealed and incubated at 25°C. Radial hyphal growth of the fungus was measured after 1 and 2 days of exposure to single or a mixture of the 5 VOCs and compared to control (empty top of a Petri dish). To check whether the solvent itself had any effect on growth of the fungus, *R. solani* was also exposed to methanol alone. Student's *t*-Test was performed to determine statistically significant differences compared to the control ($p < 0.05$, $n = 3 − 5$).

VOC-mediated Plant Growth Promotion

To determine whether *Streptomyces* VOCs had an effect on plant growth, *Arabidopsis thaliana* seedlings were exposed to the VOCs emitted by the different isolates. *A. thaliana* seeds (wild-type Col-0) were surface sterilized as previously described (van de Mortel et al., 2012) and sown on 90-mm-diameter Petri dishes containing 50 ml of 0.5X Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with 0.5% (w/v) sucrose. The 90-mm-diameter Petri dishes were placed

inside a 145-mm-diameter Petri dish, sealed and incubated in a climate chamber (21°C/21°C day/night temperatures; 180 μmol light m^{-2} s^{-1} at plant level during 16 h/d; 70% relative humidity). After 7 days, 35-mm-diameter Petri dishes containing *Streptomyces* isolates growing on GA medium (previously incubated at 30°C for 1 week) were added to the 145-mm Petri dishes with the *A. thaliana* seedlings. Plates were sealed and kept at 21°C. After 14 days, plant fresh weight was determined. In addition, plant dry weight was measured after drying shoots and roots overnight in an incubator at 65°C. Student's *t*-Test was performed to determine statistically significant differences compared to the control treatment (plants exposed to medium only).

Results

Diversity of Actinobacteria Isolated from Suppressive Soil

Using PhyloChip-based metagenomic analyses, we previously described the diversity of the bacterial community associated with the rhizosphere of sugarbeet plants grown in a *Rhizoctonia*-suppressive soil (Mendes et al., 2011). Actinobacteria were prominently more represented in the suppressive soil than in the non-suppressive (conducive) soil. Bacterial diversity detected by the PhyloChip used in the aforementioned study is displayed in **Figure 1A**. To select as many Actinobacterial isolates as possible, several pre-treatments of the rhizospheric soil and different selective media were used for their isolation (Supplementary Table S1). A total of 300 Actinobacterial isolates were obtained and characterized by 16S rRNA gene sequencing. Based on the sequence similarities (95–100%) to the 16S rRNA gene sequences available in the Greengenes database (used as reference in the PhyloChip analyses), 18 different genera of Actinobacteria were identified. These were *Streptomyces*, *Microbacterium*, *Rhodococcus*, *Micromonospora*, *Microbispora*, *Kribbella*, *Pseudonocardia*, *Cellulomonas*, *Mycobacterium*, *Actinoplanes*, *Arthrobacter*, *Actinomadura*, *Amycolaptosis*, *Nocardioides*, *Nonomureae*, *Streptosporangium*, *Micrococcus*, and *Rothia* (**Figure 1B**). The genus *Streptomyces* was the most abundant, representing 69% of all isolates and at least 25 different species based on 16S rRNA gene sequences (**Figure 1C**).

Phylogenetic Analysis of *Streptomyces* Isolates

To select *Streptomyces* isolates for VOC and functional analyses, 16S rRNA gene sequences of the *Streptomyces* isolates (n = 173) obtained in this study were compared with those of the representative *Streptomyces* OTUs (n = 430) originally detected by PhyloChip (Mendes et al., 2011). A phylogenetic tree was constructed using these sequences and the sequences of different *Streptomyces* type strains (**Figure 2**). This comparison led to the selection of 11 isolates (**Figure 3**). We then constructed phylogenetic trees with these 11 isolates, their closest type strains, other *Streptomyces* species with sequenced genomes and the reference strain *Streptomyces lividans* 1326 (Supplementary Figure S1A). Additionally, we sequenced the house-keeping genes *atpD* and *recA* (Supplementary Figure S1B). Concatenation of *atpD*, *recA,* and 16S sequences allowed a better resolution of
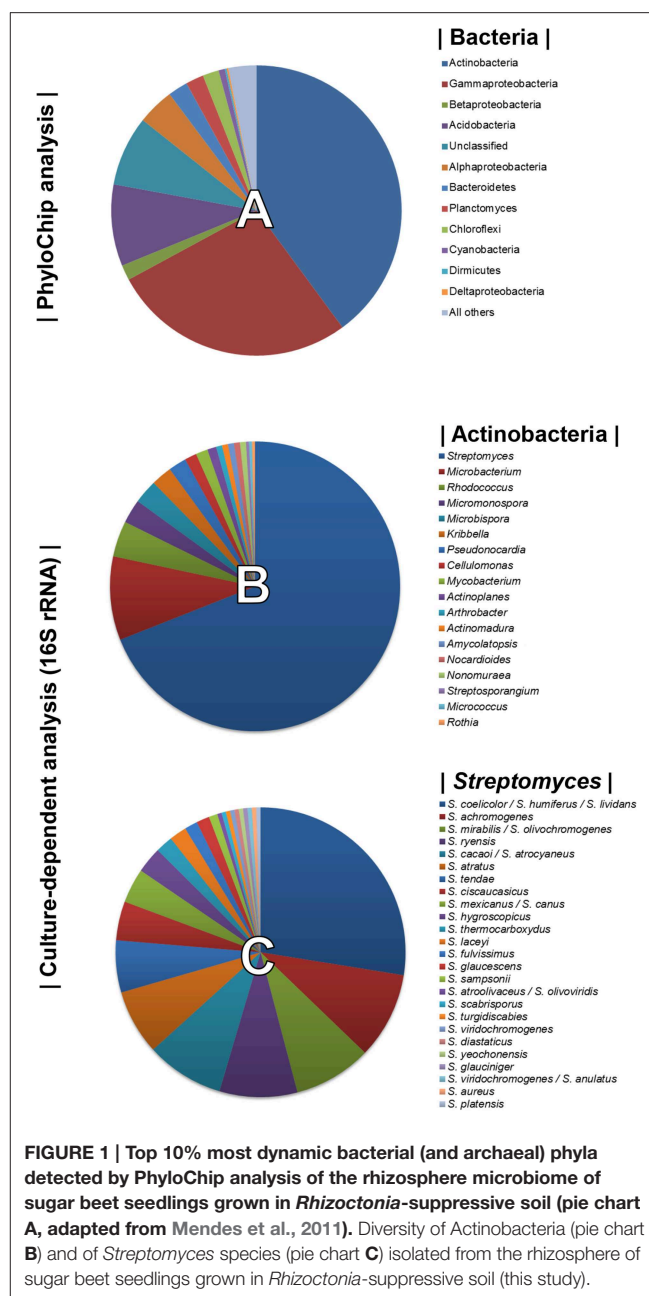
FIGURE 1 | Top 10% most dynamic bacterial (and archaeal) phyla detected by PhyloChip analysis of the rhizosphere microbiome of sugar beet seedlings grown in *Rhizoctonia*-suppressive soil (pie chart A, adapted from Mendes et al., 2011). Diversity of Actinobacteria (pie chart **B**) and of *Streptomyces* species (pie chart **C**) isolated from the rhizosphere of sugar beet seedlings grown in *Rhizoctonia*-suppressive soil (this study).

the different *Streptomyces* isolates than based on 16S sequences only. However, closely related but phenotypically different isolates, like *Streptomyces* strains W75.5 and W126 (**Figure 3**), could not be distinguished based on these three molecular markers.

VOC Profiling of *Streptomyces* Isolates

For the 12 *Streptomyces* isolates (11 rhizosphere isolates and reference strain *S. lividans* 1326) grown on GA medium and the medium alone (control), a total of 536 VOCs were detected in the headspace. Out of these, 381 VOCs that were significantly different (ANOVA, $p < 0.05$) and detected at intensities at least twice as high as in the control were considered for further

FIGURE 2 | Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences of the *Streptomyces* collection obtained in this study (in blue), *Streptomyces* detected by Phylochip analysis (in pink), and *Streptomyces* type strains (in green). *Streptomyces* isolates selected for VOC analysis are indicated in bold.
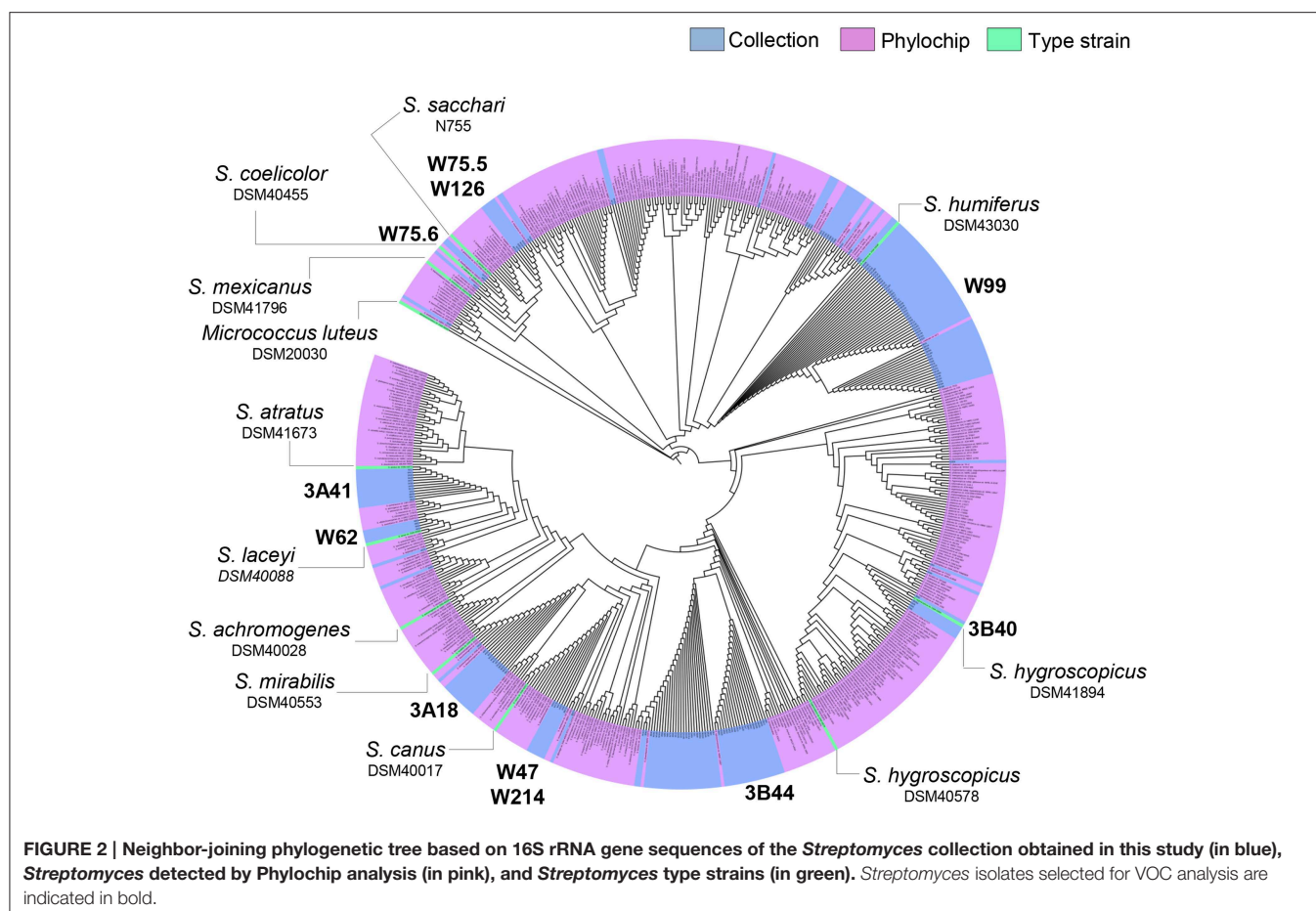
analyses. The diversity of VOCs produced by the different *Streptomyces* isolates is shown in Supplementary Table S2 and highlighted in the heat-map (**Figure 4**). The VOCs detected belong to diverse classes of compounds such as alcohols, aldehydes, carboxylic acids, esters, ketones, sulfur compounds, and several terpenes (Supplementary Table S2). Most VOCs were found to be specific for some *Streptomyces* isolates and 45 VOCs were found to be commonly produced by all isolates tested. Geosmin (trans-1,10-dimethyl-trans-9-decalol, RI 1423; Supplementary Table S2) was one of these common VOCs. HCA of the VOC profiles resulted in a similar clustering of the 12 *Streptomyces* isolates as the clustering based on the different molecular markers (**Figure 5**). In contrast to the molecular markers, however, VOC profiling allowed differentiation between closely related *Streptomyces* isolates such as *Streptomyces* strains W75.5 and W126 as well as *Streptomyces* strains W47 and W214.

Effect of *Streptomyces* VOCs on Fungal and Plant Growth

To test the antifungal activity of VOCs produced by the *Streptomyces* isolates from disease suppressive soil, hyphal growth of *R. solani* was measured during exposure to VOCs from each of the isolates. In the control, fungal hyphae reached the edge of the agar plates after 2 days of incubation. All *Streptomyces*

strains were able to significantly retard the growth of *R. solani*. *Streptomyces* strains W47 and W214 were the most inhibitory. When exposed for 2 days to the VOCs produced by these isolates, radial hyphal growth was reduced by 57 and 41%, respectively (**Figure 6A**).

Additionally, we tested whether *Streptomyces* VOCs could promote plant growth. To that end, we exposed 7-day-old *A. thaliana* seedlings to VOCs from each of the isolates and determined root and shoot biomass. After 2 weeks of exposure to *Streptomyces* VOCs, no negative effects on plant growth were observed. Ten out of 12 isolates significantly increased shoot biomass, and 8 significantly increased root biomass compared to the control (**Figure 6B**). *S. lividans* 1326, and *Streptomyces* strains W47 and W62 led to the largest increase in plant biomass, whereas *Streptomyces* strains W214 and 3A41 did not increase shoot and root biomass.

Identification of *Streptomyces* VOCs Contributing to Antifungal Activity

Since *Streptomyces* strains W47 and W214 are phylogenetically closely related and both showed strong antifungal activity, these isolates were selected to identify VOCs with activity against *R. solani*. Screening of VOCs with potential antifungal activity was computed with One-way ANOVA [$p < 0.05$; with false discovery

FIGURE 3 | Characterization of Streptomyces isolates used in this study. Species names are based on 16S rRNA gene sequence comparison using the Greengenes database. Pictures depict 4–7 day-old isolates grown on GA medium. *S. lividans 1326 refers to John Innes Center collection number and corresponds to S. lividans 66 (Hopwood et al., 1983).
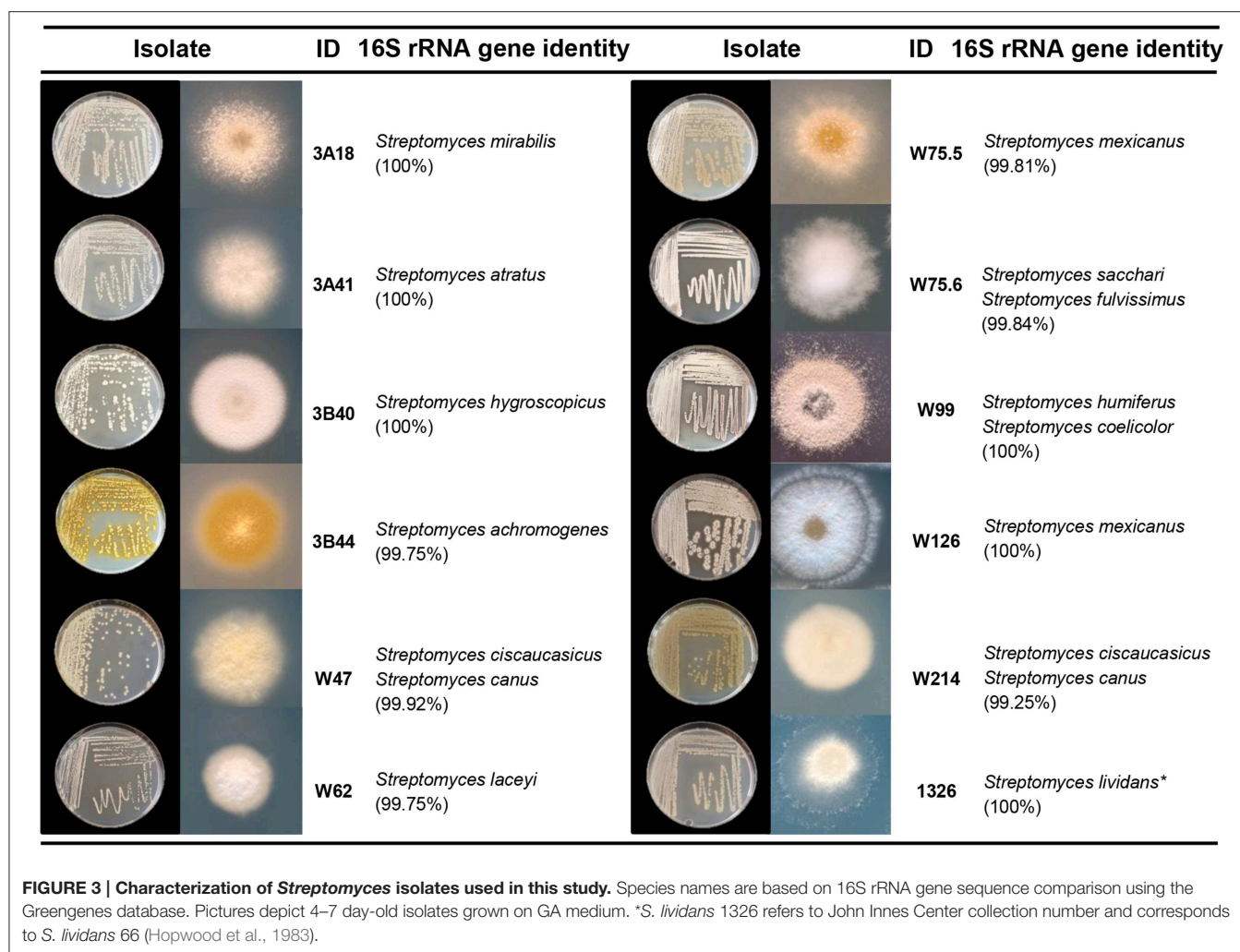
rate (FDR) correction] and a fold change >2 using the peak intensity of VOCs from W214/control and W47/control. For the selection of VOCs for *in vitro* antifungal activity, three criteria were used: (1) match factor and reverse match factor higher than 850, (2) reliable annotation based on retention indices and, (3) availability of pure (synthetic) reference compounds.

A comparison of the VOC profiles of *Streptomyces* strains W47 and W214 with the control (medium only) pinpointed VOCs potentially involved in antifungal activity (**Figure 7A**). A total of 96 VOCs were shared between these two isolates; 65 and 7 VOCs were unique for *Streptomyces* strains W47 and W214, respectively (**Figures 7A,B**). Since both *Streptomyces* strains W47 and W214 showed antifungal activity, we looked into the VOCs detected for both strains. We selected five common VOCs (methyl butanoate, methyl 2-methylpentanoate, methyl 3-methylpentanoate, 1,3,5-trichloro-2-methoxy benzene, and 3-octanone) which could be reliably annotated based on RI and mass spectral similarity and which were commercially available as authentic reference standards. The identity of these compounds was verified by analyzing pure standards by the GC-MS and comparing their mass spectra and RI with those of the VOCs detected for *Streptomyces* strains W47

and W214. Subsequently, different concentrations of these five VOCs were used to test their inhibitory effect on hyphal growth of *R. solani* (**Figure 7C**). The VOC 1,3,5-trichloro-2-methoxy benzene completely inhibited radial hyphal growth of *R. solani* at concentrations of 1 M and 100 mM (**Figure 7D**). Exposure to this VOC led to melanization of *R. solani* hyphae (**Figure 7E**). The VOC methyl 2-methylpentanoate reduced fungal growth by 47 and 25% after 1 and 2 days of exposure, respectively. Additionally, a mix of the 5 synthetic VOCs, each at a final concentration of 200 mM, inhibited hyphal growth by 58 and 42% after 1 and 2 days of exposure, respectively.

To further determine if the antifungal VOC 1,3,5-trichloro-2-methoxy benzene is typically found for *Streptomyces* isolates that inhibit hyphal growth of *R. solani*, we determined the relative amounts of this VOC produced by each of the 12 *Streptomyces* isolates tested in this study. The results show that production of this VOC is widespread among the 12 *Streptomyces* isolates. Moreover, a positive nonlinear correlation was found between the percentage of hyphal growth inhibition and the abundance (peak intensity) of 1,3,5-trichloro-2-methoxy benzene detected for the 12 isolates (**Figures 7F,G**).

FIGURE 4 | Hierarchical cluster and heat-map analyses of VOC profiles of the selected *Streptomyces* isolates. Columns represent three replicate VOC measurements of each of the 12 isolates and the medium alone (control). Rows represent the different VOCs (green, low abundance; red, high abundance), several of which were putatively annotated (see Supplementary Table S2).

FIGURE 5 | (A) Phylogenetic tree of concatenated partial sequences of 16S rRNA, *atpD* and *recA* genes of 11 *Streptomyces* isolates from the *Rhizoctonia*-suppressive soil and the reference strain *S. lividans* 1326. The tree was constructed using UPGMA method and Tamura-3 parameter calculation model with gamma distribution and 1000 bootstrap replicates. **(B)** Hierarchical cluster analysis (HCA) of *Streptomyces* VOCs with UPGMA method and Pearson's correlation coefficient. Different colors indicate different clusters of isolates based on VOC profiles.

Discussion

The production of VOCs by microorganisms is known for several decades. Only recently an increasing number of studies reported on the chemical diversity and possible functions of this group of microbial compounds (Schmidt et al., 2015). In comparison to plant VOCs, knowledge about the natural functions of microbial VOCs is still limited (Bitas et al., 2013). Here we studied the diversity and activities of VOCs produced by different streptomycetes from a *Rhizoctonia*-suppressive soil.

VOC profiling has been extensively used for food flavoring and aroma as well as indicators of fungal growth in buildings and in post-harvest management (Morath et al., 2012). More recently, VOC chemotyping allowed not only to identify species- and strain-specific VOCs but also to study soil microbial activity and shifts in microbial community compositions (McNeal and Herbert, 2009; Müller et al., 2013; Trefz et al., 2013). We showed that VOC profiling can be used for chemotyping

different streptomycetes. Most of the 381 VOCs detected for the different streptomycetes from the *Rhizoctonia*-suppressive soil were found to be specific for some isolates whereas fewer VOCs were found to be commonly produced by all isolates. The best known VOCs from streptomycetes are 2-methylisoborneol (MIB) and trans-1,10-dimethyl-trans-9-decalol (geosmin) which are responsible for the characteristic musty or earthy smell of moist soils (Gerber, 1968; Jiang et al., 2007). Our results also show that these VOCs are widely produced by *Streptomyces* isolates from the rhizosphere of sugar beet plants grown in *Rhizoctonia*-suppressive soil. Geosmin was detected for all isolates, whereas MIB was detected for eight isolates. Members of the *Streptomyces* genus differ greatly in their morphology, physiology, and biochemical characteristics (Anderson and Wellington, 2001). Taxonomic delineation of this genus remains complex and leads to over- or under-classified groups. Current approaches for classification of *Streptomyces* as well as other prokaryotes rely on genetic and phenotypic traits, mainly on 16S rRNA gene sequences. This molecular marker, however,

FIGURE 6 | Inhibition of fungal growth after 1 and 2 days of exposure to *Streptomyces* VOCs (A) and growth of *Arabidopsis thaliana* seedlings after 2 weeks of exposure to *Streptomyces* VOCs (B). The controls are displayed in green and isolates with the strongest antifungal activity in red. Bars represent standard errors of the mean of 3 independent biological replicates. Asterisks indicate a statistical difference as compared to controls (exposed to medium only) using Student's *t*-Test ($p < 0.05$, $n = 3$). Pictures of antifungal activity and plant growth promotion were made after 3 and 14 days of exposure, respectively.

is not always sufficient to discriminate between closely related species and between strains of a given species (Girard et al., 2013). We showed that concatenation of *atpD*, *recA,* and 16S rRNA gene sequences displayed a better phylogenetic delineation of the different streptomycetes than 16S rRNA gene sequences alone, although closely related isolates could not be distinguished. We revealed that VOC profiling allowed discrimination of *Streptomyces* isolates that are phylogenetically close but phenotypically different, such as *Streptomyces* strains W75.5/W126 and W47/W214.

The genus *Streptomyces* is well-known for the production of several antifungal and antiviral compounds and accounts for 80% of the currently available antibiotic compounds (Watve et al., 2001). *Streptomyces* also produces VOCs which reduce the incidence and/or the severity of several plant diseases caused by fungi and cause morphological abnormalities in different fungi (Moore-Landecker and Stotzky, 1973; Wan et al., 2008; Boukaew et al., 2013; Wang et al., 2013; Wu et al., 2015). VOCs produced by the streptomycetes tested here exhibited antifungal and plant growth promoting properties. Several

isolates inhibited hyphal growth, with *Streptomyces* strains W47 and W214 showing the strongest inhibitory effect. Given that these streptomycetes were obtained from a *Rhizoctonia*-suppressive soil suggests that VOCs may contribute to disease suppressiveness. This suggestion needs to be further investigated *in situ* but fits well with one of the initial hypotheses of Lockwood (Lockwood, 1977) for the potential role of microbial VOCs in soil fungistasis. To provide more conclusive proof of the role of these *Streptomyces* VOCs in disease suppression in the soil ecosystem, specific soil bioassays are needed where the VOC producers and the pathogen are physically separated. However, there are several technical limitations to accomplish this. First, the strains used here are rhizospheric bacteria that need to be positioned in their ecological context (the rhizosphere) to provide meaningful results. Given the need for the localization of the *Streptomyces* strains in the rhizosphere where also the pathogen colonizes and infects, it has not been possible yet to physically separate the *Streptomyces* strains from the fungal pathogen. This is due in part to the prolific growth of this particular fungus. The physical separation *in*
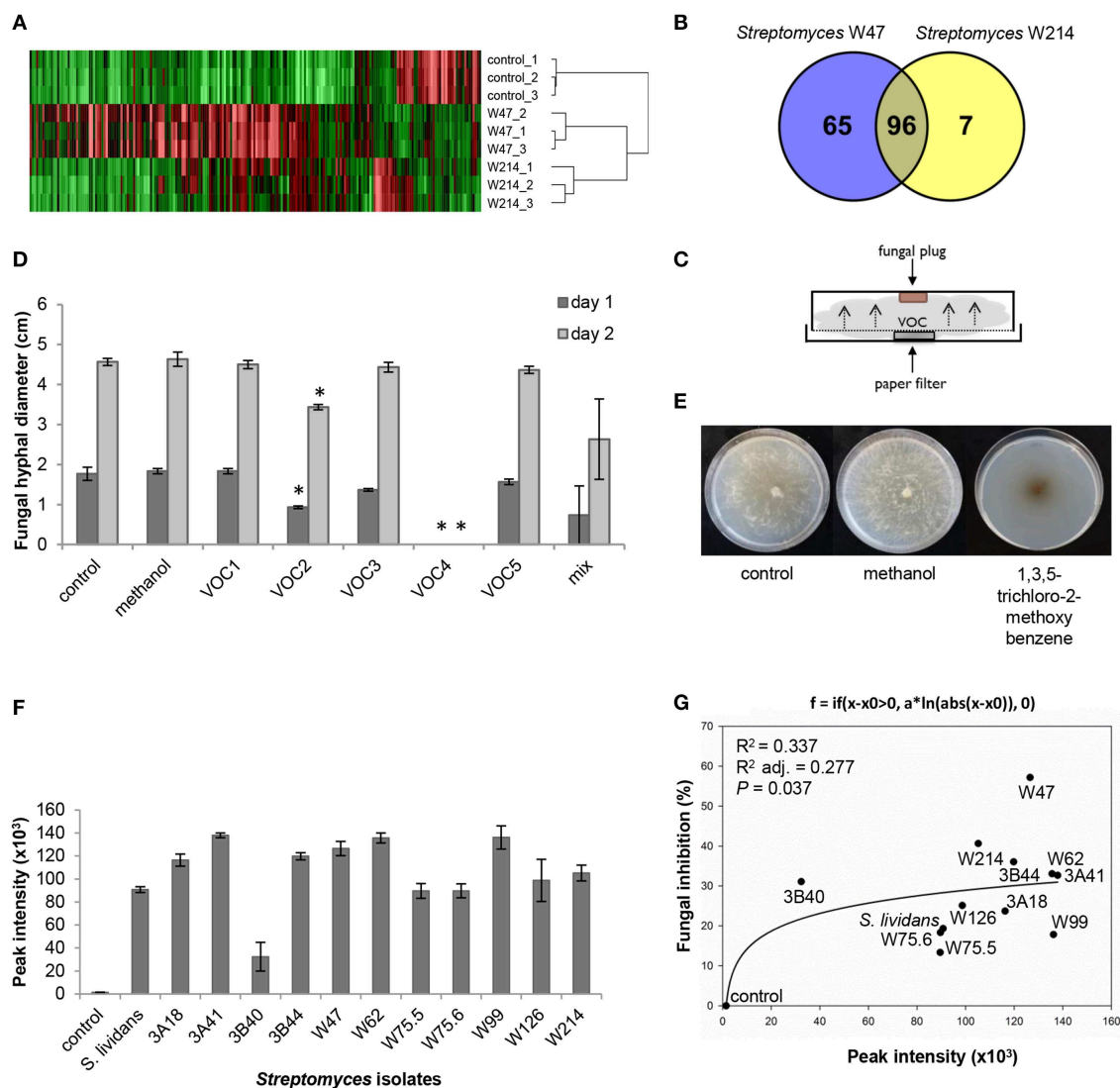
FIGURE 7 | (A) VOC profiles of *Streptomyces* strains W47 and W214 compared to control (medium only). **(B)** Venn diagram for common and unique VOCs produced by *Streptomyces* strains W47 and W214. **(C)** Experimental set-up for *in vitro* antifungal activity assay with synthetic VOCs. **(D)** *In vitro* antifungal activity with synthetic VOCs at 1 M [control, methanol, VOC1 (methyl butanoate), VOC2 (methyl 2-methylpentanoate), VOC3 (methyl 3-methylpentanoate), VOC4 (1,3,5-trichloro-2-methoxy benzene), VOC5 (3-octanone]. Methanol was used to dilute all VOCs. Bars represent standard errors of the mean of 3 independent replicates. Asterisks indicate statistical differences compared to control according to Student's *t*-Test ($p < 0.05$, $n = 3$). **(E)** Fungal growth after exposure to 1,3,5-trichloro-2-methoxy benzene. **(F)** Abundance of 1,3,5-trichloro-2-methoxy benzene produced by different *Streptomyces* isolates based on GC-MS peak intensities. **(G)** Nonlinear relationship between fungal growth inhibition and abundance of 1,3,5-trichloro-2-methoxy benzene.

situ is needed to exclude a possible role of mechanisms other than VOCs. An alternative approach would be to generate site-directed mutants of the *Streptomyces* strains that do not produce one or more of the specific VOCs identified in this study. Comparison of the activity of these mutants with their wildtype strains would then more conclusively resolve the role of specific VOCs in disease suppression *in situ*. For this alternative approach, however, we have not yet been able to generate mutants as many environmental *Streptomyces* species/strains are not or very difficult to access for genetic modification.

Several studies have described antifungal activity by bacterial VOCs, however, few have identified single or blends of VOCs responsible for the antifungal activity (Kai et al., 2007; Wang et al., 2013). For *Pseudomonas*, six VOCs (cyclohexanal, decanal, 2-ethyl 1-hexanol, nonanal, benzothiazole, and dimethyl trisulfide) were found to inhibit mycelial growth and sclerotial germination of *Sclerotinia sclerotiorum* at tested volumes of 100 and 150 μl (Fernando et al., 2005). Regarding VOCs produced by *Streptomyces* species, butanone (methyl vinyl ketone) and dimethyl disulfide were described to inhibit the spore germination in *Cladosporium cladosporioides* and mycelial

growth of *Fusarium moniliforme*, respectively (Herrington et al., 1987; Wang et al., 2013). Here we showed that two out of five VOCs detected for *Streptomyces* strains W47 and W214 (methyl 2-methylpentanoate and 1,3,5-trichloro-2-methoxy benzene) as well as the mix of these VOCs exhibited antifungal activity, albeit at high concentrations. The VOC 1,3,5-trichloro-2-methoxy benzene completely inhibited fungal growth and caused melanization of the fungal hyphae. 1,3,5-Trichloro-2-methoxy benzene is also known as 2,4,6-trichloroanisole (TCA) and causes off-flavor in wine, coffee and water (Spadone et al., 1990; Jensen et al., 1994). Anisole produced by *S. albulus* has recently been described for activity against *S. sclerotiorum* and *F. oxysporum* (Wu et al., 2015). Derivatives of anisole have been described to be produced by bacteria and fungi (Mauriello et al., 2004; Blom et al., 2011), but no function has been ascribed to this specific VOC yet. To our knowledge, this is the first time that 1,3,5-trichloro-2-methoxy benzene is described for its antifungal activity. The VOC methyl 2-methylpentanoate, which also exhibited antifungal activity, is known for other streptomycetes, but also for this VOC no specific function has been described so far (Wilkins and Scholler, 2009; Dickschat et al., 2011). For both 1,3,5-trichloro-2-methoxy benzene and methyl 2-methylpentanoate, the concentrations needed to inhibit fungal growth were high. However, in the experimental setup used here, we do not know how much of the applied VOCs actually contact the fungal hyphae, which part of the fungal hyphae are the most VOC sensitive and how long VOC exposure is necessary to exert the antifungal activity. These aspects will be subject of future studies. Also, the identification of *Streptomyces* VOCs involved in plant growth promotion was not further pursued in this study but a possible candidate is acetoin (3-hydroxy-2-butanone) which was detected for several isolates tested here. Acetoin and 2,3-butanediol were the first bacterial VOCs described for their role in plant growth promotion (Ryu et al., 2003). More recently, other VOCs have been identified for their role in plant growth promotion such as indole, 1-hexanol, pentadecane, 13-tetradecadien-1-ol, 2-butanone, and 2-methyl-n-1-tridecene (Blom et al., 2011; Park et al., 2015). Plant growth-promoting effects can also be, at least partially, due to CO_2 accumulation as products of microbial metabolism when using closed Petri dishes (Kai and Piechulla, 2009). In the experimental set-up used in our study, however, CO_2 appears

to have only a minor role since two isolates (3A41 and W214) out of the 12 tested isolates did not promote shoot and root growth, and two isolates (3A18 and W75.5) did not promote root growth.

In conclusion, VOCs produced by rhizosphere-associated streptomycetes are chemically diverse and display antifungal and plant growth-promoting properties. Hence, VOC profiling can provide a new resource of novel metabolites and biochemical pathways involved in antifungal activity and plant growth promotion by streptomycetes. We identified two VOCs with antifungal activity, but it remains to be determined whether these compounds are produced *in situ* at the biologically relevant concentrations. Our work further demonstrated the utility of VOC profiling for the characterization of streptomycetes, providing an additional tool for phylogenetic delineation of closely related strains.

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Biocide effects of volatile organic compounds produced by potential biocontrol rhizobacteria on *Sclerotinia sclerotiorum*

Annalisa Giorgio[1], Angelo De Stradis[2], Pietro Lo Cantore[1] and Nicola S. Iacobellis[1]*

[1] *Scuola di Scienze Agrarie, Forestali, Alimentari ed Ambientali, Università degli Studi della Basilicata, Potenza, Italy,* [2] *Istituto per la Protezione Sostenibile delle Piante, Consiglio Nazionale delle Ricerche, Bari, Italy*

Six rhizobacteria isolated from common bean and able to protect bean plants from the common bacterial blight (CBB) causal agent, were *in vitro* evaluated for their potential antifungal effects toward different plant pathogenic fungi, mostly soil-borne. By dual culture assays, the above bacteria resulted producing diffusible and volatile metabolites which inhibited the growth of the majority of the pathogens under study. In particular, the latter substances highly affected the mycelium growth of *Sclerotinia sclerotiorum* strains, one of which was selected for further studies either on mycelium or sclerotia. Gas chromatographic analysis of the bacterial volatiles led to the identification of an array of volatile organic compounds (VOCs). Time course studies showed the modification of the VOCs profile along a period of 5 days. In order to evaluate the single detected VOC effects on fungal growth, some of the pure compounds were tested on *S. sclerotiorum* mycelium and their minimal inhibitory quantities were determined. Similarly, the minimal inhibitory quantities on sclerotia germination were also defined. Moreover, observations by light and transmission electron microscopes highlighted hyphae cytoplasm granulation and ultrastructural alterations at cell organelles, mostly membranes, mitochondria, and endoplasmic reticulum. The membranes appeared one of the primary targets of bacterial volatiles, as confirmed by hemolytic activity observed for the majority of pure VOCs. However, of interest is the alteration observed on mitochondria as well.

Keywords: rhizobacteria, phytopathogenic fungi, *Sclerotinia sclerotiorum*, volatile organic compounds, transmission electron microscope, ultrastructures, hemolysis

Introduction

In recent years biological control, through the application of antagonistic microorganisms from the rhizosphere (Paulitz and Bélanger, 2001; Minuto et al., 2006), has raised great research interest as a possible alternative for plant protection because it seems to be eco-friendly and offers a secure long-term protection of the crops (Fernando et al., 2005). Bacterial antagonists can negatively affect the growth of plant pathogens by several mechanisms such as the excretion of antifungal metabolites e.g., antibiotics, toxins, and bio-surfactants (Raaijmakers et al., 2002). Recently, it was demonstrated that volatile organic compounds (VOCs) of soil bacteria can influence the growth of phytopathogenic fungi as well (Alström, 2001; Wheatley, 2002).

The VOCs are generally lipophilic substances with high vapor pressure which freely pass through biological membranes and are released into the atmosphere or in the soil where the producers are (Pichersky et al., 2006). Therefore, the volatiles produced by soil bacteria may have a role above the ground, but also within the soil. Some VOCs may act as signal substances for inter- and intra- organisms communication as well as between cells of the same organism (Kai et al., 2009). Over the past years, attention has focused on the study of the production of VOCs by microorganisms as a weapon of defense against pathogenic fungi (Mackie and Wheatley, 1999; Strobel et al., 2001; Fernando et al., 2005; Gu et al., 2007; Zou et al., 2007; Liu et al., 2008; Wan et al., 2008; Arrebola et al., 2010). However, research on VOCs, in the interactions between potential biological control agents (BCAs), plant pathogens and host or non-host plants, as well as the biotic and abiotic factors that influence these relationships is still in its infancy (Campos et al., 2010). Therefore, in this regard, continuous research is needed.

In our previous studies six rhizobacteria, isolated from bean in southern Italy, resulted to protect bean plants from *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans*, the causal agent of common bacterial blight (CBB). Then, three of them were demonstrated triggering induced systemic resistance (Van Loon, 2007) in the plant model *Arabidopsis thaliana* toward *Xanthomonas campestris* pv. *armoraciae* either when they were inoculated in soil or via bacterial volatiles (Giorgio et al., 2014). Furthermore, rhizobacteria showed some typical features of plant growth promoting rhizobacteria (Giorgio et al., 2015), hence they appeared as potential BCAs. As a consequence, it seemed interesting to investigate about the activity of rhizobacteria against other bean pathogens with a lifestyle different from the bacterial pathogens ones. Indeed, the aim of the present work was to evaluate the possible antifungal properties of the mentioned bacterial potential BCAs toward several, mostly soil-borne, phytopathogenic fungi, focusing the attention on the biological effects of bacterial volatiles. Among the pathogenic fungi used in this study, strains of *Sclerotinia sclerotiorum*, a cosmopolitan necrotrophic fungal pathogen characterized by a broad host range (Purdy, 1979), resulted highly sensitive to both diffusible and volatile antimicrobial substances and, for that reason, one strain of the above pathogen was selected for further studies. Here we report *in vitro* fungal growth inhibition by diffusible and volatile substances produced by six bean rhizobacteria. In particular, some pure VOCs, identified by GC-MS, were evaluated, in comparison to the natural volatile blends, for their specific antifungal activity on *S. sclerotiorum* mycelium and sclerotia germination, for hemolytic activities and, for their effects at cellular and ultrastructural levels on the pathogen mycelium in order to figure out the probable action mechanisms of the above mentioned volatiles.

Material and Methods

Bacteria and Fungi Growth Conditions

Bacteria were isolated from the rhizosphere of bean plants in the National Park Agri valley, in southern Italy and preliminarily identified on the basis of their nutritional profiles by BIOLOG

system (Biolog, Inc. Hayward, CA, USA) and partial 16S rDNA sequencing. Three of them showed an elevated sequence homology with strain NFM421 of *Pseudomonas brassicacearum* (Ortet et al., 2011) (USB2101: ID HE981747; USB2102: ID HE981748; USB2104: ID HE981749, EMBL, 2013); two isolates resulted similar to the strain W619 of *P. putida* (Copeland et al., unpublished data) (USB2105: ID HE981750; USB2106: ID HE981751, EMBL, 2013) and one isolate revealed high similarity with strains DSM 319 and QMB 1551 (Eppinger et al., 2011) of *Bacillus megaterium* (USB2103: ID HE981752, EMBL, 2013). Since the above identification is not yet definitive along this manuscript the five fluorescent pseudomonads have been reported as *Pseudomonas* spp. and the Gram positive bacterium as *Bacillus* spp. The rhizobacteria were grown on King's B agar (KBA) (King et al., 1954) at 25°C for 48 h. For short term storage, bacteria were grown on glycerol nutrient agar (GNA) slants and stored at 4°C (Lelliott and Stead, 1987). For long-term storage, bacteria were lyophilized or maintained at −80°C in 30% glycerol. They were previously phenotypically characterized showing some typical biocontrol traits (Giorgio et al., 2015) (Supplementary Table 1).

Fungal strains used in the present work (**Table 1**) were grown on potato dextrose agar (PDA) for 5 days at 25°C and maintained on the same medium at 4°C.

Bacteria Production of Bioactive Substances
Diffusible Substances

Dual culture assays were performed in order to test the possible antagonistic activity of rhizobacteria toward 17 strains of phytopathogenic fungi of different origin. In particular, two fungal plugs (5 mm ø), from a 5 days culture on PDA, were taken from the actively growing fungal colony edge and placed,

TABLE 1 | Plant pathogenic fungi used in this study.

Fungal strains	Plant host	Geographical origin
Botrytis cinerea USB-F1131	Grape	Italy
B. cinerea USB-F1636	Pepper	Italy
Fusarium equiseti USB-F2014	Soil	Italy
F. oxysporum USB-F1	Tomato	Greece
F. oxysporum USB-F111	Strawberry	Italy
F. solani USB-F607	Bean	Italy
Macrophomina phaseolina USB-F918	Pepper	Italy
Phytophthora cactorum USB-F876	Chestnut	Italy
P. cactorum USB-F1168	Strawberry	Italy
P. nicotianae USB-F172	Chestnut	Italy
Pythium ultimum USB-F2	Potato	Greece
Rhizoctonia solani USB-F3	Pepper	Greece
Rosellinia necatrix USB-F587	Cherry	Italy
Sclerotinia sclerotiorum USB-F593	Bean	Italy
S. sclerotiorum USB-F853	Cantaloupe	Italy
Verticillium dahliae USB-F464	Chicory	Italy
V. dahliae ITM-1910	Tomato	Italy

USB, Università degli Studi della Basilicata, Potenza, Italy; ITM, Istituto Tossine e Micotossine, Consiglio Nazionale delle Ricerche, Bari, Italy.

to the opposite sides, on the surface of 20 ml KBA in Petri dishes. Then, a droplet of 50 µl of bacterial suspension (10^8 CFU ml^{-1}), from an overnight culture on KBA, was inoculated in the middle of the Petri dish, between the two fungal plugs. The assay was performed also on minimal medium agar (MMA) (Lavermicocca et al., 1997) and PDA. Controls were prepared in a similar manner without bacterial suspension in the middle of plates. After 5 days incubation at 25°C the diameter of fungal colony was measured. For the determination of the inhibitory effect of the bacterial isolates on pathogenic fungi the inhibition rate (IR%) was calculated according to the following formula: IR% = $100 \times [(C-B)/C]$, where C is the diameter of the control fungal mycelium and B the diameter of the fungal mycelium grown in the presence of the antagonistic bacteria. The experiments were performed three times with three replicates.

Volatiles Compounds

Rhizobacteria were tested for their ability to produce volatile substances inhibiting fungal growth using the double plate technique. One hundred microliters of bacterial suspensions, prepared as described above, were spread on KBA in Petri dishes. Three plugs of mycelium (5 mm ø) were placed on PDA surface for each plate. Petri dishes containing fungal mycelium plugs were then placed inverted over the KBA plates inoculated with the bacteria. Each pair of plates was sealed with Parafilm® to prevent the leak of bacterial volatiles compounds. The plates were incubated at 25°C for 5 days. Control sets were prepared in a similar manner, but without bacteria. The diameter of the fungal colony was measured after the incubation period. The assays were performed three times with three replicates.

In order to verify whether bacterial volatiles could have fungicidal or fungistatic action, plugs of fungal mycelium of the strain USB-F593 of *S. sclerotiorum* exposed to volatiles substances for 5 days were taken and re-inoculated on fresh PDA incubated at 25°C for 5 days. The assay was performed three times with three replicates.

With the aim of testing bacterial volatiles effects on *S. sclerotiorum* sclerotia, three sectors Petri dishes were used filling two out three parts with PDA, on which sclerotia (one sclerotium per sector) were placed, and the third one was inoculated with a droplet of 50 µl of bacterial suspension (10^8 CFU ml^{-1}) on KBA. Sclerotia were produced inoculating 100 ml potato dextrose broth (PDB) with three agar plugs of *S. sclerotiorum* in 250 ml flasks. After incubation at 20°C for 4 weeks, the flasks were incubated at 4°C for further 4 weeks to condition the sclerotia (Dillard et al., 1995). The sclerotia of 2–4 mm ø size were selected, then washed, dried overnight in a stream of sterile air, and used for the assay. The radial growth of the mycelium growing from the sclerotia was measured after 5 days incubation at 25°C. The experiment was performed three times with three replicates per treatment. In order to verify sclerotia viability after bacterial volatiles exposure for 5 days, sclerotia were re-inoculated on fresh PDA and incubated at 25°C for 5 days. The assay was performed three times with three replicates.

All data of bioassays described above were statistically analysed for the determination of standard errors, for ANOVA and P was calculated by F-test of Fisher-Snedecor. All statistical analysis were carried out using the SPSS version 17.0 software program package (SPSS Inc., Chicago, IL).

GC-MS Analysis

One hundred microliters of bacterial suspensions (10^8 CFU ml^{-1}) were inoculated on the surface of KBA slants in glass tubes equipped with silicone septa (Supelco 12345-U) caps and incubated for 5 days at 25°C. Volatile compounds were collected from the head space of the tubes by solid phase micro-extraction (SPME) technique (Zhang and Pawliszyn, 1993; Strobel et al., 2001). For the purpose SPME fiber coated with 100 µm of a phase of polidimetilsiloxane (Supelco 57 300-U, mounted on a support 57 330 Supelco) was conditioned for 1 h at 250°C in a stream of helium and then introduced for 20 min into the head space of the tube containing bacterial suspension. Then the fiber was introduced into the injection port of a gas chromatograph HP6890 equipped with a capillary column Phenomenex Zebron ZB-5 MS (30 m × 0.25 mm ID × 0.25 mm film thickness). HP5973 mass spectrometer (mass range: 15–300 amu, scan speed: 1.9 scans s^{-1}, the voltage EM: 1435) was used as mass selective detector. Helium was used as carrier gas (0.8 ml min^{-1} flow rate) and the desorption time was 1 min. The injection port was maintained at 250°C while the detector at 230°C. The oven was maintained at a temperature of 40°C for 2 min to increase up to 250°C (8°C min^{-1}). The run method was set at 33 min.

All peaks were identified by their mass spectra in comparison with the spectra present in Wiley6N and NIST98 databases.

The isolates USB2103, USB2104, and USB2105 attributable to strains of *B. megaterium*, *P. brassicacearum*, and *P. putida*, respectively, were chosen to perform a time course GC analysis at 1, 3, 5 days incubation. VOCs collection, analysis and identification have been achieved as described above.

All the GC-MS analysis were performed three times.

Effects of Pure VOCs on *Sclerotinia sclerotiorum* Mycelium and Sclerotia

Double plate technique and three sectors Petri dishes experiments described above for mycelia and sclerotia, respectively, were used, with some modifications, to carry out pure VOCs assay, in order to verify individual VOC action on the strain USB-F593 of *S. sclerotiorum*. In particular, two plugs of mycelium, grown on PDA at 25°C for 5 days, were placed in Petri dishes on PDA. A watch glass, previously sterilized (121°C for 20 min), was placed on the lid of each plate and filled with 100 µl of pure VOC. Then each plate was inverted on its cover containing the watch glass and the Petri dish was Parafilm® sealed to prevent leakage of VOCs. For sclerotia assay, the watch glass was placed in one out three sectors of Petri dishes; while sclerotia were put as already described for natural bacterial volatiles exposure.

Petri dishes were incubated at 25°C for 5 days.

Starting from a volume of pure VOCs of 100 µl, 1:1 serial and, when necessary, intermediate dilutions were made in order to define the fungal growth minimal inhibitory quantity (MIQ) (expressed in milligrams) for mycelium growing either from the fungal plug or from the germinated sclerotia. Pure substances

were diluted in water, DMSO or methanol depending on their solubility. The assay was carried out as described above using watch glasses and Petri dishes were incubated at 25°C for 5 days.

Hemolytic Activity of Pure VOCs

According to the bacterial volatiles profile results obtained via GC-MS analysis, some VOCs, chosen on the basis of their systematic detection and availability on the market (Sigma-Aldrich, Milan, Italy), were individually used to investigate their possible activity on biological membranes via hemolytic assay according to Lo Cantore et al. (2006).

One drop of 2 μl of pure VOCs and their dilutions, in solvents such as water, DMSO or methanol, depending on their solubility, were placed, equally distanced, on blood agar base medium. After 48 h at 25°C, the minimal hemolytic quantity (MHQ), expressed in milligrams, which causes an evident hemolytic spot in correspondence of the application point was recorded.

The assay was performed three times with three replications.

Microsopic Observations and Ultrastructural Studies

In order to evaluate bacterial volatiles action on fungal cell structures, samples of mycelium were collected from the tip of growing mycelium after 5 days of exposure to bacterial volatiles as well as to pure VOCs. Mycelium was analyzed by light microscope (Zeiss Axioskop 40, Carl Zeiss Microscopy, Thornwood, NY, United States), at a resolution 100x. The images

were captured with a digital camera Olympus C-7070 Imaging software by Delta System IAS2000.

For thin sectioning, mycelium fragments were excised from the tip of growing mycelium and processed for transmission electron microscopy (TEM) analysis according to embedding standard procedures (Martelli and Russo, 1984). Briefly, mycelium was fixed in 4% glutaraldehyde in 0.05 M potassium phosphate buffer (pH 7.2) for 2 h and then it was post-fixed at 4°C in 1% osmium tetroxide in the same buffer for 2 h. Overnight bulk staining in 0.5% aqueous uranyl acetate, dehydration in graded ethanol dilutions, and embedding in TAAB Spurr resin followed. Thin sections were stained with lead citrate before observations with a Philips Morgagni 282 D (FEI Company, Hillsboro, OR) transmission electron microscope at 60 KV accelerating voltage.

Results

Bacterial Production of Bioactive Substances
Diffusible Substances

In dual culture assays on KBA rhizobacterial diffusible substances resulted to inhibit, albeit with different effectiveness, almost all the phytopathogenic fungi under study (**Table 2**). In general, the *Pseudomonas* spp. isolates USB2101 and USB2104 resulted more active in the fungi growth inhibition, whereas the isolate USB2103 of *Bacillus* spp. was the less effective (**Table 2**). Moreover, the strains USB-F1131 of *Botrytis cinerea*, USB-F876, and USB-F1168 of *Phytophthora cactorum* and USB-F587 of

TABLE 2 | Growth inhibition, determined in dual plate assays, of phytopathogenic fungi exposed to diffusible substances produced by six bacteria isolated from bean rhizosphere.

Phytopathogenic fungal strains[a]	Treatments[b,c]						
	Control	USB2101	USB2102	USB2103	USB2104	USB2105	USB2106
Botrytis cinerea USB-F1131	1.6 ± 0.04	0*	1.1 ± 0.03*	1.5 ± 0.03	0*	0.8 ± 0.05*	1.1 ± 0.03*
B. cinerea USB-F1636	1.6 ± 0.06	1.1 ± 0.03*	1.1 ± 0.03*	1.5 ± 0.02	0.8 ± 0.06*	1.3 ± 0.03*	1.3 ± 0.04*
Fusarum equiseti USB-F2014	2.8 ± 0.03	2.0 ± 0.06*	2.6 ± 0.03**	2.6 ± 0.02	2.7 ± 0.03	2.7 ± 0.05	2.7 ± 0.04
F. oxysporum USB-F1	2.8 ± 0.04	2.1 ± 0.03*	2.3 ± 0.04*	2.6 ± 0.04**	2.1 ± 0.04*	2.4 ± 0.04*	2.3 ± 0.03*
F. oxysporum USB-F111	5.8 ± 0.04	2.7 ± 0.03*	3.4 ± 0.05*	5.6 ± 0.04**	4.7 ± 0.06*	4.4 ± 0.04*	4.7 ± 0.05*
F. solani USB-F607	5.6 ± 0.03	3.4 ± 0.05*	3.2 ± 0.02*	4.6 ± 0.05*	3.3 ± 0.06*	4.5 ± 0.07*	4.6 ± 0.06*
Macrophomina phaseolina USB-F918	5.9 ± 0.04	3.1 ± 0.03*	3.3 ± 0.04*	5.8 ± 0.03	3.4 ± 0.04*	4.2 ± 0.04*	4.3 ± 0.04*
Phytophthora cactorum USB-F876	1.7 ± 0.02	0.6 ± 0.06*	1.1 ± 0.03*	1.8 ± 0.05	1.1 ± 0.03*	1.3 ± 0.04*	1.3 ± 0.05*
P. cactorum USB-F1168	1.8 ± 0.03	0.5 ± 0.03*	1.1 ± 0.03*	1.8 ± 0.05	1.1 ± 0.04*	1.3 ± 0.05*	1.3 ± 0.06*
P. nicotianae USB-F172	2.2 ± 0.07	2.1 ± 0.04	1.5 ± 0.02*	2.0 ± 0.04**	2.0 ± 0.03	2.0 ± 0.03	2.1 ± 0.05
Pythium ultimum USB-F2	6.0 ± 0.05	5.1 ± 0.03*	5.3 ± 0.03*	5.5 ± 0.05*	5.3 ± 0.04*	5.3 ± 0.07*	5.3 ± 0.05*
Rhizoctonia solani USB-F3	6.5 ± 0.05	5.6 ± 0.05*	5.6 ± 0.03*	6.6 ± 0.06	5.5 ± 0.05*	6.5 ± 0.04	6.4 ± 0.06
Rosellinia necatrix USB-F587	5.5 ± 0.04	3.1 ± 0.03*	1.7 ± 0.05*	5.4 ± 0.03	3.1 ± 0.04*	5.3 ± 0.03	4.5 ± 0.05*
Sclerotinia sclerotiorum USB-F593	5.6 ± 0.04	2.7 ± 0.07*	3.4 ± 0.05*	5.5 ± 0.06	3.4 ± 0.05*	4.8 ± 0.03*	3.4 ± 0.03*
S. sclerotiorum USB-F853	3.6 ± 0.04	1.6 ± 0.05*	1.6 ± 0.06*	3.6 ± 0.03	1.6 ± 0.04*	3.5 ± 0.05	1.6 ± 0.03*
Verticillium dahliae USB-F464	1.7 ± 0.02	1.2 ± 0.03*	1.5 ± 0.03*	1.6 ± 0.03*	1.1 ± 0.03*	1.5 ± 0.04*	1.6 ± 0.03*
V. dahliae ITM-1910	1.7 ± 0.03	1.1 ± 0.03*	1.5 ± 0.03**	1.6 ± 0.03	1.0 ± 0.02*	1.3 ± 0.03*	1.4 ± 0.03*

[a]*Microorganisms were grown on KBA (King et al., 1954).*

[b]*Results are shown in centimeters ± standard error of mycelium growth.*

[c]*Control, fungus inoculated without bacteria; bacterial isolates attributable, on the basis of the partial 16SrDNA sequencing, to Pseudomonas brassicacearum (USB2101, USBB2102, and USB2104); Bacillus megaterium (USB2103) and Pseudomonas putida (USB2105 and USB2106).*

$*P \leq 0.001$. $**0.001 < P < 0.05$.

Rosellinia necatrix were the most sensitive fungi to bacteria diffusible metabolites action; on the contrary, strain USB-F2014 of *Fusarium equiseti* was the less sensitive to the above mentioned substances (**Table 2**). A similar antifungal activity, though reduced, was observed when bacteria were grown on MMA (data not shown). On this latter medium only the isolate USB2103 *Bacillus* spp. showed an higher activity when compared to its own action on KBA. In contrast, bacteria grown on PDA presented a very low or completely lacked inhibitory activity (data not shown).

Volatile Compounds

In vitro assays have shown that bacterial volatiles are able to inhibit fungal growth as well. In general, volatiles of all rhizobacteria highly inhibited the growth of almost all the target fungi used in this study when grown on KBA (**Table 3**). In particular, all the isolates, apart from USB2103 of *Bacillus* spp., showed strong fungal growth inhibition activity via volatiles (**Table 3**). The strains USB-F1131 and USB-F1636 of *B. cinerea*, USB-F172 of *P. nicotianae*, USB-F3 of *Rhizoctonia solani*, USB-F593 of *S. sclerotiorum* and ITM-1910 of *Verticillium dahliae* were the most inhibited fungi by bacterial volatiles. On the other side, the strains USB-F111 and USB-F1 of *F. oxysporum* and the strain USB-F918 of *Macrophomina phaseolina* resulted the less inhibited in their growth (**Table 3**), even though bacterial volatiles determined a loss of pigmentation of the three pathogenic fungi mycelia (data not shown). Among the most inhibited fungi the strain USB-F593 of *S. sclerotiorum* was

selected for further studies to check bacterial volatiles toxic effect on fungal mycelium and sclerotia. However, the inhibition of fungal growth by bacterial volatiles on the strain USB-F593 of *S. sclerotiorum* resulted apparently fungistatic. Indeed, when plugs of mycelium were removed from PDA plates exposed to bacterial volatiles for 5 days, and placed on fresh PDA, the fungus was able seemingly to grow at the same development rate of the control plug, although the growth showed, by eye, a thinning of the mycelium compared to the fluffy mycelium of the control (**Table 4**).

Sclerotia Viability Assays

Bacterial volatiles caused the total lack of germination of sclerotia in the treatments with the *Pseudomonas* spp. isolates USB2101, USB2102, and USB2104; on the contrary, mycelium growth from sclerotia was reduced of about 80% by volatiles of the *Pseudomonas* spp. isolates USB2105 and USB2106 and about 40% by the isolate USB2103 of *Bacillus* spp. (**Table 4**). Moreover, after treatments with rhizobacteria volatiles, when re-inoculated on fresh PDA, sclerotia germinated forming mycelium at the same radial growth rate than the control (**Table 4**), even though thinner and not fluffy as the control, confirming the apparent fungistatic nature of bacterial volatiles.

GC-MS Analysis

The results of the qualitative GC-MS analysis of the VOCs produced by rhizobacteria after 5 days incubation are listed in **Table 5**. Time course GC-MS analysis revealed that bacteria

TABLE 3 | Growth inhibition of phytopathogenic fungi exposed to bean rhizobacteria volatiles assessed by double plate technique.

Phytopathogenic fungal strains[a]	Treatments[a,b,c]						
	Control	USB2101	USB2102	USB2103	USB2104	USB2105	USB2106
Botrytis cinerea USB-F1131	1.6 ± 0.03	0.1 ± 0.02*	0.1 ± 0.03*	1.5 ± 0.02	0.1 ± 0.03*	0.1 ± 0.02*	0.1 ± 0.03*
B. cinerea USB-F1636	1.6 ± 0.06	0.1 ± 0.02*	0.1 ± 0.03*	0.9 ± 0.05*	0.1 ± 0.03*	0.1 ± 0.03*	0.1 ± 0.02*
Fusarum equiseti USB-F2014	2.7 ± 0.02	1.1 ± 0.03*	0.9 ± 0.02*	2.7 ± 0.05	1.2 ± 0.04*	0.9 ± 0.03*	0.8 ± 0.05*
F. oxysporum USB-F1	2.8 ± 0.03	2.8 ± 0.03	2.8 ± 0.02	2.9 ± 0.03	2.8 ± 0.02	1.7 ± 0.03*	2.0 ± 0.03*
F. oxysporum USB-F111	5.8 ± 0.05	5.0 ± 0.03*	4.9 ± 0.04*	5.8 ± 0.03	5.0 ± 0.03*	4.9 ± 0.05*	4.9 ± 0.03*
F. solani USB-F607	5.6 ± 0.03	3.9 ± 0.02*	3.3 ± 0.03*	4.7 ± 0.02*	3.6 ± 0.03*	3.9 ± 0.03*	3.9 ± 0.05*
Macrophomina phaseolina USB-F918	5.9 ± 0.05	4.2 ± 0.05*	5.9 ± 0.04	6.0 ± 0.03	5.0 ± 0.03*	5.0 ± 0.05*	5.1 ± 0.05*
Phytophthora cactorum USB-F876	1.8 ± 0.03	1.8 ± 0.02	0.6 ± 0.04*	1.8 ± 0.03	1.2 ± 0.02*	1.2 ± 0.05*	1.2 ± 0.03*
P. cactorum USB-F1168	2.0 ± 0.02	2.0 ± 0.02	1.2 ± 0.03*	1.9 ± 0.02	1.1 ± 0.05*	1.1 ± 0.03*	1.1 ± 0.04*
P. nicotianae USB-F172	2.2 ± 0.07	0.2 ± 0.02*	0.3 ± 0.04*	2.1 ± 0.04	0.3 ± 0.02*	0.3 ± 0.03*	0.2 ± 0.03*
Pythium ultimum USB-F2	5.9 ± 0.06	4.3 ± 0.05*	4.9 ± 0.03*	5.0 ± 0.05*	3.5 ± 0.03*	0*	0*
Rhizoctonia solani USB-F3	6.5 ± 0.05	0.5 ± 0.03*	0.5 ± 0.04*	3.5 ± 0.05*	0.3 ± 0.05*	0.5 ± 0.02*	3.5 ± 0.05*
Rosellinia necatrix USB-F587	5.4 ± 0.03	4.3 ± 0.11*	0.7 ± 0.03*	5.4 ± 0.04	2.2 ± 0.03*	0.7 ± 0.05*	3.5 ± 0.03*
Sclerotinia sclerotiorum USB-F593	5.6 ± 0.05	0*	0*	4.4 ± 0.03*	0*	0.6 ± 0.05*	0.5 ± 0.03*
S. sclerotiorum USB-F853	3.6 ± 0.04	0*	0*	3.6 ± 0.05	0*	2.8 ± 0.03*	2.3 ± 0.05*
Verticillium dahliae USB-F464	1.8 ± 0.03	1.9 ± 0.04	0.4 ± 0.03*	1.4 ± 0.03*	0.9 ± 0.03*	1.4 ± 0.05*	0.4 ± 0.03*
V. dahliae ITM-1910	1.7 ± 0.03	0*	0*	0.9 ± 0.02*	0*	0*	1.0 ± 0.02*

[a]*Bacteria were grown on KBA (*King et al., 1954*), while fungi on PDA.*

[b]*Results are shown in centimeters ± standard error of mycelium growth.*

[c]*Control, fungus inoculated without bacteria; bacterial isolates attributable, on the basis of the partial 16SrDNA sequencing, to Pseudomonas brassicacearum (USB2101, USBB2102, and USB2104); Bacillus megaterium (USB2103) and Pseudomonas putida (USB2105 and USB2106).*

*P ≤ 0.001.

TABLE 4 | Mycelia growth of *Sclerotinia sclerotiorum* USB-F593 from fungal plug and sclerotia after 5 days exposure to rhizobacteria volatiles and from fungal plug and sclerotia re-inoculated on fresh PDA.

Treatments[a]	Mycelium diameters (cm)[b]			
	VOCs assay on mycelium	Viability assay of mycelium	VOCs assay on sclerotia	Viability assay of sclerotia
Control	5.6 ± 0.05	5.8 ± 0.08	3.2 ± 0.08	3.5 ± 0.06
USB2101	0*	5.7 ± 0.08	0*	3.4 ± 0.04
USB2102	0*	5.8 ± 0.07	0*	3.4 ± 0.06
USB2103	4.4 ± 0.03*	5.9 ± 0.04	2.6 ± 0.06*	3.7 ± 0.06
USB2104	0*	5.9 ± 0.05	0*	3.4 ± 0.05
USB2105	0.6 ± 0.05*	5.7 ± 0.03	0.3 ± 0.03*	3.4 ± 0.06
USB2106	0.5 ± 0.03*	5.7 ± 0.05	0.2 ± 0.03*	3.5 ± 0.05

[a]*Control = fungus inoculated without bacteria; bacterial isolates attributable, on the basis of the partial 16SrDNA sequencing, to Pseudomonas brassicacearum (USB2101, USBB2102, and USB2104); Bacillus megaterium (USB2103) and Pseudomonas putida (USB2105 and USB2106).*
[b]*Results are shown in centimeters ± standard error of mycelium growth.*
*P ≤ 0.001.

under study produce VOCs among which several ones were found in every time points, while other VOCs were detected in a specific time point. In other words, bacteria produce a typical volatiles blend depending on their growth stage. VOCs identified at the three time point considered are listed in **Table 6**.

Among the VOCs identified, 1-undecene, 2-nonanone, 2-undecanone, 2-propanone, 1-tetradecanol, acetic acid, m-cymene, dl-limonene, dimethyl disulfide, and dimethyl trisulfide were selected in order to assess their single biological activity. The selection was based on their detection at the 5th day of bacterial incubation, on their systematic detection in all the three times that the analysis was performed, on the concentration of the components evaluated as peak area and their availability on the market.

Effects of Pure VOCs on *Sclerotinia sclerotiorum* Mycelium and Sclerotia

The results from assays performed using pure VOCs toward strain USB-F593 of *S. sclerotiorum* are showed in **Table 7**.

The acetic acid and 2-nonanone, with MIQs of 4.19 and 4.92 mg, respectively, resulted the most active compounds in reducing mycelium growth arising from the fungal plug (**Table 7**). On the contrary, 2-propanone, 1-tetradecanol, and 1-undecene appeared to be completely inactive in inhibiting fungal growth at all the quantities applied (**Table 7**). Higher MIQ with values comprised between 13.77 and 31.38 mg were observed for the other VOCs (**Table 7**).

A similar effects trend was observed in the sclerotia germination assay, though the MIQs were higher than those observed for the mycelium growth. An exception was observed for dimethyl trisulfide whose MIQ resulted similar either on mycelium growth or sclerotia germination (**Table 7**).

Hemolytic Activity of Pure VOCs

All the pure VOCs used in this work, apart from 1-tetradecanol, were able to lyse red blood cells (**Table 7**). Dimethyl disulfide and acetic acid resulted the most active since they showed MHQs of 0.065 and 0.066 mg, respectively (**Table 7**). On the contrary,

TABLE 5 | Volatile organic compounds (VOCs) produced by rhizobacteria and detected by GC-MS analysis after 5 days incubation.

VOCs[a]	Producers[b]
acetic acid	USB2104
m-cymene	USB2104
dimethyl disulfide	USB2105, USB2106
dimethyl trisulfide	USB2105, USB2106
dl-limonene	USB2104
2-nonanone	USB2101, USBB2102, USB2104, USB2105
2-propanone	USB2103
1-tetradecanol	USB2103
2-undecanone	USB2101, USBB2102, USB2104, USB2105
1-undecene	USB2101, USBB2102, USB2104, USB2105

[a]*VOCs identified with a score > 80%.*
[b]*Bacterial isolates attributable, on the basis of the partial 16SrDNA sequencing, to Pseudomonas brassicacearum (USB2101, USBB2102, and USB2104); Bacillus megaterium (USB2103) and Pseudomonas putida (USB2105 and USB2106).*

1-undecene and 2-propanone showed hemolytic activity with MHQ of 75.1 and 79 mg, respectively (**Table 7**).

Microsopic Observations and Ultrastructural Studies

Observations at light microscope of the strain USB-F593 of *S. sclerotiorum* mycelium, exposed to bacterial volatiles of the six rhizobacteria, showed hyphae morphological abnormalities compared to the control treatment. In fact, in all the treatments, hyphae, observed by optical light microscope, appeared thinner and characterized by the presence of vacuolization of cytoplasm when compared to the control (**Figure 1**). Further observations at TEM of the mycelium treated with bacterial volatiles showed the alterations of the hyphae ultra-structures confirming the hyphae morphology alteration observed by light microscopy. In particular, thin sections of the *S. sclerotiorum* mycelium not exposed to bacterial volatiles, observed at TEM, revealed characteristic ascomycete hyphae ultra-structures. Indeed,

TABLE 6 | Volatile organic compounds (VOCs) time-course profiles produced by selected rhizobacteria isolated from common bean as determined by GC-MS analysis.

VOCs	Bacterial isolates[a,b]								
	USB2103			USB2104			USB2105		
	1	3	5	1	3	5	1	3	5
acetic acid						x			
2-bromo-dodecane		x			x		x		
2-bromo-tetradecane	x								
4-butyl-cicloexene								x	
m-cymene						x			
3-decen-1-ol acetate				x	x				
3,6- dimethyl-decane	x			x	x			x	
dimethyl disulfide							x	x	x
dimethyl trisulfide									x
4,7-dimethyl-undecane		x			x				
dl-limonene						x			
eicosane		x					x		
2-ethyl-2-methyl-tridecanol		x							
5-ethyl-2-methyl-octane	x								
heneicosane	x	x		x	x		x	x	
hentriacontane				x					
heptacosane	x	x		x	x		x	x	
heptadecane	x				x		x		
hexacosane	x							x	
hexadecane	x							x	
1-iodo-dodecane					x				
1-iodo-octadecane	x						x		
1-iodo-tridecane								x	
methanethiol							X		
2-methyl-heptadecane	x						x		
8-methyl-heptadecane	x						x	x	
4-methyl-tetradecane	x				x				
2-nonanone						x		x	x
octacosane	x	x		x	x		x	x	
octadecane				x					
pentacosane	X			x			x		
pentadecane					x			x	
2-pentadecanone					x				
2-propanone			x						
1-tetradecanol			x						
triacontane				x			x		
2-tridecanone					x			x	
2,3,7-trimethyl-decane					x				
2,6,11-trimethyl-dodecane					x			x	
2-undecanone				x	x	x		x	x
1-undecene				x	x	x	x	x	x

[a]Bacterial isolates attributable, on the basis of the partial 16SrDNA sequencing, to *Bacillus megaterium* (USB2103); *Pseudomonas brassicacearum* (USB2104) and *Pseudomonas putida* (USB2105).
[b]Time points considered for the analysis: 1, 3, and 5 days of bacteria incubation.

TABLE 7 | Antifungal activity toward *Sclerotinia sclerotiorum* strain USB-F593 mycelium and sclerotia, and hemolytic activity of representative volatile organic compounds produced by the six rhizobacteria isolated from common bean.

VOCs	MIQ on mycelium	MIQ on sclerotia	MHQ
acetic acid	4.19	9.44	0.066
m-cymene	13.77	17.22	0.215
dimethyl disulfide	31.38	73.22	0.065
dimethyl trisulfide	24.04	24.04	0.075
dl-limonene	17.20	30.10	0.215
2-nonanone	4.92	16.4	0.102
2-propanone	NA	NA	79.1
1-tetradecanol	NA	NA	NA
2-undecanone	14.85	16.5	0.206
1-undecene	NA	NA	75.1

MIQ, Minimal Inhibitory Quantity, expressed in milligrams, able to reduce mycelium growth and sclerotia germination. MHQ, Minimal Hemolytic Quantity, expressed in milligrams, able to lyse red blood cells. MIQ and MHQ were calculated considering the applied volumes and the densities of each compound. The values reported stand for the average of the values from three experiments. NA, No detected activity applying 100 μl of pure compounds.

a typical septum showing a single hole, normo-functional cytoplasm, good adhesion between the cytoplasmic membrane and the outer wall, various organelles, distinct, and normal in their constituent elements, normal size vacuoles, regularly shaped mitochondria and portions of rough endoplasmic reticulum were observed (**Figures 2A,B**). In the samples exposed to volatiles of the *Pseudomonas* spp. isolate USB2104 some hyphae showed very condensed cytoplasm, others were empty, in some other cases hyphae appeared duplicated and were apparently included in wall-like structures. It was also noticed cytoplasmic membrane detachment from the cell wall, numerous and hyper-crested mitochondria, multi-vesiculation, cytoplasmic accumulations of material (protein or lipid), hypertrophy of the endoplasmic reticulum (**Figures 2C,D**). Volatiles of *Pseudomonas* spp. isolate USB2105 caused the thickening of the hyphae cell wall and hyphae showed very condensed and granulated cytoplasm. Furthermore, hyphae presented numerous mitochondria with iper-vesiculated and hypertrophic ridges and multi-vesiculation and cytoplasmic accumulation of material (protein or lipid) (**Figures 2E,F**). The exposure to volatiles of *Bacillus* spp. isolate USB2103 led to cytoplasm thickening, to the partial detachment of outer nuclear membrane, to hypo-crested mitochondria with denser matrix as well as accumulation of lipids and proteins in the cytoplasm and into the endoplasmic reticulum which also showed hyperplasia (**Figures 2G,H**).

TEM observation of the samples treated with the pure VOCs 2-nonanone, dl-limonene and dimethyl disulfide applied at their MIQ confirmed their involvement in the alteration of hyphae ultrastructures noticed in the samples exposed to the natural blend of bacterial volatiles. In particular, 2-nonanone caused complete or partial hyphae emptying due to the damage of cytoplasmic membrane which, in fact, resulted detached from the outer wall; strong vacuolization with internal residues of membranes and cytoplasmic matrix in the cytoplasm were also noticed (**Figures 3A,B**). dl-Limonene treatment led to granulation of hyphae cytoplasm, cytoplasmic membrane detachment from the cell wall that resulted thickened; it was also noticed absence of organelles, multi-vesciculation
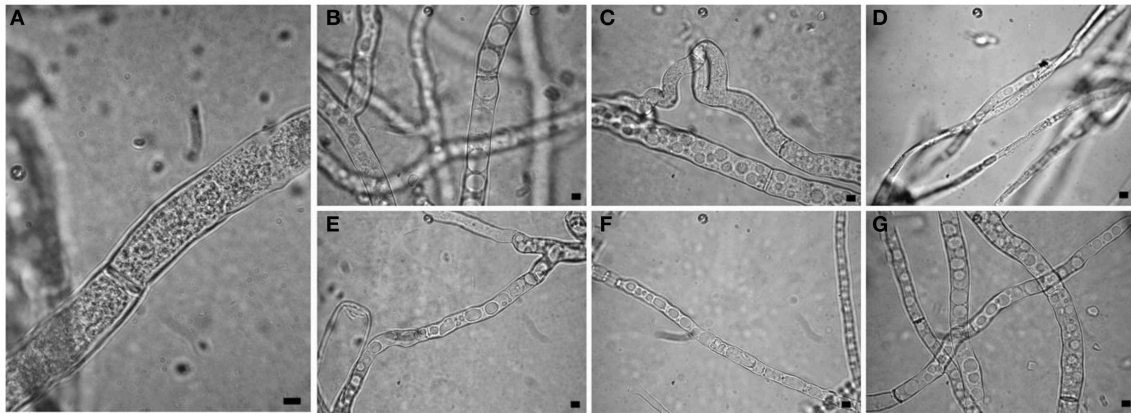
FIGURE 1 | Light observations of *Sclerotinia sclerotiorum* hyphae exposed to bacterial volatiles. (A) control; **(B,C,E–G)** strains USB2101, USB2102, USB2104, USB2105, and USB2106 of *Pseudomonas* spp.; **(D)** strain USB2103 of *Bacillus* spp.. Bars = 2 μm.

and accumulations of proteic and lipidic material in the cytoplasm (**Figures 3C,D**). Lastly, dimethyl disulfide treated samples showed strongly marked ultrastructural modification: hyphae mostly appeared with missing or altered cytoplasm, hyper-vesiculation, hypocrested and vesiculated mitochondria which resulted fewer than the control, and accumulations of proteic and lipidic material in the cytoplasm (**Figures 3E,F**). Nevertheless, in the all TEM treated samples apparently normal hyphae were also observed, even though very few compared to the damaged ones.

Discussion

Recent our studies showed the potential ability of six rhizobacteria, isolated from bean plants, to control *in vitro* and greenhouse CBB and to have several typical important phenotypic traits of biocontrol agents (Giorgio et al., 2015) (Supplementary Table 1). In this study the above rhizobacteria were evaluated for their capability to inhibit *in vitro* the growth of several phytopathogenic fungi, mostly soil-borne pathogens, for their possible use as antagonists in fungal disease protection of bean as well as other horticulture crops. The six rhizobacteria strongly affected fungal growth, though a different sensitivity among the phytopathogenic fungi was observed, not only via direct diffusible substances but also via volatiles-mediated action. In this work particular emphasis has been given to bacterial volatiles effects since they may play a pivotal role in the interaction between organisms living the same ecological niche (Popova et al., 2014). Among the phytopathogenic soil borne fungi screened in this study, the strain USB-F593 of *S. sclerotiorum* isolated from bean, resulted mostly affected by bacterial volatiles and for that selected for further studies. Furthermore, of interest is the fact that in the volatiles assays a loss of pigmentation in *F. oxysporum* and *M. phaseolina* strains was also noticed. The alteration observed could play an important significance not only for metabolic aspects of the fungi but also for some features related to the pathogen virulence/pathogenicity as well as to the reduction/loss of its

antimicrobial weapon arsenal. Indeed, the observed loss of melanin by *M. phaseolina* may negatively influence survival, pathogenicity, and recovery functions of the pathogen from radiation and oxidizing agents (Dhingra and Sinclair, 1978; El Bassam et al., 2002; Cao et al., 2006). Similarly, pinkish-purple naphthoquinones produced by *F. oxysporum* were demonstrated to have antimicrobial activity (Visconti et al., 1983; Baker et al., 1990; Medentsev and Akimenko, 1998).

Observations with light microscope of *S. sclerotiorum* mycelium, exposed to the six rhizobacteria volatile blends, revealed thinner hyphae characterized by the presence of vacuolization in the cytoplasm, compared to control, indicating cytoplasmic membrane as a possible target of the volatile mixtures. TEM observations of *S. sclerotiorum* mycelium exposed to three out the six rhizobacteria (namely USB2103, USB2104, USB2105) confirmed the hyphae ultrastructural alterations due to the loss of cell membranes integrity and the consequent cell permeability alteration, as confirmed by the hemolytic activity shown by most of the pure VOCs. Furthermore, the alteration of mitochondrial membranes confirmed the membrane system as one of volatiles target. To our knowledge, this is the first work in which the individual activity of bacterial VOCs on phyto-pathogenic fungi is investigated at the ultrastructural level. Moreover, in the case of *Pseudomonas* spp. isolate USB2105 volatiles exposure a proliferation of mitochondria or mitochondria cristae hypertrophy was noticed indicating possible increased respiratory requirements as a result of bacterial volatiles toxic action.

GC-MS analysis of bacterial volatiles, after 5 days incubation, showed some similarity of the volatile profiles of the five isolates of *Pseudomonas* spp. under study; indeed four out the five isolates (USB2101, USB2102, USB2105, USB2106) produced 1-undecene, 2-nonanone, and 2-undecanone. However, the *Pseudomonas* spp. isolate USB2104 produced in addition acetic acid, m-cymene and dl-limonene. 1-Undecene is a terminal olefin demonstrated to have strong toxic effects on *Drosophila melanogaster* and the nematode *Caernorhabditis elegans* but no effect on bacteria or pathogenic fungi growth has been reported (Popova et al.,
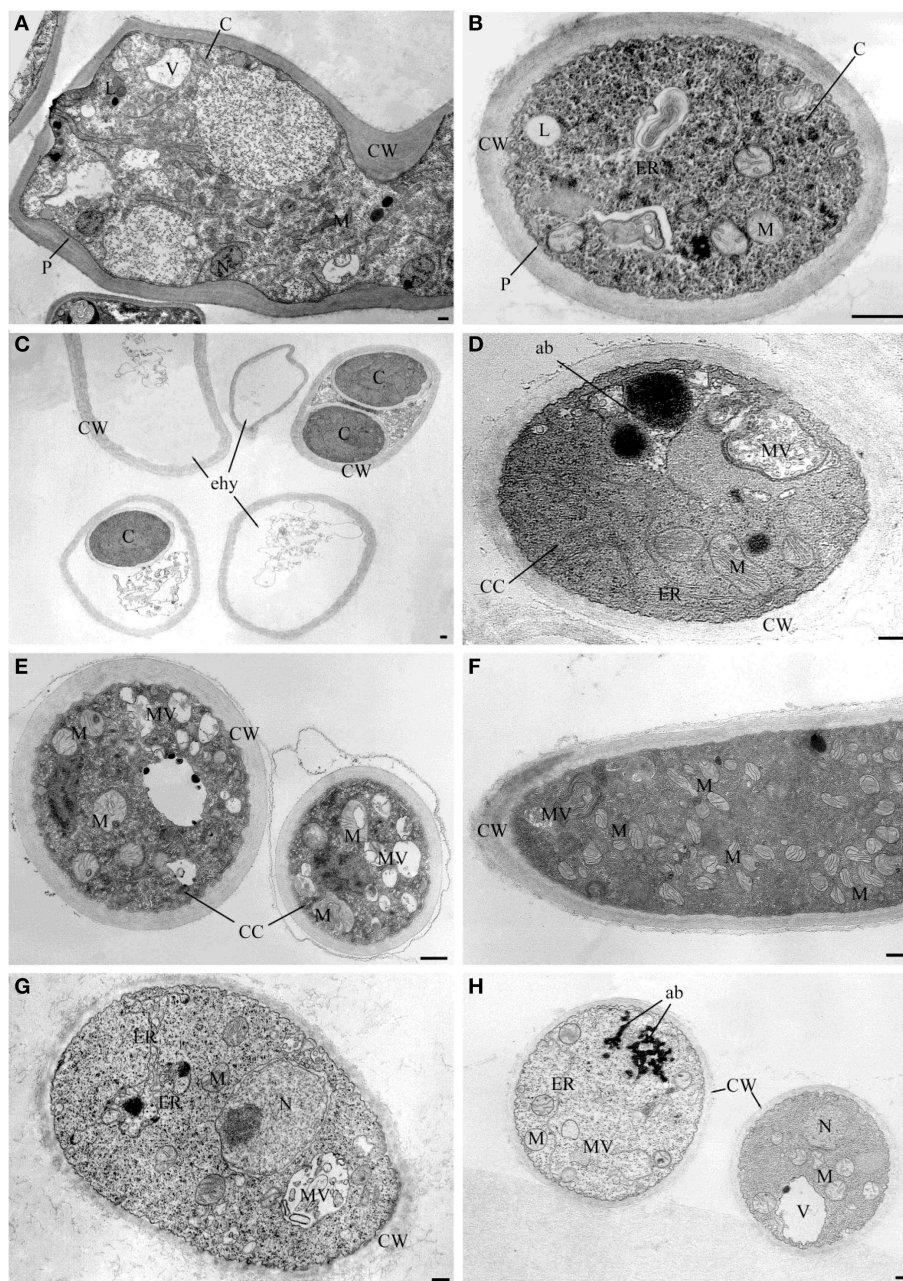
FIGURE 2 | TEM observations of *Sclerotinia sclerotiorum* hyphae exposed to bacterial volatiles. (A,B) TEM micrographs showing hyphae ultrastructures of the strain USB-F593 *S. sclerotiorum* mycelium not exposed to bacterial volatiles. TEM micrographs showing ultrastructural changes of *S. sclerotiorum* USB-F593 mycelium exposed to volatiles of the *Pseudomonas* spp. USB2104 **(C,D)**, USB2105 **(E,F)** and *Bacillus* spp. USB2103 **(G,H)**. ab, accumulation body; C, cytoplasm; CC, condensed cytoplasm; CW, cell wall; ER, endoplasmic reticulum; ehy, empty hyphae; M, mitochondria; MV, multivesicular; N, nucleus; P, plasmalemma. Bars: 250 nm.

2014). In fact, even in this study, this substance showed no apparent effect on fungal growth. On the contrary, the methyl ketones 2-nonanone and 2-undecanone, coming from the same metabolic pathway (Park et al., 2012), showed strong fungal growth inhibition. Methyl ketones, commonly found in nature as constituent of essential oils of several plants and produced by bacteria, fungi, insects (Cavill et al., 1956; Cavill and Hinterberger, 1960; Bernardi et al., 1967; Moser et al., 1968), and

mammalian cells (Forney and Markovetz, 1971), present a variety of biological properties and potential commercial exploitations (Forney and Markovetz, 1971; Bolster et al., 2001; Antonious et al., 2003; Guo et al., 2008; Shi et al., 2008; Zhu and Hua, 2009). For example, 2-nonanone, released by red raspberries and strawberries during ripening, was demonstrated to inhibit postharvest decay fungi in strawberry fruit (Vaughn et al., 1993) and both 2-nonanone and 2-undecanone from soil bacteria
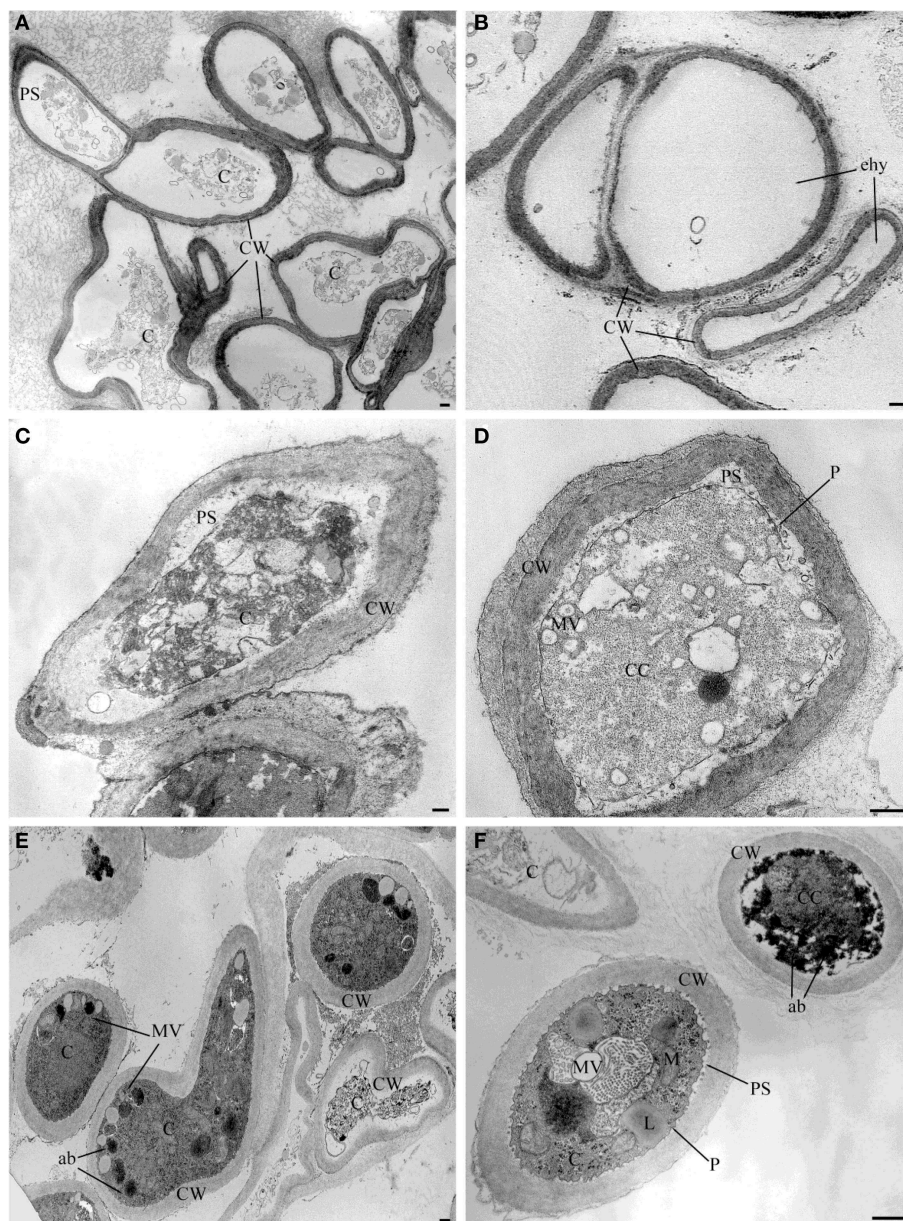
FIGURE 3 | TEM observations of *Sclerotinia sclerotiorum* hyphae exposed to pure VOCs. Ultrastructural changes of the strain USB-F593 of *S. sclerotiorum* mycelium treated with pure VOCs 2-nonanone **(A,B)**, dl-limonene **(C,D)** and dimethyl disulfide **(E,F)**. ab, accumulation body; C, cytoplasm; CC, condensed cytoplasm; CW, cell wall; ehy, empty hyphae; MV, multivesicular; P, plasmalemma: PS, periplasmic space. Bars: 250 nm.

have nematicidal action (Gu et al., 2007). The activity of such molecules is related to their ability to cross lipid layers of cell membranes in relation to their lipophilicity which depends on the acyl chain length appearing optimal when is 9–14 carbons long (Dimock et al., 1982). In our hemolytic experiments pure VOCs 2-nonanone and 2-undecanone demonstrated to lyse red blood cells confirming their action on biological membranes; ultrastructural hyphae alterations at the membranes level was confirmed in TEM observations of mycelium treated with 2-nonanone. Furthermore, the observed fungal cell wall darkening, probably due to chitin condensation, suggests that this ketone

may alter chitin chemical structure possibly via nucleophilic addition between its carbonyl group and the acetyl amine groups of fungal chitin (Carey, 2003). Acetic acid, m-cymene and dl-limonene inhibited fungal growth and showed hemolytic activity as well stating their action on cell membranes. Acetic acid is a natural compound found throughout the biosphere and has long been known for its flavoring and preservative properties against microorganisms in a variety of food products (Davidson et al., 2002; Alawlaqi and Alharbi, 2014). Its anti-microbial activity is exerted by the capacity to penetrate the microbial cell interfering with transport of metabolites and maintenance

of potential at membrane level (Banwart, 1981). m-Cymene is a benzene derivative terpene, constituent of a number of essential oils such as cumin and thyme, whose antimicrobial properties were noticed for the first time by Prasad et al. (2010). The other terpene detected, dl-limonene, is more common in nature as the main constituent of citrus essential oil and it has antifungal (Chee et al., 2009) but limited antibacterial activities (Lo Cantore et al., 2009) and extremely wide industrial applications (Pakdel et al., 1991; Dambolena et al., 2008). In the present work TEM observations showed visible ultrastructural damage of *S. sclerotiorum* hyphae apparently due to limonene toxic action (Chee et al., 2009).

The shorter chain methyl ketone 2-propanone, detected in the volatiles natural blend of the isolate USB2103 of *Bacillus* spp., did not apparently affect the fungal growth when tested alone confirming the previously observed low toxicity. Isolate USB2103 resulted to produce 1-tetradecanol as well which did not show fungal growth inhibitory activity; this is not surprising since the compound is generally considered having low toxicity (Wigaeus et al., 1981; Gorsuch et al., 1990; Hernandez, 1999) because of its limited amphipathic properties that lead to low interference with membranes.

The isolates USB2105 and USB2106 of *Pseudomonas* spp. produced, besides the above mentioned volatiles, dimethyl disulfide and dimethyl trisulfide, that exhibited antifungal action on *S. sclerotiorum* mycelium and inhibited sclerotia germination according to literature data (Fernando et al., 2005; Kai et al., 2009). The bacterial VOCs action on sclerotia germination is important since the control and eradication of this pathogen is difficult due to the environmental resistance of these structures that can survive in soil for years without hosts or favorable condition for development (Coley-Smith and Cooke, 1971). It has been suggested that dimethyl disulfide and dimethyl trisulfide play a role in plant protection, acting in the control of plant-pathogenic fungi (Kai et al., 2009), weeds (Freeman et al., 2009), and nematodes (Coosemans, 2005). For the first time here the hemolytic activity of the above substances has been unraveled. TEM observations of dimethyl disulfide treated mycelium revealed a high damage on organelles and the cytoplasmic organization. According to literature data, dimethyl disulfide is a small molecule capable to cross over the cell wall and membranes and it was demonstrated that, in different organisms, it acts as a powerful inhibitor of complex IV of cytochrome oxidase in mitochondria (Dugravot et al., 2003). This malfunction may contribute to explain the decrease of mitochondria number that appeared hypocrested and vesiculated in our TEM observations.

Even though volatiles natural blend of isolate USB2103 of *Bacillus* spp. affected to a lesser extent *S. sclerotiorum* mycelia growth, TEM observations noticed structural damages suggest possible synergy between the various constituents of natural volatile mixture. Moreover, it is not to be excluded that other bioactive volatiles were, for some reasons, not detected in GC-MS analysis condition.

Summarizing, it can be stated that individual VOCs showed, at least for most of them, the involvement in growth suppression of *S. sclerotiorum* and the pathogen membranes are one of their targets, as suggested by their hemolytic activities and membranes alteration, beside other observed ultrastructural changes on cell organelles such as mitochondria and endoplasmic reticulum.

The apparent fungistatic action of VOCs, as suggested by the fact that mycelium plugs of *S. sclerotiorum* radial grow at a rate similar to the control when transferred on fresh PDA, is in apparent contradiction with the observed alterations on hyphae morphology and cell ultrastructures. This can be explained by the fact that in the mycelium masses some hyphae not altered yet and alive, as observed in TEM analysis, are present. This may be due to differences in the hyphae response to the VOCs exposure in relation to hyphae different physiological state. Similar consideration may be done in the case of not germinated sclerotia after exposure to bacterial volatiles. Nevertheless, the VOCs appears good candidates for the control of this soil fungal pathogen as fumigants.

Apart from the inhibitory activity that bacterial VOCs could have to prevent the development of fungal mycelium, some other volatiles can contribute to the observed effects, being an integrative part of the pool of biologically active volatiles produced by the tested bacteria. This is the case of hydrogen cyanide or ammonia produced by the three *Pseudomonas* spp. attributable to *P. brassicacearum* and the *P. putida* likely strains. Although our study was mostly focused on the investigation on the effects of VOCs detected and identified after 5 days incubation, it is not to be excluded that the final effects on fungal structures may be the outcome of the action of all the volatiles produced along the whole incubation period. Indeed, in our GC-MS time course analysis some other VOCs were detected and this finding deserves to be deepened in future studies.

Acknowledgments

Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.01056

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Bioactivity of volatile organic compounds produced by *Pseudomonas tolaasii*

Pietro Lo Cantore*, Annalisa Giorgio and Nicola S. Iacobellis

Scuola di Scienze Agrarie, Forestali, Alimentari e Ambientali, Università degli Studi della Basilicata, Potenza, Italy

Pseudomonas tolaasii is the main bacterial pathogen of several mushroom species. In this paper we report that strains of *P. tolaasii* produce volatile substances inducing *in vitro* mycelia growth inhibition of *Pleurotus ostreatus* and *P. eryngii*, and *Agaricus bisporus* and *P. ostreatus* basidiome tissue blocks brown discoloration. *P. tolaasii* strains produced the volatile ammonia but not hydrogen cyanide. Among the volatiles detected by GC–MS, methanethiol, dimethyl disulfide (DMDS), and 1-undecene were identified. The latter, when assayed individually as pure compounds, led to similar effects noticed when *P. tolaasii* volatiles natural blend was used on mushrooms mycelia and basidiome tissue blocks. Furthermore, the natural volatile mixture resulted toxic toward lettuce and broccoli seedling growth. In contrast, pure volatiles showed different activity according to their nature and/or doses applied. Indeed, methanethiol resulted toxic at all the doses used, while DMDS toxicity was assessed till a quantity of 1.25 µg, below which it caused, together with 1-undecene ($\geq$10 µg), broccoli growth increase.

Keywords: **Pseudomonas tolaasii, mushrooms, broccoli, lettuce, volatile organic compounds, methanethiol, dimethyl disulfide, 1-undecene**

Introduction

Pseudomonas tolaasii (Paine, 1919) is the brown blotch disease's causal agent in several economically important edible mushrooms such as *Agaricus bisporus* (Lange) Imbach (Tolaas, 1915), *A. bitorquis* (Quél.) Sacc. and other *Agaricales* (Gandy, 1979), *Pleurotus ostreatus* (Jacq. : Fr.) Kum (Gill, 1995), *P. eryngii* (D. C. : Fr.) Quél (Ferri, 1985; Rodriguez and Royse, 2007), *Lentinula edodes* (Berkeley) Singer (Tsuneda et al., 1995), and *Flammulina velutipes* (Curtis) Singer (Lee et al., 1996). Brown blotch is considered to be the cultivated mushrooms's main disease because of the important economic losses (Fermor, 1987; Iacobellis, 2011) and the difficulty to control it. Compost and casing soil, required for mushroom cultivation were found as a primary source of *P. tolaasii* (Wong and Preece, 1980).

Pseudomonas tolaasii is also described as a pathogen of some plants, indeed it causes diseases on cauliflowers (*Brassica oleracea* L. var. *botrytis*) (Talame and Piccirillo, 1995), tobacco (*Nicotiana tabacum* L.), *Solanum* sp. (Rainey et al., 1991; Shirata et al., 1995) and strawberry (*Fragaria* × *ananassa* Duchesne) (Tanprasert and Reed, 1997). Moreover, it has been reported as a saprophytic bacterium associated to pear (*Pyrus communis* L.) phylloplane (Noval et al., 1993) and bean (*Phaseolus vulgaris* L.) and sugar beet (*Beta vulgaris* L.) rhizosphere (Zdor and Anderson, 1992).

Pseudomonas tolaasii produces tolaasins, considered the primary factors involved in the virulence and interaction between the pathogen and the hosts (Rainey et al., 1992;

Soler-Rivas et al., 1999; Largeteau and Savoie, 2010; Iacobellis, 2011). The toxic lipodepsipeptides tolaasins (Nutkins et al., 1991) are the only molecules whose role in the virulence of *P. tolaasii* has been unequivocally ascertained (Cutri et al., 1984; Rainey et al., 1992; Grewal et al., 1995; Lo Cantore et al., 2006). In fact, when applied directly on mushrooms, tolaasins can reproduce the symptoms of the disease.

Besides tolaasins, other compounds have been reported to be produced by *P. tolaasii* and considered as potential factors responsible for bacterial botch symptoms. Park et al. (1994) isolated a compound from a *P. tolaasii* strain, characterized as an aminobenzene, containing an amylamine group, able to induce the symptoms of the disease on *A. bisporus* caps. Furthermore, Shirata (1996) described the ability of *P. tolaasii* strains to produce volatile organic compounds (VOCs) called tovsins, different from tolaasins, which are not volatiles, and able to induce the alteration of *P. ostreatus* and *F. velutipes* basidiomes.

Volatile organic compounds are small molecules (molecular masses lower 300 Da) with low polarity and high vapor pressure that are produced by both eukaryotes and prokaryotes. The biological activities and role of many bacterial VOCs, so far identified, are partly known; in fact, they have been demonstrated to act as infochemicals for inter- and intra-organism communication (Effmert et al., 2012), attraction, and defense (Ryu et al., 2004; Farag et al., 2006). They can be detected in small amounts by the organisms and diffused in the atmosphere and soil (Kai et al., 2009; Morath et al., 2012).

The bacterial volatile blends have been proved to interact with plants and fungi by inhibiting or stimulating their growth (Kai et al., 2007; Vespermann et al., 2007; Effmert et al., 2012). To date, many studies have been performed with the aim of identifying bacterial volatile substances, including those produced by *Pseudomonas* sp.. However, the knowledge on their biological activities is still limited. In fact, only in recent years the inhibition and/or stimulation of fungal and plant growth by carbon dioxide (CO_2), hydrogen cyanide (HCN), ammonia (NH_3), 2,3-butanediol, acetoin, dimethyl disulfide (DMDS), and 1-undecene, has been reported (Howell et al., 1988; Ryu et al., 2003; Haas and Défago, 2005; Afsharmanesh et al., 2006; Farag et al., 2006; Kai and Piechulla, 2009; Kai et al., 2009, 2010; Blom et al., 2011; Meldau et al., 2013; Weise et al., 2013; Hunziker et al., 2015).

Based on the above consideration, it seemed worthy of interest to confirm the production of volatiles by *P. tolaasii* and establish their identity and biological role on the host mushroom and plants. In this work we report a study on the bioactivity of volatile compounds, produced by three virulent strains of *P. tolaasii*, on *P. eryngii* and *P. ostreatus* mycelia and *A. bisporus* and *P. ostreatus* basidiome tissue blocks. Some of the pure VOCs, identified by GC–MS, were *in vitro* evaluated for their toxicity, respectively, on the above mycelia growth and tissue blocks. Furthermore, since *P. tolaasii* is also a natural rhizosphere inhabitant of some plants, in the present study is also reported the bioactivity of both natural volatile mixture and pure VOCs on lettuce and broccoli seeds germination and seedling growth.

Materials and Methods

Bacterial, Fungal Cultures, and Seeds

Bacterial type strain NCPPB2192 and the strains USB1 (ICMP13791), USB66 (ICMP13792) of *P. tolaasii*, and fungal strains DS256, DS270 of *P. eryngii* and DS226, DS284 of *P. ostreatus*, have been used in this study. Bacterial strains were maintained lyophilised at 4°C and subcultures were obtained on King's B agar medium (KBA; King et al., 1954) for 48 h at 25°C. *P. ostreatus* and *P. eryngii* strains were maintained on Malt Extract Agar (MEA, Oxoid, Milan, Italy) slants at 4°C and subcultures were obtained by growing the fungi on the same medium at 25°C for 72 h.

Broccoli (*Brassica oleracea* L. var. *italica*; variety "Cima di rapa sessantina", Blumen, Milan, Italy) and lettuce (*Lactuca sativa* L.; variety "Lattuga romana lentissima a montare", Blumen, Milan, Italy) seeds were used for the evaluation of bioactivity of bacterial volatiles on plant material.

Mushroom Bioassays

Mycelia of *P. eryngii* and *P. ostreatus* strains, grown as reported above, and tissue blocks of *A. bisporus* and *P. ostreatus*, obtained from freshly harvested basidiomes, prepared according to Ercolani (1970), were used.

The assay was performed inoculating 100 µl of about 10^8 CFU ml^{-1} ($OD_{590} = 0.2$) bacterial suspension of *P. tolaasii* strains on KBA in each of two out of three sectors Petri dishes. In the third sector a *Pleurotus* sp. mycelium plug (6 mm diameter) was placed on 5 ml of MEA. In the case of basidiome tissue assays, one tissue block of *A. bisporus* or *P. ostreatus* was placed onto the plate bottom. After inoculation, Petri dishes were incubated at 25°C for 5 days and then mycelia growth was measured (colony diameter subtracted the mycelium agar plug diameter) and the effects on tissue blocks were visually evaluated.

The assays as above described were performed either sealing Petri dishes with parafilm (Pechiney Plastic Packaging Company, Chicago, IL, USA) to minimize volatile compounds exchange during the incubation period or in non-sealed Petri dishes to simulate what happens in nature in an open system such as the rhizosphere.

Controls were obtained placing mycelium plugs or tissue blocks as above, but no bacterial suspensions were inoculated on KBA in the remaining Petri dishes sectors. All mushroom bioassays were performed twice with three replicates and, in the case of mycelia growth assays, the final data were reported in percentage compared to the control (100%).

Seed Bioassays

Broccoli and lettuce seeds were surface disinfected by treating for 1 min with 0.5% chlorine, washed three times with sterile distilled water and then dried under an air flow cabinet at room temperature for 20 min. One hundred disinfected seeds were transferred on filter paper soaked with 1.5 ml of sterile distilled water in one out of three sectors of Petri dishes. The other two sectors, containing each 5 ml of KBA, were inoculated with 100 µl of about 10^8 CFU ml^{-1} bacterial suspension of *P. tolaasii* strains. Petri dishes containing 100 broccoli or lettuce seeds in

one out three sectors, while in the other two just KBA were used as controls. The assay was performed as above both in parafilm-sealed and non-sealed dishes. After 5 days incubation at 25°C in the dark, broccoli and lettuce seeds germination was assessed and the whole seedlings, epicotyls, main rootlets were measured with a ruler. The seed bioassays were performed at least twice with three replicates and the final growth data were reported as percentage compared to the control growth (100%).

GC–MS Analysis

The three strains of *P. tolaasii,* plus *P. eryngii* strain DS256 and *P. ostreatus* strain DS284, were analyzed for their volatile compounds profile after a 5 days incubation period both in sealed and non-sealed conditions. The bacterial volatile analysis was carried out in Petri plates, prepared as reported in the previous paragraph, but without fungal inoculum or seeds, respectively, while the fungal volatile analysis was carried out in Petri plates prepared as reported in the mushroom bioassays, but without bacterial inoculum. Petri dishes with non-inoculated KBA and MEA were used as negative control.

Volatiles produced by bacteria and fungi were collected with SPME fiber, coated with 100 μm of polydimethylsiloxane (PDMS) phase (Supelco 57300-U, mounted on a Supelco 57330 support). The fiber was introduced in the headspace of Petri dishes through a hole previously obtained by piercing the parafilm layers, and kept inside the plate for 20 min. The fiber was then introduced into the injection port of a HP6890 plus gas-chromatograph equipped with a Phenomenex Zebron ZB-5 MS capillary column (30 m × 0.25 mm ID × 0.25 μm film thickness). A HP5973 mass selective detector (mass range: 15–300 amu; scan rate: 1.9 scans s^{-1}; EM voltage: 1435) with helium at 0.8 ml min^{-1} as carrier gas was used. The injection port, equipped with glass insert (internal diameter 0.75 mm) was split less at 250°C. The desorption time of 1.0 min was used. Detector was maintained at 230°C. Oven was maintained at 40°C for 2 min, then the temperature increased to 250°C (8°C min^{-1}) which was maintained for 10 min. All the analysis were performed twice with three replicates. A blank run was performed after each analysis in order to check for residual compounds contaminating the fiber.

All the peaks were identified by comparison with spectra in Wiley6N and NIST98 libraries. Furthermore, the identity of some of the VOCs components was confirmed by GC–MS analysis of reference substances [acetaldehyde (Sigma–Aldrich, 402788); methanethiol (MT) (Sigma–Aldrich, 295515); DMDS (Sigma–Aldrich, W353604); *p*-cymene (Sigma–Aldrich, C121452); 1-undecene (Sigma–Aldrich, 242527); 2-undecanone (Sigma–Aldrich, U1303)] used as control. The volatile relative concentrations in each Petri plate were calculated based on GC–MS peak areas without using correction factors. The results were reported as percentage average of total peak area (±SE).

NH₃ and HCN Production

Strains of *P. tolaasii* were screened for NH₃ production for which freshly grown cultures were grown in 10 ml peptone water

(peptone 10 g l^{-1}, NaCl 5 g l^{-1}, pH 7.2) tubes for 72 h at 25°C. After incubation period 0.5 ml of Nessler's reagent [0.09 mol l^{-1} solution of $K_2(HgI_4)$ in 2.5 mol l^{-1} of KOH] was added in each tube. The development of brownish color of bacterial liquid cultures was positive for NH₃ production (Cappuccino and Sherman, 2010).

Hydrogen cyanide production was assessed inoculating bacteria in nutrient sucrose agar (sucrose 5 g l^{-1}, yeast extract 4 g l^{-1}, peptone 4 g l^{-1}, beef extract 2 g l^{-1}, agar 18 g l^{-1}) amended of glycine (4.4 g l^{-1}) Petri dishes. Before incubation a Whatman No. 1 filter paper disk, soaked with a solution of 0.5% picric acid in Na_2CO_3 (2%) aqueous solution was placed on the upper lid of each plate. The plates were finally incubated for 4 days at 25°C. Filter paper color switch from yellow to red-brown was positive for HCN production (Lorck, 1948).

Strains USB2106 of *P. putida* and USB2102 of *P. brassicacearum* were used as positive control for the production of NH₃ and HCN, respectively. Non-inoculated media were used as negative controls. The assays were performed twice with three replicates.

VOCs Bioassays

In order to evaluate VOCs bioactivity, identified by GC-MS, belonging to *P. tolaasii* strains volatile mixture, three pure VOCs, DMDS, MT, and 1-undecene (Sigma–Aldrich, Milan, Italy), selected on the basis of their detection for all the *P. tolaasii* strains, were used in mushrooms and seeds bioassays. In these bioassays *P. ostreatus* mycelium plugs, *A. bisporus* and *P. ostreatus* basidiome tissue blocks and 100 broccoli seeds, were placed in two out three sectors of Petri dishes, while in the third one were dropped or injected, according to their physical state, DMDS, 1-undecene, and MT. In particular, liquid DMDS and 1-undecene were dropped in glass slide posed in one of the three sectors; then the dishes were rapidly sealed with parafilm, to minimize volatile compound exchange, able to alter the volatile quantity introduced in the system. In the bioassay with gaseous MT, at first, the plates were pierced in correspondence of the empty sector and the hole closed with a triple layer of parafilm. Then the gas were taken with a GC syringe (Supelco, Milan, Italy) from a gas Pyrex pipette device (Microglass Heim s.r.l., Naples, Italy) and injected through the hole; then the dishes were immediately re-sealed and incubated for 5 days at 25°C in the dark. Petri dishes without the VOCs, were used as controls. Then mycelia and seedlings growth was measured with a ruler as above said and the effects on tissue blocks were visually evaluated. All bioassays were performed twice with three replicates and the final data were reported in percentage compared to the control.

Statistical Analysis

All GC–MS analysis and bioassays data were statistically evaluated for the determination of standard errors and the latter subjected to analysis of variance (ANOVA) and *P* calculated by F test of Fisher–Snedecor. All statistical analysis was carried out using software program package SPSS version 17.0 (SPSS Inc., Chicago, IL, USA).

Results

Mushroom Bioassays

Volatile compounds produced by *P. tolaasii* strains caused a highly significant reduction, in non-sealed Petri dishes, ($P \leq 0.014$) of *P. eryngii* and *P. ostreatus* mycelia growth (**Figure 1A**). In fact, the mycelia growth of the strains DS256 and DS270 of *P. eryngii*, in the presence of volatile compounds of the strains NCPPB2192, USB1, and USB66 of *P. tolaasii* was only 16 and 19% (NCPPB2192), 34 and 33% (USB1) and 20% for both fungi (USB66) compared to the control (100%), respectively (**Figure 1A**). In the same assay conditions, mycelia growth of the strains DS226 and DS284 of *P. ostreatus* was only 16 and 10% (NCPPB2192), 12 and 25% (USB1), 12 and 31% (USB66) of the control, respectively (**Figure 1A**).

When the bioassay was performed in sealed Petri dishes, instead, (**Figure 1B**) it came out that *P. tolaasii* strains volatiles caused a significant *P. eryngii* and *P. ostreatus* strains mycelia growth increase ($P \leq 0.038$) (**Figure 1B**). Indeed, fungal growth of the *P. eryngii* strains DS256 and DS270, co-incubated with the

P. tolaasii strains NCPPB2192, USB1, and USB66, was assessed to 131%, and 122% (NCPPB2192), 143 and 129% (USB1), 143 and 129% (USB66), respectively, compared to the control (**Figure 1B**). In a similar way, the effect of the above examined volatiles on mycelia growth of *P. ostreatus* strains DS226 and DS284 was 145 and 166% (NCPPB2192), 153 and 139% (USB1), 131 and 144% (USB66), respectively, compared to the control (**Figure 1B**).

In non-sealed Petri dishes, volatiles from *P. tolaasii* strains caused a noticeable brown discoloration of *A. bisporus* and *P. ostreatus* tissue blocks (**Figure 2**); when Petri dishes were sealed, instead, no apparent alteration of the *A. bisporus* tissue blocks was noticed and the presence of mycelium growth surrounding *P. ostreatus* tissue blocks was observed (**Figure 3**).

Seed Bioassays

In non-sealed Petri dishes assays, volatile compounds produced by *P. tolaasii* strains NCPPB2192, USB1 and USB66 caused a highly significant reduction ($P < 0.001$) of broccoli seedlings growth, compared to the water control (**Figure 4A**). Indeed, the
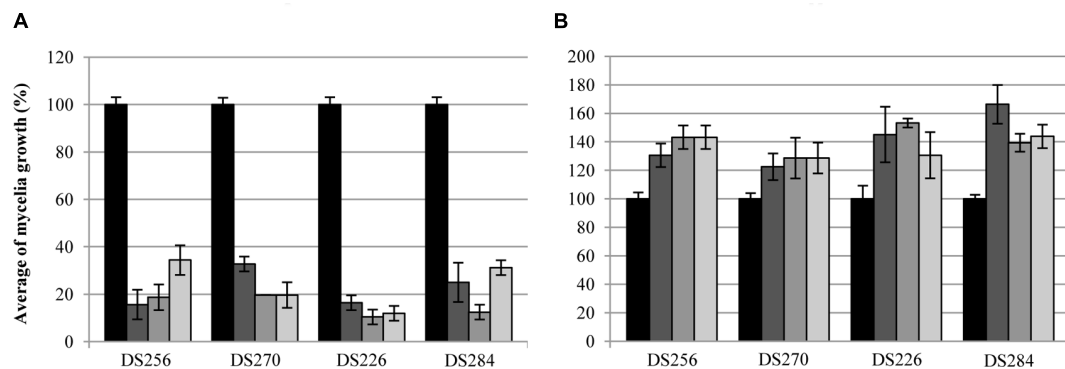
FIGURE 1 | Average of mycelia growth (%) of strains DS256, DS270, and DS226, DS284 of *Pleurotus eryngii* and *P. ostreatus*, respectively, in presence of water (■) and volatile compounds produced by strains NCPPB2192 (■), USB1 (■), and USB66 (■) of *Pseudomonas tolaasii* in Petri dishes not sealed (A) and sealed (B) with parafilm. Bars on the columns correspond to the standard error of the mean in percentage.
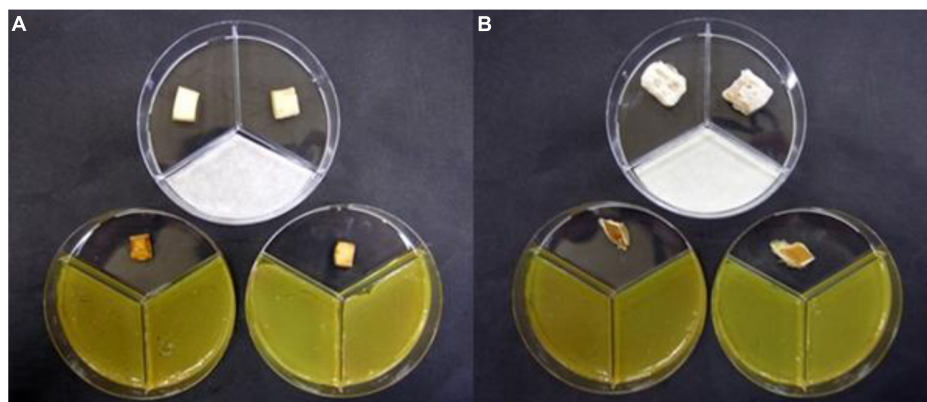
FIGURE 2 | *Agaricus bisporus* (A) and *Pleurotus ostreatus* (B) basidiome tissue blocks showing marked brown discoloration caused by volatile compounds blend produced by *Pseudomonas tolaasii* NCPPB2192 when the Petri dishes were not sealed with parafilm. At the top the Petri dishes containing the blocks treated with water.
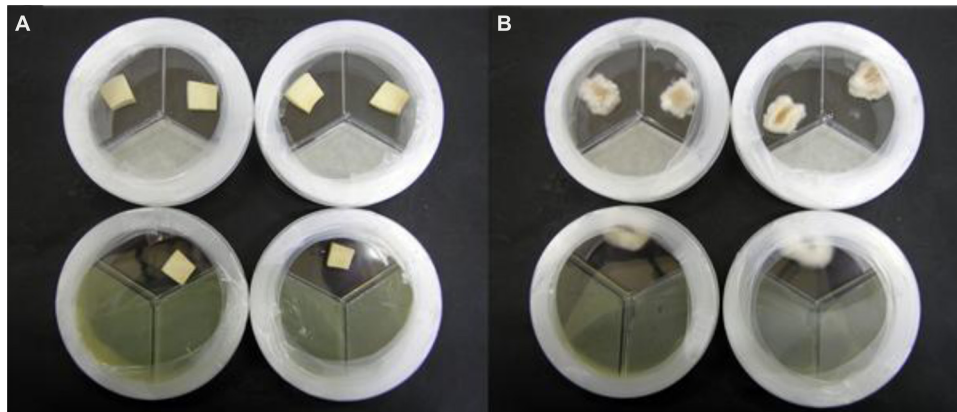
FIGURE 3 | *Agaricus bisporus* **(A) and** *Pleurotus ostreatus* **(B) basidiome tissue blocks showing apparently no change (A) or a slight increase in mycelia growth (B) when they were exposed, in sealed Petri dishes, to volatile compounds blend produced by** *Pseudomonas tolaasii* **NCPPB2192.** At the top the Petri dishes containing the blocks treated with water.
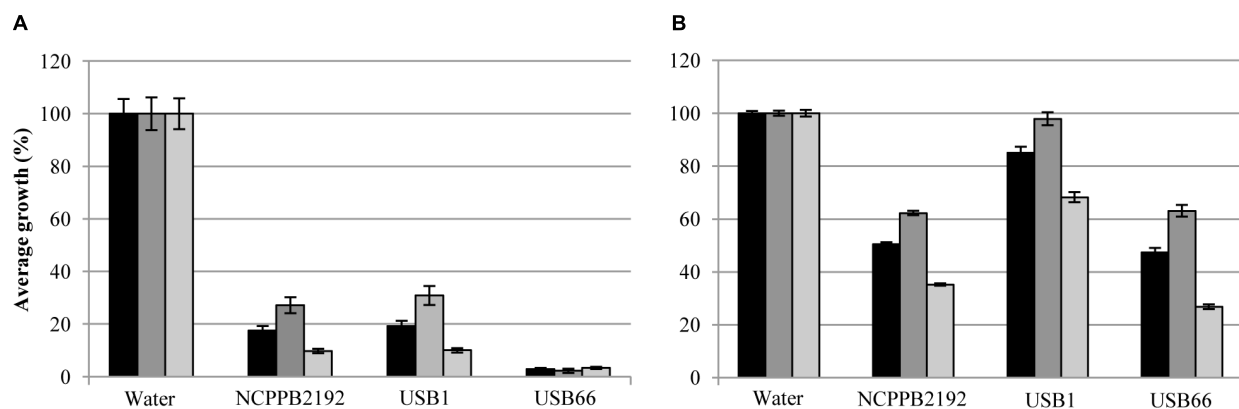
FIGURE 4 | Average growth (%) of whole seedlings (■), epicotyls (■), and main rootlets (□) of broccoli (A) and lettuce (B) in presence of volatile compounds blend produced by strains NCPPB2192, USB1 and USB66 of *Pseudomonas tolaasii* **in non-sealed Petri dishes.** Bars on the columns correspond to the standard error of the mean in percentage.

growth of whole seedlings, epicotyls and main rootlets was highly reduced, resulting in 17, 27, and 10% (NCPPB2192), 19, 31, and 10% (USB1), 3, 2, and 3% (USB66), respectively, compared to the control (100%) (**Figure 4A**). The above bioassays on lettuce seeds produced a similar toxic effect, though more limited. In fact, the growth of whole seedlings, epicotyls and main rootlets was highly reduced ($P < 0.001$) to 51, 62, 35, 47, 63, and 27 in presence of the NCPPB2192 and USB66 strains, respectively, compared to the control (**Figure 4B**). When the USB1 strain was used, the whole seedlings and epicotyls length of the plantlets (85 and 98%, respectively) resulted not significantly ($P > 0.05$) different from the controls, while the main rootlets length resulted highly reduced ($P < 0.001$) at 68% compared to the control (**Figure 4B**).

In sealed Petri dishes assays, the reduction of broccoli and lettuce plantlets growth was more pronounced (**Figure 5**) and the statistical analysis confirmed that volatile compounds from the above bacterial strains led to a highly significant reduction ($P < 0.001$) of broccoli and lettuce seedlings growth in comparison to the control (**Figure 5**). Actually, the growth of

the whole broccoli seedlings, epicotyls and main rootlets resulted highly reduced being only 1, 0, 2% for all *P. tolaasii* strains, compared to the control (**Figure 5A**). Similarly, the growth of the whole lettuce seedlings, epicotyls, and main rootlets was 25, 23, 27% (NCPPB2192), 6, 1, 13% (USB1), and 21, 24, 16% (USB66), respectively, compared to the control (**Figure 5B**).

Furthermore, the inspection of broccoli and lettuce seedlings made evident root browning and, in the case of lettuce, the absence of root hairs, as it was observed in the negative control (**Figure 6**).

GC–MS Analysis and NH$_3$ and HCN Production

Pseudomonas tolaasii strains, in sealed Petri dishes, produced MT, DMDS, *p*-cymene, 1,4-undecadiene, 1-undecene, 2-undecanone, and 4,7-dimethylundecane, even though not all the volatile compounds identified have been systematically produced by the three *P. tolaasii* strains (**Table 1**; **Figure 7**). In addition, CO$_2$ was also present in the headspace of these samples. The strains DS256 and DS284, respectively, of *P. eryngii* and *P. ostreatus*, in the same
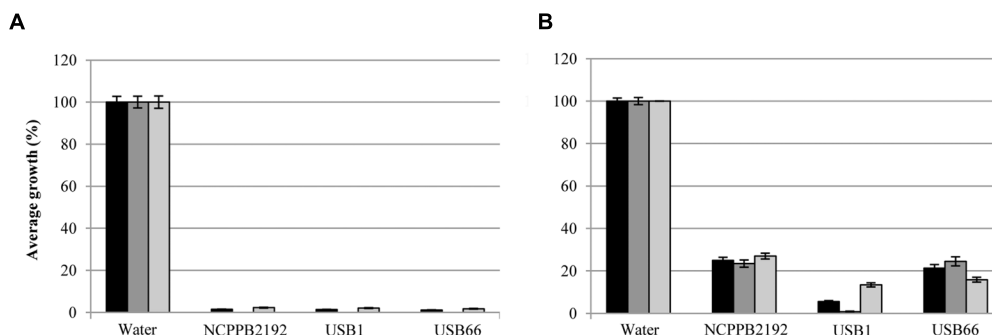
FIGURE 5 | Average growth (%) of whole seedlings (■), epicotyls (■), and main rootlets (■) of broccoli (A) and lettuce (B) in presence of volatile compounds blend produced by strains NCPPB2192, USB1, and USB66 of *Pseudomonas tolaasii* in sealed Petri dishes. Bars on the columns correspond to the standard error of the mean in percentage.
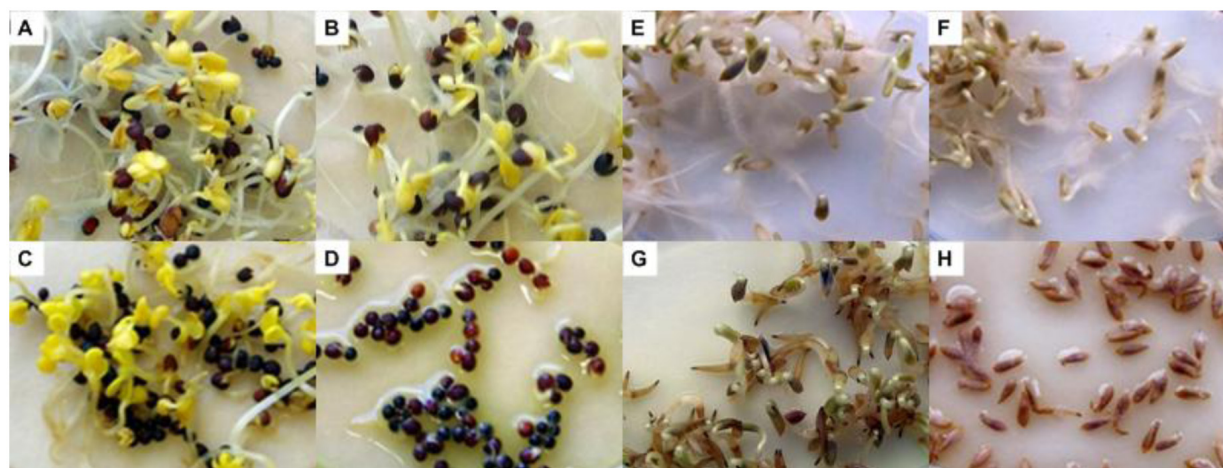
FIGURE 6 | Growth of broccoli (A–D) and lettuce (E–H) seedlings in absence (A,B,E,F, respectively) and in presence (C,D,G,H, respectively) of volatile compounds produced by strain NCPPB2192 of *Pseudomonas tolaasii* in Petri dishes not sealed (A,C,E,G) and sealed (B,D,F,H) with parafilm.

TABLE 1 | Percentage average of total peak area (±SE) of volatile compounds in the overhead space of sealed Petri dishes, as determined by SPME–GC analysis, produced by pure cultures of *Pseudomonas tolaasii*, *Pleurotus eryngii*, and *P. ostreatus* strains and by non-inoculated KBA and MEA media.

Volatile compounds[a]	*P. tolaasii* strains[b]			*P. eryngii* DS256[b]	*P. ostreatus* DS284[b]	Media	
	NCPPB 2192	**USB1**	**USB66**			**KB[c]**	**MEA[d]**
Carbon dioxide (CO_2)	63.66 ± 2.20	44.71 ± 5.22	42.73 ± 4.34	69.86 ± 1.26	6.75 ± 0.88	5.04 ± 0.22	5.31 ± 0.59
Nitrous oxide (NO_2)	–	–	–	1.10 ± 0.11	–	–	–
Ethylene oxide	–	–	–	–	5.41 ± 0.32	–	45.25 ± 1.94
Acetaldehyde	–	–	–	–	69.42 ± 2.44	52.55 ± 1.98	–
Propane	–	–	–	–	–	2.68 ± 0.32	–
Methanethiol (MT)	2.12 ± 0.19	1.89 ± 0.19	2.03 ± 0.27	–	–	–	–
Dimethyl disulfide (DMDS)	0.25 ± 0.04	0.22 ± 0.04	0.26 ± 0.04	–	–	–	–
p-cymene	Traces	0.11 ± 0.04	0.10 ± 0.03	–	–	–	–
1,4-undecadiene	–	0.49 ± 0.18	0.20 ± 0.04	–	–	–	–
1-undecene	11.86 ± 2.34	17.04 ± 2.73	14.45 ± 2.08	–	–	–	–
2-undecanone	1.98 ± 0.71	–	0.40 ± 0.04	–	–	–	–
4,7-dimethylundecane	Traces	0.13 ± 0.04	0.15 ± 0.03	–	–	–	–

[a]*Volatile compounds are reported according to their molecular weight.* [b]*Pseudomonas tolaasii, Pleurotus eryngii, and P. ostreatus strains were grown on KB and MEA, respectively; NCPPB, National Collection of Plant Pathogenic Bacteria; USB, Microorganism collection of Università (U) degli Studi (S) della Basilicata (B).* [c]*KB, medium B of King et al. (1954).* [d]*MEA, Malt Extract Agar. –, no volatile compounds detected.*

FIGURE 7 | Volatile compounds produced by *Pseudomonas tolaasii* strains (NCPPB2192, USB1, and USB66). 1- carbon dioxide (CO_2); 2- methanethiol (MT); 3- dimethyl disulfide (DMDS); 4- *p*-cymene; 5, 1,4-undecadiene; 6- 1-undecene; 7- 2-undecanone; 8- 4,7-dimethylundecane.
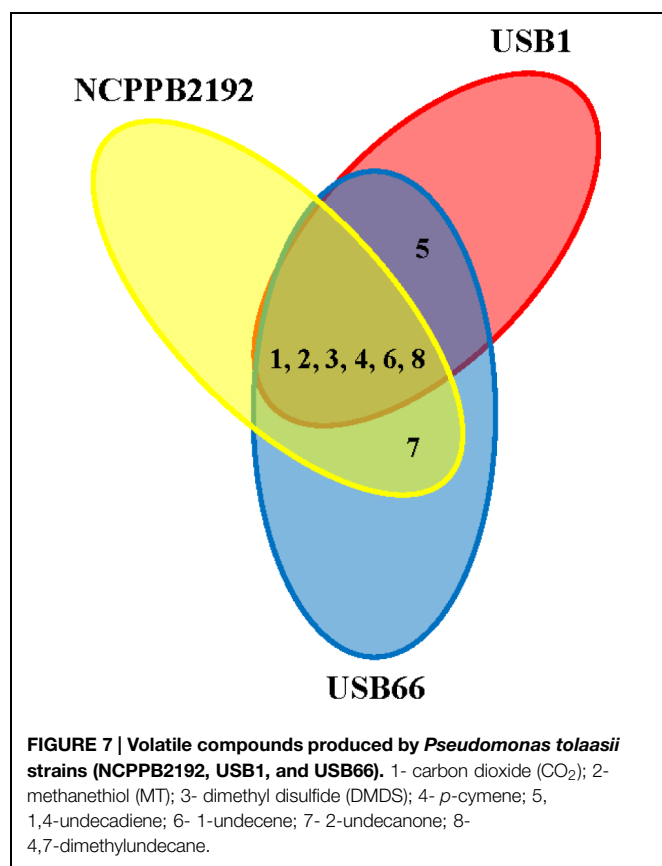
experimental conditions, produced CO_2 and nitrous oxide (NO_2) and CO_2, ethylene, and acetaldehyde, respectively (**Table 1**). In the headspace of plates containing non-inoculated KBA or MEA media, CO_2, acetaldehyde, and propane, and CO_2 and ethylene oxide, respectively, were detected (**Table 1**).

In non-sealed conditions no volatiles were detected.

The three *P. tolaasii* strains resulted to produce NH_3 but not HCN.

VOCs Bioactivity
Mushroom Bioassays

Treatments with 1,500 µg of MT per Petri dish caused a noticeable inhibition of *P. eryngii* and *P. ostreatus* strains mycelia growth (**Figure 8A**). In particular, *P. eryngii* strain DS256 resulted less sensitive than DS270 strain to MT effects. Indeed, DS256 strain growth was significantly reduced ($P \leq 0.001$) by doses ≥ 500 µg [mycelia growth $\leq 69\%$ when compared to the control (100%)], below which no significant growth reduction values were observed. *P. eryngii* strain DS270 resulted more affected by MT inhibiting action; using doses ≥ 50 µg mycelia growth was $\leq 88\%$ ($P = 0.01$). The diverse sensitivity between two strains of the same mushroom was noticed also in the case of *P. ostreatus* strains DS226 and DS284, where quantities ≥ 500 µg of MT were necessary to cause significant growth inhibition of DS226 (mycelia growth $\leq 65\%$, $P \leq 0.001$), and ≥ 100 µg for DS284 strain (mycelia growth $\leq 87\%$, $P = 0.001$) (**Figure 8A**).

The total inhibition of *P. eryngii* and *P. ostreatus* strains mycelia growth was observed when they were treated with doses ≥ 20 µg per Petri dish of DMDS (**Figure 8B**). At lower doses of DMDS, the effect on mycelia growth was reduced but still highly significant. In particular, at doses ≥ 0.625 µg per Petri dish mycelia growth of the strains DS256 and DS270 of *P. eryngii* was $\leq 76\%$ ($P = 0.013$) and $\leq 86\%$ ($P = 0.002$), respectively, when compared to the control. A similar effect on *P. ostreatus* DS226 and DS284 strains growth was observed applying doses ≥ 1.25 µg of DMDS per Petri dish. Indeed, mycelia growth of the latter strains was $\leq 75\%$ ($P = 0.001$) and $\leq 80\%$ ($P \leq 0.002$), respectively, compared to the control (**Figure 8B**).

1-undecene treatments on *P. eryngii* strains did not affect mycelia growth at doses ≤ 10 µg while, above the mentioned quantity, it caused a significant reduction ($P \leq 0.001$) (**Figure 8C**). In particular, *P. eryngii* mycelia growth at doses ≥ 25 µg was $\leq 74\%$ for both *P. eryngii* strains compared to the control (**Figure 8C**). The same treatments on *P. ostreatus* strains DS226 and DS284 were ineffective in mycelia growth inhibition at doses ≤ 2.5 and ≤ 5 µg, respectively; on the contrary, using quantity ≥ 5 and ≥ 10 µg, respectively, 1-undecene caused a significant reduction of mycelia growth ($\leq 91\%$, $P = 0.002$ and $\leq 90\%$, $P = 0.038$) (**Figure 8C**).

On *A. bisporus* and *P. ostreatus* tissue blocks MT aliquots determined aerial mycelia growth reduction, brown discoloration and deliquescence, with *P. ostreatus* being the less sensitive (**Figure 9**). Brown discoloration (**Figure 9-1C**) and tissue deliquescence of *A. bisporus* tissue blocks were determined with treatments of 100 and ≥ 250 µg of MT per Petri dish, respectively (**Figure 9-1D**). When *P. ostreatus* tissue blocks were treated with 50 or 100 µg of MT, the reduction of aerial mycelia growth was solely observed (**Figure 9-2B,C**); doses ≥ 250 µg caused a marked reduction of the mycelia growth and depressed brown discoloration (**Figure 9-2D,E**). The deliquescence of *P. ostreatus* tissue blocks was observed with treatments ≥ 1500 µg of MT (**Figure 9-2G**).

Aerial mycelia growth reduction, yellowing, brown discoloration and deliquescence of *A. bisporus* and *P. ostreatus* tissue blocks were observed when they were treated with DMDS aliquots, even though *P. ostreatus* tissue resulted, also in this case, less sensitive than *A. bisporus* (**Figure 10**). The yellowing of *A. bisporus* tissue blocks was observed with 0.156 µg of DMDS per Petri dish treatment (**Figure 10-1B**), while the tissue deliquescence was observed with doses ≥ 0.625 µg (**Figure 10-1D**). Treatments with 1.25 µg of DMDS caused, on *P. ostreatus* tissue blocks, a reduction of the aerial mycelia growth (**Figure 10-2C**), whereas 2.5 µg caused brown discoloration (**Figure 10-2D**). A marked depressed brown discoloration and deliquescence of tissue was observed with doses of DMDS ≥ 20 µg (**Figure 10-2G**).

1-undecene treatments also caused aerial mycelia growth reduction, yellowing, brown discoloration, and deliquescence of *A. bisporus* and *P. ostreatus* tissue blocks and, in this case too, *P. ostreatus* tissue was found less sensitive than *A. bisporus* (**Figure 11**). In fact, *A. bisporus* tissue blocks showed marked depressed brown discoloration and deliquescence of tissue at all 1-undecene doses used in this work (**Figure 11-1**). On the other
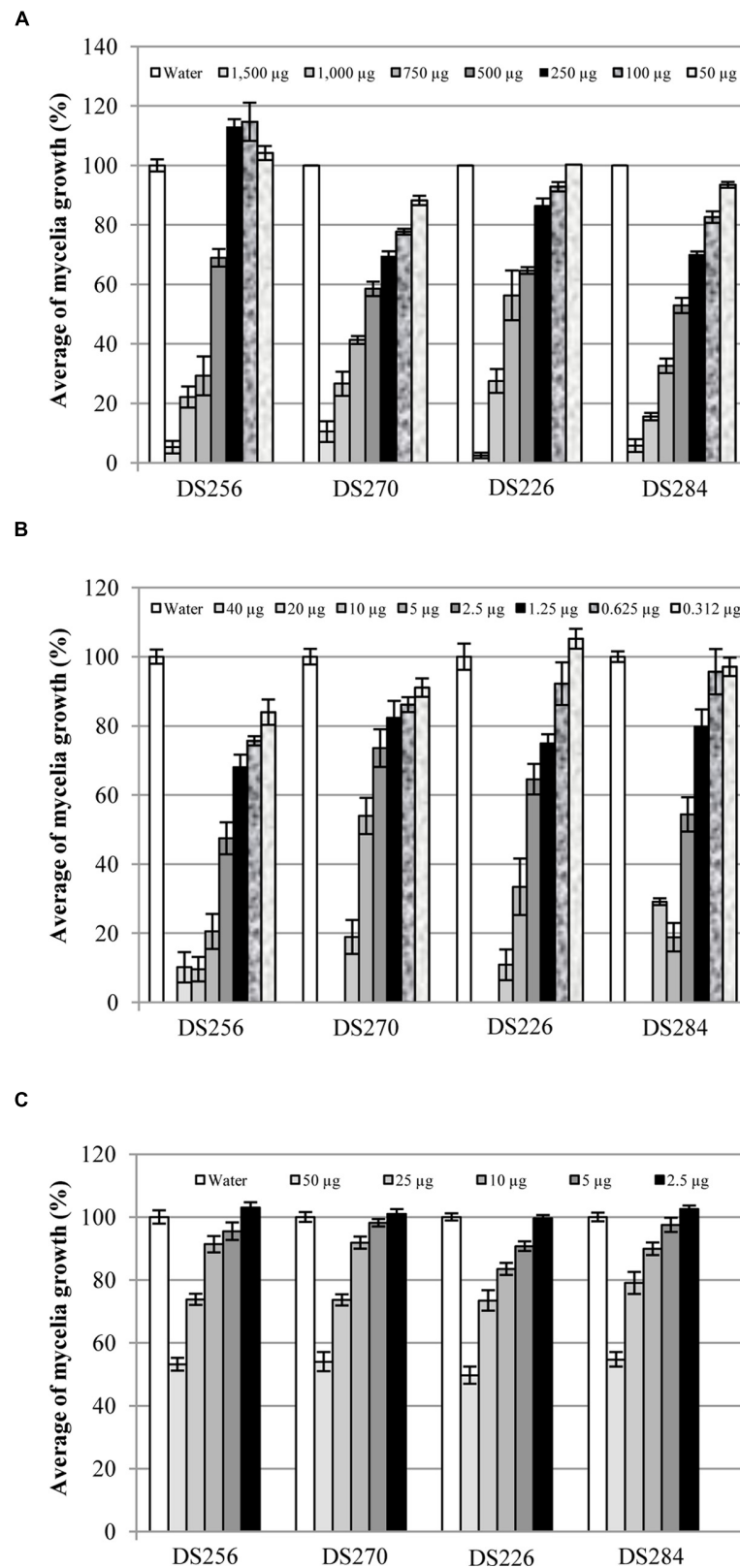
FIGURE 8 | Average of mycelia growth (%) of strains DS256, DS270, and DS226, DS284 of *Pleurotus eryngii* and *P. ostreatus*, respectively, in presence of pure MT (A), DMDS (B), and 1-undecene (C) aliquots. Bars on the columns correspond to the standard error of the mean in percentage.
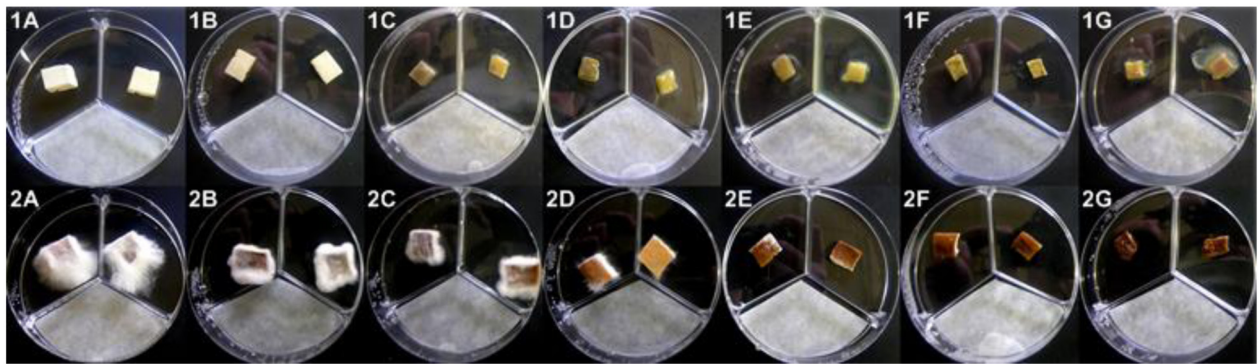
FIGURE 9 | *Agaricus bisporus* (1) and *Pleurotus ostreatus* (2) basidiome tissue blocks treated with pure MT aliquots (1A and 2A = H_2O; 1B and 2B = 50 μg; 1C and 2C = 100 μg; 1D and 2D = 250 μg; 1E and 2E = 500 μg; 1F = 750 μg; 1G and 2F = 1,000 μg; 2G = 1,500 μg).
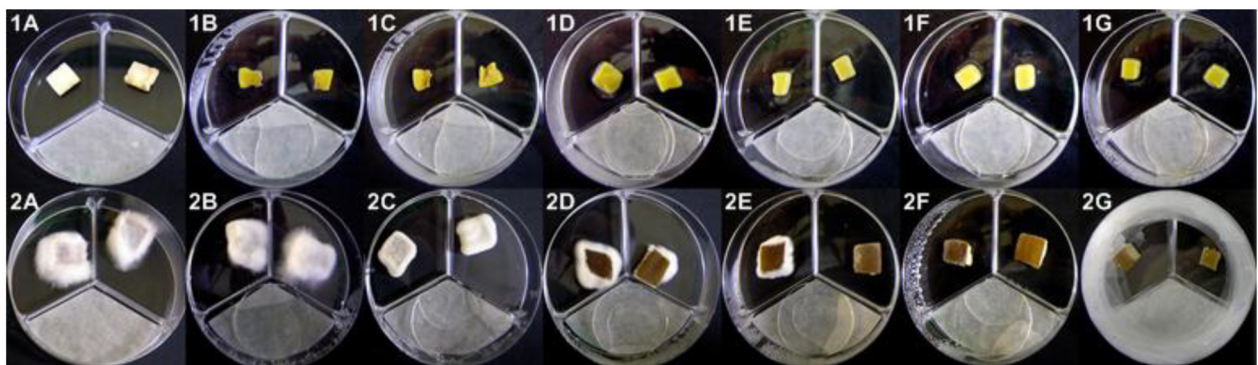
FIGURE 10 | *Agaricus bisporus* (1) and *Pleurotus ostreatus* (2) basidiome tissue blocks treated with pure DMDS aliquots (1A and 2A = H_2O; 1B = 0.156 μg; 1C = 0.312 μg; 1D and 2B = 0.625 μg; 1E and 2C = 1.25 μg; 1F and 2D = 2.5 μg; 1G and 2E = 5 μg; 2F = 10 μg; 2G = 20 μg).
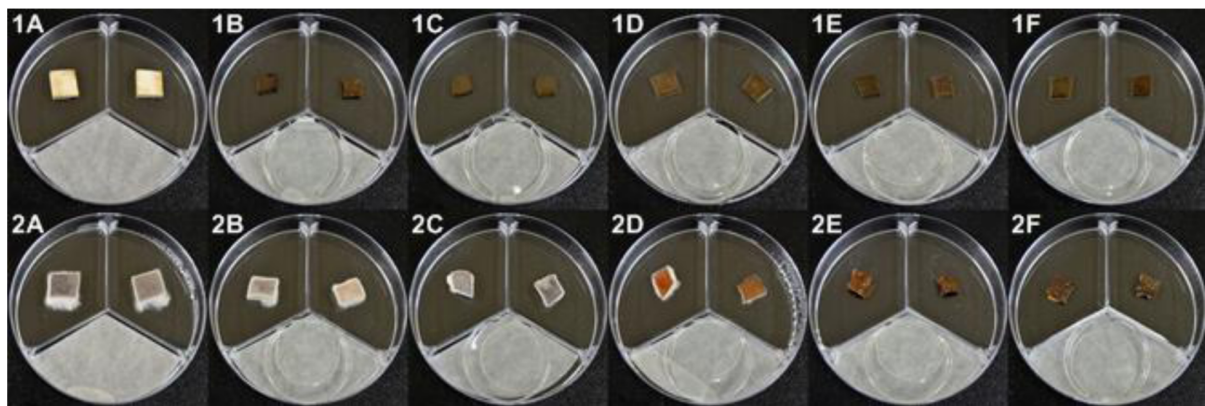
FIGURE 11 | *Agaricus bisporus* (1) and *Pleurotus ostreatus* (2) basidiome tissue blocks treated with pure 1-undecene aliquots (1A and 2A = H_2O; 1B and 2B = 2.5 μg; 1C and 2C = 5 μg; 1D and 2D = 10 μg; 1E and 2E = 25 μg; 1F and 2F = 50 μg).

side, *P. ostreatus* tissue blocks at doses ≤5 μg of the VOC caused aerial mycelia growth reduction (**Figure 11-2B,C**), at doses of 10 μg determined brown discoloration (**Figure 11-2D**), and at doses ≥25 μg induced a marked depressed brown discoloration and deliquescence of tissue (**Figure 11-2E,F**).

Seed Bioassays

Methanethiol and DMDS at doses >500 and 5 μg per Petri dish, respectively, inhibited broccoli seeds germination. At lower doses, different effects on seedlings growth were observed (**Figure 12**). Doses of 500, 250, and 100 μg of MT led to highly

FIGURE 12 | Average growth (%) of whole seedlings (■), epicotyls (■), and main rootlets (■) of broccoli in presence of pure MT (A), DMDS (B), and 1-undecene (C) aliquots. Bars on the columns correspond to the standard error of the mean in percentage.
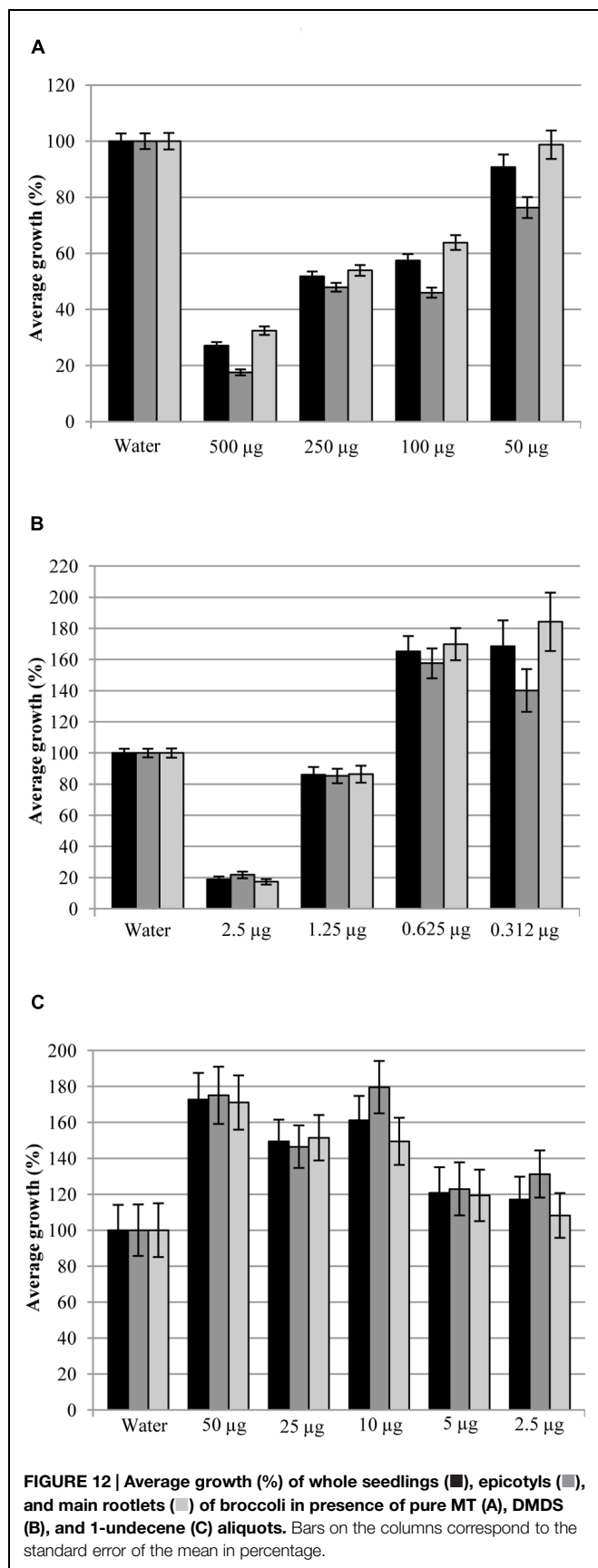
significant reduction ($P < 0.001$) of whole seedling (27, 52, and 57%), epicotyl (18, 48, and 46%), and main rootlet growth (32, 54, and 64%), respectively, when compared to the control (100%) (**Figure 12A**). Treatment with 50 μg of MT caused a significant reduction ($P \leq 0.002$) of the whole seedling (9%) and epicotyl (24%) growth but the main rootlet growth was not significantly different (99%) from the control (**Figure 12A**).

Significant reduction ($P < 0.001$) of whole seedling (19%), epicotyl (22%) and main rootlet (17%) growth, in comparison with water seed treatment (100%) used as control (**Figure 12B**), was observed after seeds treatment with 2.5 μg of DMDS. Doses of 1.25 μg of DMDS caused significant decrease ($P < 0.001$) of the whole seedling (86%) and epicotyl length (85%) while the main rootlet growth (86%) was not statistically different ($P > 0.05$) from the control (**Figure 12B**). Highly significant increase ($P \leq 0.002$) of seedlings growth (whole seedling, epicotyl, and main rootlet) was obtained by treatments with 0.625 μg (165, 158, and 170%, respectively) and 0.312 μg (169, 140, and 184%, respectively) of DMDS, compared to the control (**Figure 12B**).

Broccoli seedlings growth stimulation was also noticed in 1-undecene treatments (**Figure 12C**). In fact, significant increase of whole seedlings, epicotyls and main rootlets growth was caused by treatments with 10 μg (161, 180, and 149%, respectively, with $P \leq 0.013$), 25 μg (150, 147, and 151%, respectively, with $P \leq 0.021$), and 50 μg (173, 175, and 171%, respectively, with $P \leq 0.05$) of 1-undecene, compared to the control (**Figure 12C**).

Discussion

In this paper, it was demonstrated that *P. tolaasii*, an ubiquitous bacterium mainly recognized as a mushroom pathogen but also reported as associated to plant in either parasitic or commensal positions (Rainey et al., 1991; Zdor and Anderson, 1992; Noval et al., 1993; Shirata et al., 1995; Talame and Piccirillo, 1995; Tanprasert and Reed, 1997), is able to produce *in vitro* volatiles which were identified by GC–MS. They were demonstrated to affect mushrooms, seed germination, and seedling growth features confirming previous evidences (Shirata, 1996). Indeed, in assays performed in non-sealed Petri dishes, *P. eryngii* and *P. ostreatus* strains mycelia growth was greatly reduced when exposed to *P. tolaasii* strains volatile metabolites. In the same experimental conditions *P. tolaasii* volatiles toxicity was explicated by pronounced *A. bisporus* and *P. ostreatus* basidiome tissue blocks brown lesions or yellowing. On the other side, *P. tolaasii* volatiles in sealed Petri dishes, did not have toxic effect: *P. eryngii* and *P. ostreatus* mycelia showed a significant growth increase and *A. bisporus* and *P. ostreatus* blocks presented even mycelia proliferation on their surfaces. These apparently divergent and contradictory results may be explained as follows. In *in vitro* experimental systems performed, there is the strictly aerobic bacterium *P. tolaasii* and *Agaricus* and *Pleurotus* sp. which are able to live also in limited oxygen conditions (Rast and Bachofen, 1967a,b; San Antonio and Thomas, 1972; Zadražil, 1975). In fact, in the latter condition *Pleurotus* sp. mycelia increase their biomasses because of their ability to assimilate CO_2 when available in the environment at

high concentrations (Rast and Bachofen, 1967a,b; San Antonio and Thomas, 1972; Zadražil, 1975). This seems the case of the sealed Petri dishes assay conditions. On the other hand the oxygen limitation may lead the bacterium to produce VOCs, which levels may be under the toxicity threshold; it could also be that the simultaneous accumulation of CO_2, produced by both organisms, may counteract VOCs toxic effects (Rast and Bachofen, 1967a,b; San Antonio and Thomas, 1972; Zadražil, 1975).

Pseudomonas tolaasii, when grown in non-sealed Petri dishes, expresses its aerobic metabolism and inorganic and organic volatile compounds are produced better than the bacterium could do in limited oxygen conditions (sealed plates) (Ferchichi et al., 1986). In such state, despite the fact that part of the volatile compounds produced may be dispersed outside of the Petri dish, the VOCs still reach toxic concentrations for *Pleurotus* sp. mycelia and *A. bisporus* and *P. ostreatus* basidiome tissue blocks.

Lettuce and broccoli seeds exposure to bacterial volatiles led to negative effect on both germination and seedling growth. The highest effects were observed when the assays were carried out in sealed Petri dishes. In non-sealed dishes, with normal oxygen concentrations and gas exchange, seeds germination is just partly inhibited due to the possible sub-lethal volatiles dose achievement. In sealed plates system, the toxic effects of volatiles, since there is a progressive decrease of oxygen concentration and an accumulation of gaseous catabolites, considering also that both aerobic organisms are stressed in this conditions, may depend on possible additional/synergistic action of volatiles (Segal and Starkey, 1969; Ferchichi et al., 1986; van Leerdam et al., 2008). Moreover, the above results suggest roots as being the most sensitive plantlet part to the *P. tolaasii* toxic action volatiles since they showed some brown-necrotic areas beside growth reduction. Furthermore, despite the fact that *P. tolaasii* produced high quantities of CO_2 and it is known that this compound is able to stimulate plant growth (Kai and Piechulla, 2009), no beneficial effect on plant biostimulation was observed.

The GC–MS analysis, despite the fact that in non-sealed conditions no volatiles were detected probably because of their dispersion, indicated that *P. tolaasii* strains are able to produce different volatiles, among which MT, DMDS, and 1-undecene have been produced by all the three strains of the bacterium. Moreover, GC–MS analysis of non-inoculated KBA or MEA and *Pleurotus* sp. strains inoculated on MEA clearly demonstrated that no one of the above mentioned volatiles were detected, confirming their exclusive origin from *P. tolaasii*. The same GC-MS analysis stated, as expected, the presence of a quite important level of CO_2 produced by the growing microorganisms.

Finally, all *P. tolaasii* strains did not produce HCN, although this substance is produced by several bacteria including some *Pseudomonas* sp. inhabiting plants rhizosphere (Blom et al., 2011; Weise et al., 2013). However, *P. tolaasii* strains produce NH_3 and this volatile compound has a significant role on the plants and fungi growth (Mikeš et al., 1994; Weise et al., 2013). In fact, it is known that NH_3 inhibits the growth of *Arabidopsis thaliana*

through the alkalization of the neighboring plant medium and, on the other hand, it stimulates the fungal growth via its assimilation by the glutamine synthetase and glutamate dehydrogenase NAD-dependent enzymes (Mikeš et al., 1994; Weise et al., 2013). This may contribute to explain the growth inhibition of broccoli and lettuce seedlings and increased mycelia growth in biological assays performed in this study.

On the basis of these results the VOCs MT, DMDS, and 1-undecene were selected for further assays.

The *in vitro* assays results, performed using pure VOCs showed that MT, DMDS, and 1-undecene, whose toxic action on pathogenic fungi is well known (Lewis, 1985; Fritsch, 2005; Groenhagen et al., 2013; Wang et al., 2013; Popova et al., 2014; Hunziker et al., 2015), are also toxic for the *P. eryngii* and *P. ostreatus* mycelia and *A. bisporus* and *P. ostreatus* tissue blocks. Furthermore, higher MT and DMDS doses (from 50 to 500 µg and ≥ 1.25 µg, respectively) inhibited the broccoli seeds germination while the DMDS and 1-undecene (at doses ≤ 0.625 and ≥ 10 µg, respectively) have also caused an increase of whole broccoli seedlings growth. The results concerning the bioactivity of the above mentioned VOCs and, in particular, on plant growth stimulation action by 1-undecene have here been reported, to the best of our knowledge, for the first time.

These findings clearly indicate that MT and DMDS have an important role in the bioactivity of bacterial volatiles natural blend toward fungal and plant systems. The toxic effect of these VOCs may have cellular respiration as target, since it is known that MT and DMDS are able to inhibit mitochondrial activity (Waller, 1977; Dugravot et al., 2003). Our recent electron microscopy studies (Giorgio et al., 2015) revealed that DMDS and other VOCs, such as 2-nonanone and DL-limonene, are also able to determine structural alterations of cell membranes on phytopathogenic fungi that can contribute, along with the alteration of mitochondrial activity, to cell death. DMDS also proved capable, at doses ≤ 0.625 µg, of stimulating the growth broccoli seedlings. These results are not very different from the ones obtained by other authors. In fact, Kai et al. (2009) showed that DMDS inhibits *A. thaliana* (L.) Heynh growth and Groenhagen et al. (2013) have highlighted that the same molecule, at doses ranging from 1 ng to 1 mg, significantly increased the same plant biomass. Recently, Meldau et al. (2013) demonstrated that DMDS is able to make available its organic sulfur to the roots of *Nicotiana attenuata* Torr. ex S. Watson favoring its growth.

1-undecene's ability to inhibit *Pleurotus* sp. strains growth, to alter *A. bisporus* and *P. ostreatus* blocks and increase the growth of broccoli seedlings are, to our knowledge, new biological properties for this compound. Antimicrobial activity of 1-undecene has been only recently determined (Kai et al., 2009; Groenhagen et al., 2013; Wang et al., 2013; Popova et al., 2014) on some target microorganisms [*Agrobacterium tumefaciens* Smith & Townsend, *Synechococcus* sp., *Rhizoctonia solani* (Cooke) Wint. and *Fusarium culmorum* (Wm. G. Sm.) Sacc.] among which only the fungus *F. culmorum* was weakly inhibited; biostimulation ability on plants of this molecule, on the other side, remains totally unexplored. Some authors

(Nisenbaum et al., 2013) have previously shown that hydrocarbons, including 1-undecene, can be assimilated or transformed by microorganisms, but to our knowledge, no one has proven whether broccoli or plants in general, are able to utilize 1-undecene as carbon source. This outcome certainly highlights the need for further studies to understand this result.

Conclusion

Pseudomonas tolaasii volatile blend, as well as pure VOCs used in the present work, may have an important role in the interaction between pathogen and cultivated mushrooms. Shirata (1996) reported that *P. tolaasii* volatiles were able to contribute to the disease symptoms and interfere with mushrooms mycelia development, during substrate colonization, and thus, indirectly on mushrooms production. The fact that MT, DMDS and 1-undecene showed antifungal activity toward cultivated mushrooms mycelia and were toxic toward basidiome tissue blocks suggest the mentioned VOCs as *P. tolaasii* new potential virulent factors since they were able to reproduce, at least in part, brown blotch and yellowing typical symptoms caused by bacteria on *A. bisporus* and *P. ostreatus*, respectively. However, it is necessary to consider that, in some of the case, the above VOCs reproduced the biological effect of the whole volatile blend when applied in high, probably non-natural, concentrations. New studies would unravel whether the bacterium is able to produce *in vivo* VOCs among resulting toxic to mushrooms and if VOCs synergic actions may occur. *P. tolaasii* mutants availability in producing VOCs may highlight their contribution, beside tolaasins, on the virulence of the pathogen. Moreover, since *P. tolaasii* is also reported to be associated to plants rhizospheres (Zdor and Anderson, 1992), it is not to be excluded that VOCs, possibly produced in those niches, may interfere with the behavior of organisms dwelling the same habitat. In this regard, *P. tolaasii,* could present typical biocontrol agents traits since it is able to produce lipodepsipeptides and VOCs which are toxic for fungi. Studies to verify the rhizosphere competence of the bacterium would be very useful to assess his candidacy as a biocontrol agent.

Nevertheless, our results on these VOCs biological activity indicate that they may represent alternatives to methyl bromide for fumigation of soils infected by soil-borne fungal pathogens. As a matter of fact, DMDS is already used as a novel pre-planting soil fumigant under the commercial name PALADIN. Furthermore, it has been recently established the ability of DMDS to control plant pathogenic fungi (Fritsch, 2005; Kai et al., 2009), either directly or via the induction of systemic resistance (Huang et al., 2012), nematodes (Coosemans, 2005) and weeds (Freeman et al., 2009). Finally, since DMDS and 1-undecene were able to increase plant growth, they can also be candidate as potential plant biostimulators/fertilizers.

Acknowledgments

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Volatile Organic Compounds from Native Potato-associated *Pseudomonas* as Potential Anti-oomycete Agents

Mout De Vrieze[1,2], Piyush Pandey[3], Thomas D. Bucheli[1], Adithi R. Varadarajan[2,4], Christian H. Ahrens[2,4], Laure Weisskopf[1,2,5] and Aurélien Bailly[1,6]*

[1] Institute for Sustainability Sciences, Agroscope, Zurich, Switzerland, [2] Institute for Plant Production Sciences, Agroscope, Wädenswil, Switzerland, [3] Department of Microbiology, Assam University, Silchar, India, [4] Swiss Institute of Bioinformatics, Wädenswil, Switzerland, [5] CHANGINS, Viticulture and Oenology, University of Applied Sciences and Arts Western Switzerland, Nyon, Switzerland, [6] Microbiology, Institute of Plant Biology, University of Zurich, Zurich, Switzerland

The plant kingdom represents a prominent biodiversity island for microbes that associate with the below- or aboveground organs of vegetal species. Both the root and the leaf represent interfaces where dynamic biological interactions influence plant life. Beside well-studied communication strategies based on soluble compounds and protein effectors, bacteria were recently shown to interact both with host plants and other microbial species through the emissions of volatile organic compounds (VOCs). Focusing on the potato late blight-causing agent *Phytophthora infestans*, this work addresses the potential role of the bacterial volatilome in suppressing plant diseases. In a previous study, we isolated and identified a large collection of strains with anti-*Phytophthora* potential from both the phyllosphere and the rhizosphere of potato. Here we report the characterization and quantification of their emissions of biogenic volatiles, comparing 16 *Pseudomonas* strains differing in (i) origin of isolation (phyllosphere vs. rhizosphere), (ii) *in vitro* inhibition of *P. infestans* growth and sporulation behavior, and (iii) protective effects against late blight on potato leaf disks. We systematically tested the pharmacological inhibitory activity of core and strain-specific single compounds against *P. infestans* mycelial growth and sporangial behavior in order to identify key effective candidate molecules present in the complex natural VOCs blends. We envisage the plant bacterial microbiome as a reservoir for functional VOCs and establish the basis for finding the primary enzymatic toolset that enables the production of active components of the volatile bouquet in plant-associated bacteria. Comprehension of these functional interspecies interactions will open perspectives for the sustainable control of plant diseases in forthcoming agriculture.

Keywords: *Phytophthora*, *Pseudomonas*, *Solanum tuberosum*, volatile organic compounds, biocontrol, microbiome

INTRODUCTION

After more than a decade of exploratory work, it is now recognized that beside their well-documented, soluble anti-microbial arsenal, bacteria emit a wide range of volatile organic compounds (VOCs) that hold a strong inhibitory potential against microbial competitors (Garbeva et al., 2014; Audrain et al., 2015; Hunziker et al., 2015; Kanchiswamy et al., 2015; Schmidt et al., 2015). Therefore bacterial VOCs (bVOCs) are currently bringing an additional motivation to prospect the plant-associated microbiome with respect to its ability to confer to crop plants a natural protection against microbial pathogens. Indeed, *in natura*, these molecules are suspected to mediate or participate in intra- and interspecies communication processes such as bacterial quorum sensing, growth, differentiation or antibiotic and stress resistance (Vespermann et al., 2007; Bailly and Weisskopf, 2012; Bos et al., 2013; Groenhagen et al., 2013; Audrain et al., 2015; Kanchiswamy et al., 2015). Next, under laboratory conditions, bVOCs were also demonstrated to hinder growth and differentiation in numerous phytopathogenic fungal species (Kai et al., 2007; Athukorala et al., 2010; Ting et al., 2011; Velazquez-Becerra et al., 2011; Effmert et al., 2012; Yuan et al., 2012; Groenhagen et al., 2013; Tenorio-Salgado et al., 2013; Hunziker et al., 2015), suggesting that the complex blends of bacterial emissions represent a source of novel, naturally produced, anti-fungal substances. Finally, plants themselves were shown to directly react to microbial volatiles, resulting in direct or indirect plant health and growth promotion (Vespermann et al., 2007; Zhang et al., 2007; Kai et al., 2008; Gutierrez-Luna et al., 2010; Kai and Piechulla, 2010; Kwon et al., 2010; Blom et al., 2011a; Bailly and Weisskopf, 2012; Bitas et al., 2013; Farag et al., 2013; Weisskopf and Bailly, 2013; Bailly et al., 2014). Taken together, these insights interrogate the role of the bacterial volatilome in the dynamic interactions taking place in the plant natural environment. The plant-associated microbiome is virtually covering the entire plant surface and is especially abundant in the nutrient-rich rhizosphere, where the competition pressure between organisms is high (Berendsen et al., 2012; Mendes et al., 2013; De-la-Peña and Loyola-Vargas, 2014). On aerial organs, specialized bacteria survive in hostile niches in close-association with the leaf tissues (Vorholt, 2012; Bulgarelli et al., 2013; Junker and Tholl, 2013). On both below- and aboveground plant structures, bacterial populations have thus the possibility to convert metabolites found in the environment into volatile effectors, which are expected to be particularly effective when in close range of pathogens' invasion points. The pathosystem *Phytophthora infestans*-potato represents a good model to investigate VOCs' contributions to the microbial relationships occurring at the plant soil and air interfaces. This devastating pathogen, which causes the economically highly relevant potato late blight disease, can both infect aerial and soil organs. Furthermore, it enters the plant tissues where bacteria have the greatest potential to reside: around the root cap, on the leaf surface and in the stomatal chamber (Fry, 2008). In a previous screen for the anti-oomycete potential of potato-associated bacteria natively present in the rhizo- and phyllosphere, we isolated and characterized 16 *Pseudomonas*

strains with various degrees of VOCs-mediated efficacy against *P. infestans* radial mycelial growth. Our work advocated for the existence of effective bVOCs against *P. infestans* on top of well-recognized potent inorganic compounds such as hydrogen cyanide or ammonia (Voisard et al., 1989; Rudrappa et al., 2008; Blom et al., 2011b; Hunziker et al., 2015). Although many bacterial volatile compounds have been reported as bioactive against pathogens (Vespermann et al., 2007; Athukorala et al., 2010; Ting et al., 2011; Velazquez-Becerra et al., 2011; Effmert et al., 2012; Yuan et al., 2012; Groenhagen et al., 2013; Tenorio-Salgado et al., 2013; Wang et al., 2013; Hunziker et al., 2015), a large majority of the available literature has reported the effects of a few prominent molecules recorded from a limited number of bacterial strains only, if not single isolates. We previously followed the same logic and reported the main *Pseudomonas* sp. volatile metabolite 1-undecene as an active ingredient of the anti-oomycete properties of eight *Pseudomonas* isolates' volatilome (Hunziker et al., 2015). However, treating *P. infestans* with this single compound did not reach the full inhibition capacity of natural VOCs blends, suggesting that more volatile molecules are involved in the anti-oomycete activity of the *Pseudomonas*. Here we report the gas chromatography-mass spectrometry chemoprofiles of 16 selected *Pseudomonas* strains and the systematic testing of the activity of their individual pure chemical components against the growth and sporulation of *P. infestans*. Our goal was to determine the contribution of each chemical species present in the recorded bVOC spectra to the inhibition of *P. infestans* and consequently try to identify specific compounds or chemical families required for the anti-oomycete activity. Our results suggest that, in addition to biogenic soluble chemicals or protein effectors, the quest for bacterial bio-control agents should take into account the enzymatic traits leading to the production of VOCs as they represent a supplementary defense line against infection by plant pathogens.

MATERIALS AND METHODS

Chemicals and Culture Media

Chemicals were purchased from Sigma–Aldrich (Switzerland) with the exception of 1-dodecene (Dr. Ehrenstorfer GmbH, Germany) and 2-acetylfuran (Alfa Aesar, Germany). Luria-Bertani (LB) medium was prepared by dissolving 20 gl^{-1} of Difco LB Broth, Lennox (BD) and adding 15 gl^{-1} agar (Agar Agar, ERNE surface AG). Rye agar (RA) was prepared by gently boiling 200 g rye grains in 1.5 l tap water for 1 h. The liquid was then filtered through a sieve (1.5 mm mesh) and filled up to the end volume of 1 l with tap water and supplemented with 5 gl^{-1} D-glucose. 20 gl^{-1} agar were added. Petri dishes were filled using a plate-pouring machine (Mediajet, Integra Biosciences) with 18 ml of medium in standard Petri dishes (94 mm × 16 mm, Greiner Bio-One).

Microbial Strains and Culture Conditions

A *P. infestans* polypore isolate sampled in 2001 (provided by H. Krebs, Agroscope) was used for all experiments. This isolate had been maintained as mycelial culture on RA and

regularly transferred to potato slices for host passage. Petri dishes were sealed with Parafilm M (BEMIS Flexible Packaging) and incubated or stored in the dark at 18°C.

Most bacteria were isolated and maintained as in (Hunziker et al., 2015). *Pseudomonas protegens* CHA0 and its corresponding hcn^{A-} mutant CHA77 were obtained from Prof. Dr. Dieter Haas (University of Lausanne). Bacterial strains were routinely grown on LB and kept at $^-80$°C in 25% glycerol for long-term storage.

Multi Locus Sequence Alignments

In order to elucidate the phylogenetic relationships of the 16 candidate *Pseudomonas* sp. strains with respect to additional selected *Pseudomonas* reference strains, the sequences of four major housekeeping genes including 16s rRNA, gyrB, rpoD, and rpoB (Mulet et al., 2010; Gomila et al., 2015) were extracted from an Illumina MiSeq paired end (2x 300 bp) sequencing effort aimed at describing the gene inventory of these strains. The contigs that resulted from *de novo* genome assembly using the Spades algorithm (Bankevich et al., 2012) were then annotated with the software Prokka (Seemann, 2014), prior to retrieving the sequences of the four housekeeping genes and subsequently concatenating them in the order 16s rRNA, gyrB, rpoD, and rpoB. The MEGA (Molecular Evolutionary Genetic Analysis) software (Kumar et al., 2008) was used to construct a maximum-likelihood-based phylogenetic tree from the concatenated sequences of the four genes of 16 strains, as well as of nine additional reference *Pseudomonas* strains, including *P. protegens* CHA0. All generated sequences were deposited to GeneBank under the accession numbers KT890284 - KT890343[1].

Collection of Volatiles and GC/MS Analysis

The volatiles of sixteen selected strains were collected and analyzed by gas chromatography-mass spectrometry (GC/MS) using closed-loop-stripping analysis (CLSA) as described earlier (Hunziker et al., 2015) using a modified apparatus design (see Supplementary Figure S5). The strains were pre-grown at 18°C on LB-agar plates for 4 days before single colonies were resuspended and adjusted to a density of $OD_{570nm} = 1$ in sterile water. Bacterial samples were cultured by inoculating 50 µl of cell suspensions, spread as a layer using sterile glass beads into 4 cm glass Petri dishes to avoid plastic volatile contaminants and grown for 24 h at 25°C in the CLSA apparatus under constant air flow. Uninoculated LB-agar glass plates were used as controls. Trapped volatiles were extracted from the charcoal filter by rinsing the filter three times with 20 µl dichloromethane (≥99.8%, Merck, Germany). The headspace extracts were subsequently adjusted to 100 µl with dichloromethane and analyzed by GC/MS. Each experiment was repeated three times. Analyses by GC/MS were performed on a Varian CP3800 gas chromatograph (Varian, Walnut Creek, CA, USA) connected to a triple quadrupole mass spectrometer (Varian 1200, Varian). Separation by GC took place on a Rtx-5Sil MS capillary column (30 m, 0.25 mm i.d., 0.25 µm film thickness) from Restek (Bellafonte, PA, USA). As a retention gap, a 2 m Siltek guard

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[1]http://www.ncbi.nlm.nih.gov/genbank/

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column (0.53 mm i.d., Restek) was mounted in front of the separation column. Helium was used as a carrier gas at a constant flow of 1 ml.min^{-1}. The samples were injected on-column into a programmable temperature vaporization injector (temperature program: 50°C for 0.1 min, to 300°C at 200°C.min^{-1}, 30 min at 300°C, to 160°C at 20°C.min^{-1}, 20 min at 160°C). The oven temperature was programmed as follows: 10 min at 40°C, to 320°C at 25°C.min^{-1}, 1 min at 320°C. The transfer line temperature was set at 200°C. Analyte detection by MS was conducted in the electron impact mode with 70 eV ionization energy, at a source temperature of 250°C. Full scan monitoring (scan time 0.7 s) was performed in the m/z mass range from 35 to 350. Compounds were identified by comparison of mass spectra to database spectra (NIST 08 and pure commercial reference compounds), and comparison of the retention times and mass spectra previously obtained (Hunziker et al., 2015).

Effect of Pure Compounds on *P. infestans* Mycelial Growth and Sporulation

The effect of selected pure compounds of the VOCs blends on mycelial growth, sporangia production and germination, zoopore release, motility and germination of *P. infestans* was assessed as follows: 5 mm agar plugs from the edge of actively growing mycelial colonies were placed downward-faced in the center of fresh RA plates. Definite quantities of the test compounds or dilutions in dimethylsulfoxide were applied on PTFE/silicone septa (8 mm, Supelco) and placed in the center of the Petri dish lid so the test compounds faced the mycelial plug. Plates were sealed with Parafilm M and incubated upside-down in the dark at 18°C. Mycelial growth was monitored 7 days after inoculation by taking photographs and total mycelial area was further assessed using ImageJ. At the end of a 9-day incubation period, the plates were opened and sporangia were collected using 5 ml room temperature sterile water by gently rubbing the mycelial mat with a sterile glass rod. A 500 µl aliquot of the resulting suspension was dispensed into a 24-well polycarbonate plate. The number of produced sporangia and rate of germination was assessed under the binocular right after collection and 24 h later, respectively. The same procedure was used to produce zoospores, except that 10 ml of ice-cold water was laid on top of the mycelial mat and kept at 4°C for 2 h. After this incubation period, the suspension was left at room temperature for 20 min prior to collection in order to allow zoospore release to occur. A 500 µl aliquot of the resulting suspension was dispensed into a 24-well polycarbonate plate. The quantity of produced zoospores and zoospore motility was assessed under the binocular right after collection. Zoospore encystment and subsequent germination was assessed 24 h later.

To evaluate the effect of pure compounds on direct sporangial germination, sporangia were collected in 10 ml room temperature sterile water from 9 days-old mycelial plates and adjusted to a concentration of 200'000 sporangia.ml^{-1} in Eppendorf tubes supplemented with the adequate chemical treatment. No vortexing was applied in order to avoid triggering germination. After 1 day of incubation at 20°C in the dark, series of 10 µl

droplets of the suspension were mounted on glass slides and photographed under the microscope. The number of closed, open and germinating sporangia were counted using ImageJ. In both experimental setups, the inhibitory concentration yielding 50% inhibition (IC$_{50}$) of mycelial growth or sporangial germination for each treatment was calculated through extrapolation from the curve-fitted plots.

Zoospore Tracking

Zoospores from preparations obtained as described above were used to follow the trajectories of individual cells over time. Image sequences were taken for 10 s, using a Wild Heerbrugg MDG17 binocular (Wild Heerbrugg, Switzerland) coupled to a Leica DFC290 Camera using the LAS software v4.6.2 (Leica, Germany). Images were next filter-transformed using ImageJ to achieve high contrast. Single particle tracking over the segmented images was performed using the ImageJ TrackMate plugin[2], (Jaqaman et al., 2008) using semi-automated default parameters. Chemical treatments were performed immediately prior to observations and image capture to avoid any bias from the decrease of zoospore motility occurring in control conditions.

Fluorescent Label Microscopy and Analysis

Phytopthora infestans 208 m^2 was obtained from Prof. Dr. F. Mauch (University of Fribourg) and maintained on RA plates as described above. Sporangial yield was increased by exposing the Petri dish to natural light for 8 h at days 7 and 8 after inoculation. Sporangia were collected as described above 9 days after inoculation and GFP fluorescence was visualized and digital micrographs acquired using a Zeiss Axiovert 200 M stereomicroscope equipped with adequate fluorescent source and filters. The GFP signal mean gray intensities of individual sporangia were extracted using a custom-made segmentation macroinstruction protocol to estimate the signal intensity distribution at the population level.

Leaf Disk Assays

A 10 μL drop of a water suspension of 125'000 sporangia.mL^{-1} was applied in the middle of the abaxial side of a ⌀17 mm leaf disk cut from 1 month old potato plants *cv.* Victoria. Leaf disks were placed on a soaked filter paper in standard Petri dishes. Definite amounts of the test compounds were applied on PTFE/silicone septa (8 mm, Supelco) and placed in the center of the Petri dish, 3 cm away from each leaf disk. Petri dishes were closed with Parafilm M, placed in a lightproof plastic box and incubated at 18°C for a period of 8 days. *P. infestans* infection was then visualized and acquired under the binocular.

Data Analysis

Data were analyzed using the GraphPad Quikcalcs tools[3], GraphPad Prism 5 software and Microsoft Excel software.

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[2]imagej.nih.gov
[3]http://www.graphpad.com/quickcalcs/

RESULTS

The Plant-associated *Pseudomonas* Chemoprofiles Present a Conserved Volatilome

In order to correlate the production of specific bVOCs to the inhibitory potential of the 16 different *Pseudomonas* strains we previously screened, showing various VOCs-mediated inhibition against *P. infestans* (Hunziker et al., 2015), we trapped and identified the different chemical species they emitted. With the underlying hypotheses that the most active strains should produce either (1) a different set of compounds or (2) differential amounts of specific compounds, we cultured and further analyzed the bacterial emissions in standardized conditions. All tested strains were inoculated with the same density of cell suspension and let grow for the same amount of time under identical culture conditions. We chose to collect the bVOCs over a 24 h incubation period (i.e., until late stationary phase) with the aim to maximize the chance of recovering chemoprofiles that would best represent the *P. infestans* growth-inhibiting bVOC blends occurring in our previously published dual-assays (Hunziker et al., 2015). Furthermore, a steady closed-loop-stripping apparatus allowed us to trap and extract the emitted chemical species with high reproducibility. Consequently, although the GC/MS spectra obtained do not report quantitative amounts of individual chemical species, they capture very comparable relative profiles of the different test strains' biogenic emissions (**Figure 1** and Supplementary Figure S1).

Under these conditions, we were able to retrieve a large majority of the volatile compounds identified earlier and obtained similar chromatograms (Hunziker et al., 2015). However, slight changes in the gas chromatograph setup and parameters allowed us to get a better insight into the smallest and more hydrophilic components of the volatile emissions from plant-associated *Pseudomonas*. To our surprise, we discovered that in many of the tested strains the sulfur-containing compound dimethyldisulfide (DMDS) was produced in similar or higher amounts as 1-undecene (**Figure 1**), which we have described earlier as being the major chemical in the bacterial VOCs mixture (Hunziker et al., 2015). When GC peak area of definite amounts of pure DMDS and 1-undecene where compared to test spectra, a 24 h collection period yielded micrograms of both compounds, thus underlining the capacity of bacterial strains to accumulate high concentrations of VOCs in the headspace. In the vast majority of strains, 1-undecene, DMDS, 4-hydroxy-2-pentanone and benzaldehyde were the major peaks of the emission spectra. No particular chemical patterns could be unequivocally found between the different strains included in this study, neither by clustering the spectral data according to the strains' phylogeny (**Figure 1**, and Supplementary Figure S2) or origin of isolation (rhizosphere vs. phyllosphere, Supplementary Figure S1). We also did not observe a straight correlation between the presence or the amounts of a given chemical species and the full VOCs blends effects of the strains on *P. infestans* radial growth (Hunziker et al., 2015, **Figure 1**). While the
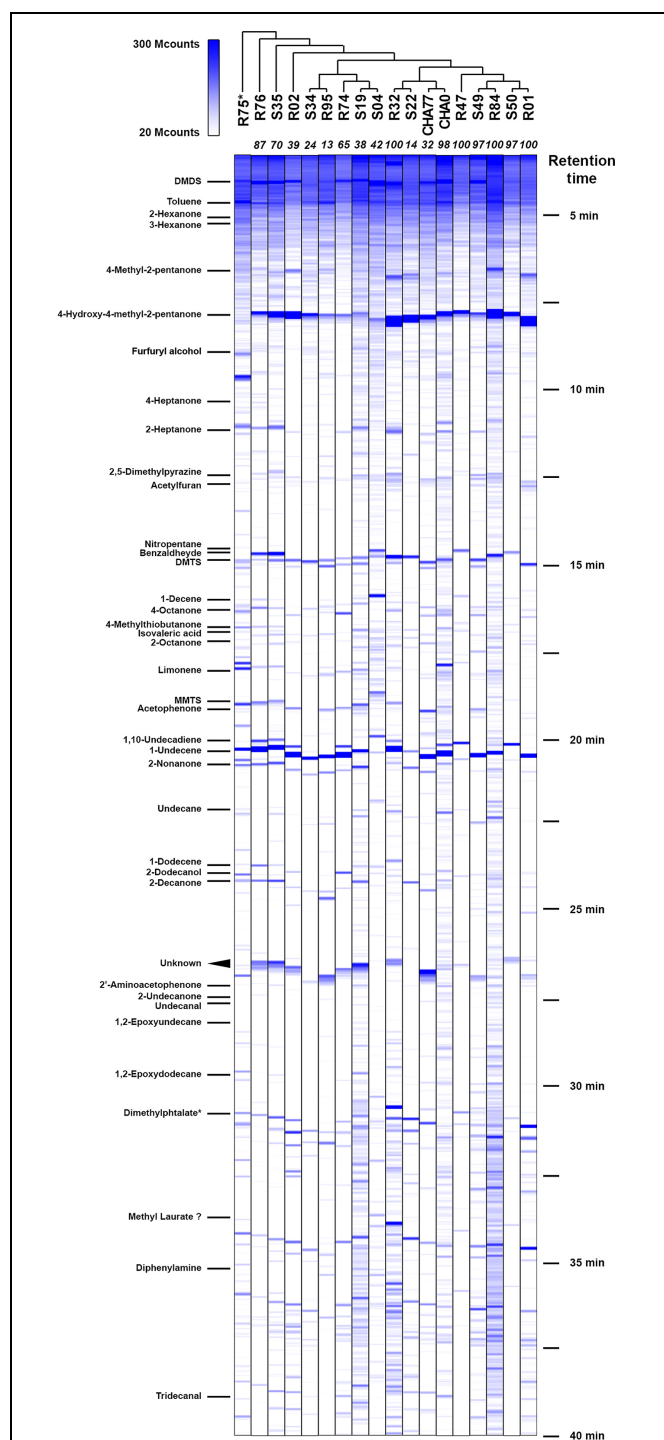
FIGURE 1 | Chemoprofiles of volatile organic compounds (VOCs) emitted by potato-associated *Pseudomonas*. Representative gas chromatography spectra for each strain are ordered according to phylogenetic clustering from multilocus sequence alignments (Supplementary Figure S2). Selected identified compounds are indicated on the left; their approximate retention times can be deduced from the right legend. Numbers in italics at the top of each spectrum represent the percentage of *Phytophthora infestans* growth inhibition triggered by the strains' VOCs blends. MS signal intensities are expressed in megacounts (Mcounts). *R75 was not identified as a *Pseudomonas* strain.

strongest *P. infestans* growth inhibition was as expected caused by HCN-producing strains (Hunziker et al., 2015), the individual volatile compounds that could contribute to the *P. infestans* inhibition triggered by non-HCN producing strains were not obvious to deduce. However, comparison of the emission spectra of the biocontrol *P. fluorescens* CHA0 (Voisard et al., 1989) with those of its corresponding HCN deficient, *hcn*A⁻ mutant CHA77 (Laville et al., 1998) revealed differences in the metabolite production (**Figure 2**). Indeed CHA77 produced lower amounts of DMTS and 1-undecene, and significantly higher amounts of DMTS and MMTS. In addition, we observed a set of two or more yet uncharacterized compounds (rt = 26.8 min) that seem to be common products from non-cyanogenic bacteria (**Figure 1**). Likewise, the CHA0 strain produced higher amounts of limonene and two supplementary compounds that await formal identification. From the same initial inoculum density, several strains such as R32, R84, CHA0, S04, or S19 had apparent higher levels of volatile production that could be explained by a higher metabolism or a better adaptation to the culture conditions (**Figure 1**). Nevertheless, the phylogenetic group comprising R74, R95, S04, S19, and S34 seemed to have repeatedly produced smaller amounts of 4-methyl-2-pentanone and 4-hydroxy-4-methyl-2-pentanone. Moreover, strains that produced the lowest, or not detectable amounts of DMDS, R01, R32, R47, S22, and S34, showed also the lowest detected amounts of DMTS and MMTS. Finally, the strain R75, that displayed a clearly dissimilar chemoprofile, was formally identified as belonging to the *Flavobacterium* genera, and not as previously reported to *Pseudomonas* (Hunziker et al., 2015), supporting the concept that bacteria could be identified at the species level via their volatile chemical signature (Thorn et al., 2011).

Effects of Pure Volatile Compounds on *P. infestans* Growth and Sporangial Behavior

The high number of sampled *Pseudomonas* volatile profiles and their high similarity prompted us to investigate the contribution of a subset of individual chemical species to the previously observed growth inhibition of *P. infestans* by VOCs inhibition. Although not exhaustive, the list of tested, commercially available compounds we present here covers a large portion of the recorded *Pseudomonas* spectra (**Figure 1** and **Table 1**). While our pharmacological screen revealed that half of the tested compounds showed mild to low growth inhibition of the plant pathogen at high applied amounts, only few compounds possessed strong inhibitory activity (i.e., an IC_{50} below 1 mg, reaching at least 30% inhibition and a $R^2 > 0.6$) against *P. infestans* mycelial growth. Nitropentane, isovaleric acid, undecanal, phenylpropanedione, propiophenone, dimethyl trisulfide (DMTS) and *S*-methyl methanethiosulfonate (MMTS) showed total inhibition of *P. infestans* growth at 1 mg. Although 1-undecene did not perform very well in hindering *P. infestans* growth within the applied amounts, its inhibitory activity was found to be close to those reported earlier (Hunziker et al., 2015). In our hands, the bacterial quorum-sensing-related molecules 2'-aminoacetophenone and acetophenone had low influence on

FIGURE 2 | Representative gas-chromatograms of *Pseudomonas protegens* CHA0 and its HCN-deficient mutant CHA77. Major peaks and associated compounds are indicated. UNK, unknown chemical species.
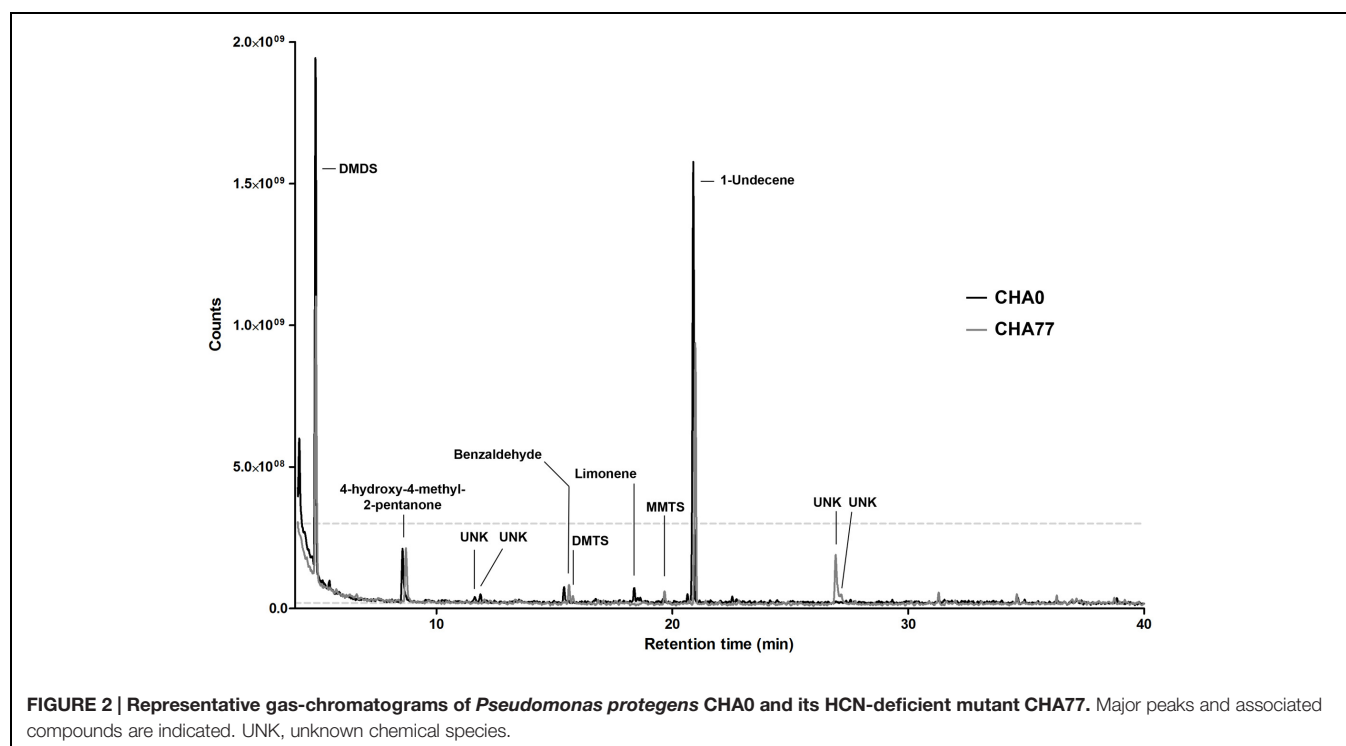
growth of the pathogen, reaching merely about 30% mycelium area reduction. In contrast, cyclic ketones sharing a similar backbone such as propiophenone, and phenylpropanedione were amongst the most potent chemicals assayed in this study, with IC50 values ranging from 10 µg to 0.5 mg. Strikingly, nearly all sulfur-containing VOCs showed a great mycelial growth inhibition potential. From all assessed chemical species, MMTS was the most potent with an estimated IC_{50} in the low nanogram range. We did not observe specific apparent phenotypic defects in *P. infestans* upon exposure to the tested pure chemicals, with the exception of 2-undecanone (and to a lesser extent 2-decanone) treatments, which resulted in a dose-dependent densification of the mycelial mat and extended mycelium aerial growth, resulting in a "fluffy" appearance of the colony (Supplementary Figure S3).

Phytophthora infestans is a complex and adaptive eukaryotic lifeform that can spread and survive in multiple diverse forms through its life cycle, inside and outside its *Solanaceae* hosts (Fry, 2008; Fry et al., 2015). While mycelium growing on nutrient rich media appeared robust to the chemical treatments, we examined the impact of the individual volatiles exposure on both asexual forms that serve as infection vectors, namely sporangia and zoospores (Fry, 2008).

We then assessed the ability of our *P. infestans* isolate to produce sporangia and discharge zoospores after the end of the mycelial growth period in presence of the pure volatiles. We also monitored the germination rate of both spore types and the motility of zoospores. Obviously chemicals that greatly impaired *Phytophthora* growth, nitropentane, isovaleric acid, undecanal, DMTS, MMTS, propiophenone and phenylpropanedione had a strong impact on sporangia production, and consequently

on zoospore numbers (**Figure 3**). However, other compounds that did not greatly impede mycelial growth, such as the simple ketones 3-hexanone, 2- and 4-heptanone, 2-decanone and 2-undecanone, 4-hydroxy-methyl-2-pentanone as well as the alkenes 1-undecene and 1-dodecene significantly impacted sporangia production (**Figure 3**), suggesting that these mid-to-long hydrocarbon chains could specifically interfere with this process. At the highest amounts tested, diphenylamine and 2-acetylthiazole also decreased sporangial formation. The same set of chemicals also decreased the germination rate of sporangia, though this measurement does not depend on sporangia production, indicating that the spores may have been hindered during their developmental process or that non negligible amounts of the supplied VOCs were extracted during sample preparation. Based on the data displayed in **Figure 3**, limonene, 1-dodecene, and *S*-methylbutanethioate seem to have a specific effect on sporangia germination after mycelium exposure. Logically, the production of zoospores largely followed the pattern of sporangia production in terms of inhibition mediated by individual VOCs. None of the tested compounds seemed to specifically block this process. Further, zoospores released from sporangia freely swam in most treatments and exhibited no particular defects, with the notable exception of treatments of the mycelium with high amounts of MMTS, where a large number of zoospores underwent cell lysis. Nevertheless, exposure to 2-undecanone, limonene, 1-undecene, diphenylamine, *S*-methylbutanethioate, acetophenone, or phenylacetone during mycelial growth significantly decreased zoospore motility. Nitropentane, isovaleric acid, undecanal, as well as propiophenone and phenylpropanedione had also a strong negative impact on swimming in the highest

TABLE 1 | *Phytophthora infestans* mycelial growth inhibition after exposure to pure volatile organic compounds (VOCs).

	No constraints				Constrained fit Top = 100, Bottom = 0[a]	
	Bottom[a]	**Top**[a]	**Rel. IC50 (mg)**	R^2	**IC50 (mg)**	R^2
Nitropentane	**−5.583**	**98.73**	**0.02563**	**0.9719**	**0.02163**	**0.9682**
Undecane	114.1	100.9	0.01501	0.2055	*	*
1,2-Epoxydodecane	39.74	90.44	2.51	0.2489	2.911	−0.3972
4-Hydroxy-4-methyl-2-pentanone	113.4	99.79	0.000272	0.122	42.59	−0.3338
2-Hexanone	69.61	99.21	0.000292	0.4418	2.809	−1.027
3-Hexanone	*	*	*	*	*	*
2-Heptanone	~−14680	98.32	~418.9	0.4586	1.754	0.446
4-Heptanone	*	*	*	*	*	*
2-Octanone	**59.42**	**99.61**	**9E-05**	**0.7706**	**0.3907**	**−1.197**
4-Octanone	**41.59**	**95.91**	**0.02942**	**0.8831**	**0.1871**	**0.4772**
2-Decanone	90.9	99.99	2.55E-05	0.1651	4.735	−0.02398
2-Undecanone	71.13	99.58	0.000398	0.3975	3.126	−0.807
4-Methylthiobutanone	−969900	100.1	47231	n.d.	3.961	0.2865
S-Limonene	132.5	100.1	0.6097	0.4483	*	*
R-Limonene	*	*	*	*	*	*
1-Undecene	75.48	98.87	0.000207	0.6268	1.739	−0.6645
1-Dodecene	*	*	*	*	59.14	−0.1474
Isovaleric acid[†]	**−62.37**	**103.9**	**0.8344**	**0.8724**	**0.3167**	**0.8337**
Methyl laurate	*	*	*	*	3.565	−2.136
Undecanal	**−5.856**	**106.3**	**0.01611**	**0.9688**	**0.01638**	**0.9562**
Tridecanal	73.23	105.6	0.001336	0.6605	3.035	−0.2182
Acetylfuran	71.97	99.26	0.000151	0.5422	1.034	−0.09331
Diphenylamine	*	*	*	*	*	*
S-Methylbutanethioate	~−5331	99.95	~102.9	0.7277	0.9715	0.7188
Bis(methylthiomethyl)sulfide	90.34	94.02	0.04948	0.004254	8.637	−0.07327
Dimethyl disulfide	~−13740	96.01	~559.2	0.6117	2.452	0.5208
Dimethyl trisulfide	**−9.093**	**99.84**	**0.06971**	**0.8518**	**0.0574**	**0.8443**
S-Methyl methanethiosulfonate[†]	**1.561**	**93.72**	**0.004302**	**0.9854**	**0.003588**	**0.9775**
Dimethylpyrazine	81.92	100.5	5.56E-05	0.3498	4.979	−0.4588
2-Dodecanol	81.48	93.01	0.2443	0.1974	4.7	−0.5748
Furfuryl alcohol	73.75	103.9	0.000571	0.5662	2.456	−0.318
Farnesyl acetone	83.45	99.95	3.27E-05	0.3287	2.512	−0.3345
2′-Aminoacetophenone	**69.61**	**100**	**1.06E-05**	**0.6025**	**2.043**	**−2.174**
Acetophenone	74.4	99.56	0.000218	0.4854	1.147	0.1975
Phenylacetone	**60.35**	**99.42**	**9.66E-05**	**0.7837**	**0.4146**	**−1.168**
Propiophenone	**−3.049**	**99.82**	**0.02381**	**0.9769**	**0.02195**	**0.9758**
Phenylpropanedione[†]	**−7.092**	**106.5**	**0.01691**	**0.9765**	**0.01677**	**0.9618**
2-Phenylethanol	**43.46**	**96.46**	**0.02484**	**0.8582**	**0.1679**	**0.36**
2-Acetylthiazole	*	*	*	*	*	*
Benzaldehyde	*	*	*	*	70.75	−0.1969

[a]% of P. infestans mycelial growth; [†]Compounds considered efficient in both mycelial growth and sporangial germination inhibition; *Curve fitting did not converge. Grayed cells indicate S-containing compounds. Relevant non-linear regression parameters [Log(inhibitor) vs. Response] are derived from pharmacological assays. All test compounds were assayed at 1 mg, 100 μg, 10 μg, 1 μg, 100 ng. In addition, DMTS was tested at 5 mg, 500 μg and 50 μg and dimethylpyrazine and MMTS were tested at 2 mg, n = 4–12. Curve fitting was performed with or without maximal constraints (top = 100% of P. infestans growth, bottom = 0% of P. infestans growth) to extrapolate IC_{50} and relative IC_{50} values for each compound, respectively. Bold indicates compounds displaying at least 30% inhibition, an IC_{50} below 1 mg and a R^2 > 0.6.

treatments, while DMDS, DMTS, and MMTS showed the greatest inhibition. As for sporangia, the influence of pure compounds on the formation of germ tubes from encysted zoospores mostly resembled the effect on zoospore production. Taken together, these results suggest that *Pseudomonas* bVOCs have the potential to alter and impede *P. infestans* development, and that bioactive compounds mostly act similarly on the different lifeforms of the pathogen (mycelium, sporangia, zoospores) rather than specifically interfering with only one or the other.
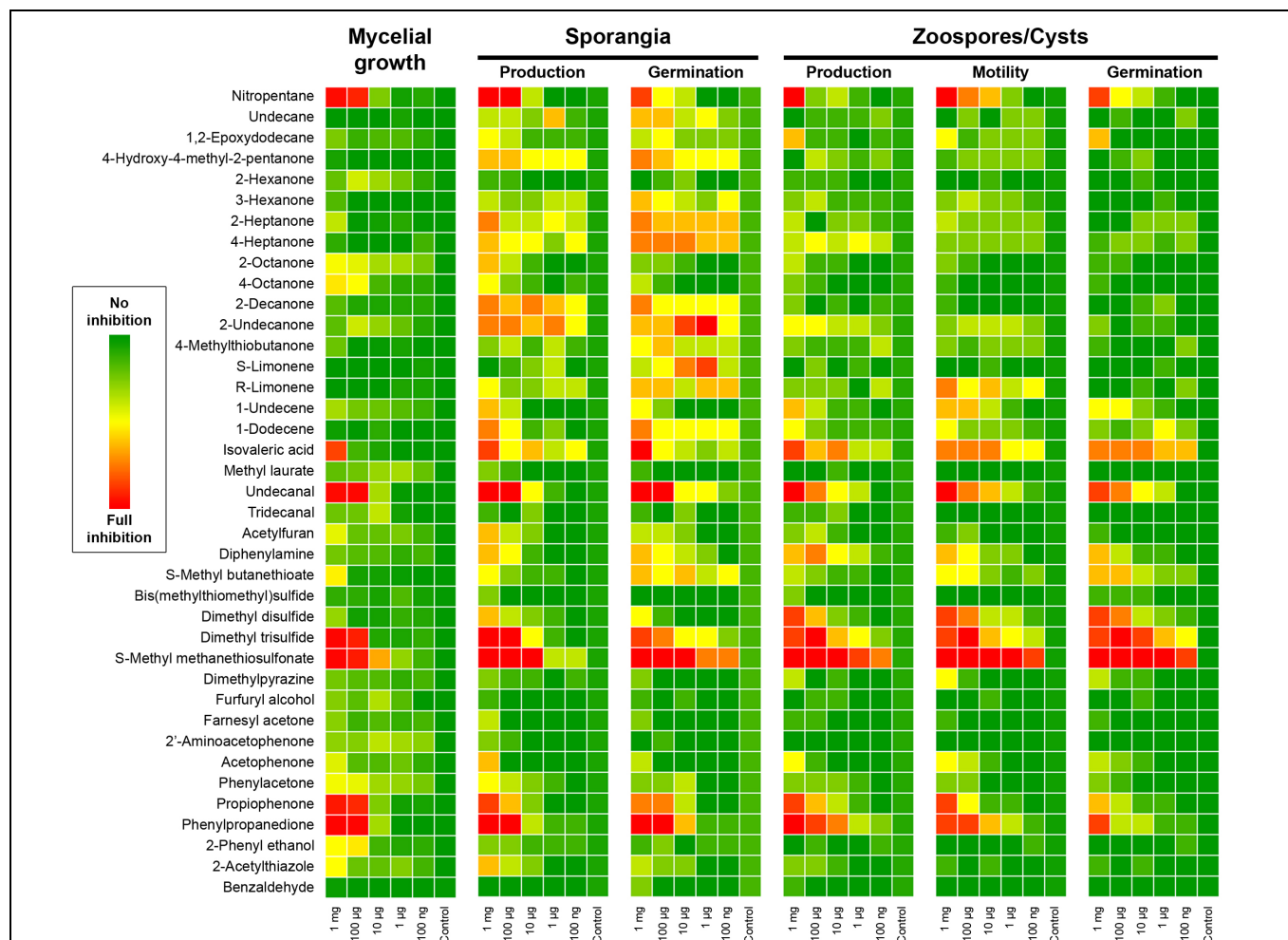
FIGURE 3 | *Phytophthora infestans* mycelial growth, sporangiogenesis, zoosporogenesis, and spore development after exposure to *Pseudomonas* pure VOCs. The heatmap color code represents the inhibitory effect obtained in each condition. For mycelial growth the percentage of total mycelium area compared to control is reported. Other parameters were scored on a 0–5 scale as follows: sporangia production, 0 = no sporangia in the field of view, 5 = 100% of control; sporangia germination, 0 = no germinated sporangia in the field of view, 5 = 100% of control; zoospore production, 0 = no zoospores in the field of view, 5 = 100% of control; Zoospore motility, 0 = no mobile zoospores, 5 = 100% of control; cystospore germination, 0 = no germinated cysts in the field of view, 5 = 100% of control. Results presented here are the average of two independent experiments with 4–5 replicates each.

Pseudomonas Volatilome Encompasses Molecules Interrupting *P. infestans* Direct Sporangial Germination

The fungicides currently in use to constrain late blight spreading in potato crops vary in their activity against the different developmental stages in the life cycle of *Phytophthora* species, acting either against mycelial growth, zoospore release, zoospore motility, or germination of spores (Gisi and Cohen, 1996; Cohen and Gisi, 2007). Therefore, in order to select for *P. infestans* biocontrol biogenic volatiles, we focused this work on direct sporangia germination for the following reasons: (1) the *P. infestans* population shift observed since the 1980's displays A2 mating type-dominated populations that tend to favor direct germination (Li et al., 2013; Fry et al., 2015), (2) sporangia are easily generated and collected in high yields, thus allowing larger

screening efforts in the laboratory; (3) sporangia germination is easy to score and requires minimal equipment. We collected mature sporangia from control-grown *P. infestans* colonies and incubated them in serial dilutions of the test chemicals. After 24 h of treatment, about half of the chemicals tested showed inhibition of the germination process at 1 mg treatments (**Table 2**). However, the inhibitory effect rapidly dropped in most cases at lower drug amounts. Volatile species that demonstrated a significant inhibition potential (i.e., $IC_{50} < 1$ mg, reaching at least 30% inhibition and a $R^2 > 0.6$) were 3-hexanone, 1-dodecene, isovaleric acid, *S*-methylbutanethioate, MMTS, furfuryl alcohol, acetophenone, phenylpropanedione, and 2-acetylthiazole (**Table 2**). Although 1-undecene and nitropentane treatments extrapolations did not meet these criteria, both molecules displayed satisfactory inhibitory power. Amongst these compounds, nitropentane, isovaleric acid and MMTS, as well

TABLE 2 | *Phytophthora infestans* sporangia germination inhibition after exposure to pure VOCs.

	No constraints				Constrained fit Top = 100, Bottom = 0[a]	
	Bottom[a]	**Top[a]**	**Rel. IC50 (mg)**	**R^2**	**IC50 (mg)**	**R^2**
Nitropentane	30.68	98	6.054	0.7341	0.000252	0.4156
Undecane	87.97	~103.4	~6.271E-12	0.1019	5.771	−0.1492
1,2-Epoxydodecane	84.19	101.3	9.437E-7	0.1212	*	*
4-Hydroxy-4-methyl-2-pentanone	22.11	88.35	0.1844	0.4354	0.2635	0.3038
2-Hexanone	81.61	101.5	6.951E-5	0.132	2.818	−0.09947
3-Hexanone	**11.26**	**100.8**	**0.1289**	**0.6432**	**0.1837**	**0.6372**
2-Heptanone	41.46	102.1	0.06601	0.4721	0.5089	0.3916
4-Heptanone	35	108	0.03331	0.485	0.1637	0.3915
2-Octanone	53.09	96.4	0.01547	0.4561	0.7168	0.03963
4-Octanone	63.7	97.51	0.03694	0.1883	1.513	0.07603
2-Decanone	84.93	101.3	0.2728	0.07671	7.492	0.07136
2-Undecanone	81.67	101.4	4.226E-5	0.1358	2.818	−0.1261
4-Methylthiobutanone	90.44	102.8	2.505E-4	0.04701	7.544	−0.00575
S-Limonene	~~−11300	97.04	~199.6	0.4006	0.7464	0.3854
R-Limonene	53.63	99.38	0.2099	0.1713	1.455	0.1585
1-Undecene	~~−14410	93.32	~182.2	0.7917	0.3083	0.7284
1-Dodecene	**24.46**	**96.56**	**0.108**	**0.7615**	**0.2333**	**0.701**
Isovaleric acid[†]	**14**	**94.64**	**5.327E-4**	**0.81**	**0.000796**	**0.7574**
Methyl laurate	83.74	100.6	2.169E-5	0.08009	2.313	−0.00186
Undecanal	30.21	97.99	3.345E-5	0.7653	0.000142	0.4182
Tridecananl	46.49	~98.06	~8.653E-10	0.63	0.001213	−0.3044
Acetylfuran	33.66	98	9.805E-6	0.4782	9.77E-05	0.1851
Diphenylamine	−82.89	84.46	1.065	0.5583	0.1593	0.4402
***S*-Methylbutanethioate**	**−18.13**	**93.34**	**0.2622**	**0.7281**	**0.1404**	**0.7005**
Bis(methylthiomethyl)sulfide	−42.27	87.04	0.6047	0.5751	0.1619	0.4925
Dimethyl disulfide	*	*	*	*	*	*
Dimethyl trisulfide	46.05	94.39	0.08824	0.2538	0.6927	0.1751
***S*-Methyl methanethiosulfonate[†]**	**4.41**	**88.1**	**0.006435**	**0.7354**	**0.005417**	**0.6915**
Dimethylpyrazine	*	*	*	*	2.1E-11	−5.633
2-Dodecanol	*	*	*	*	*	*
Furfuryl alcohol	**8.759**	**94.98**	**0.2501**	**0.65**	**0.2742**	**0.6267**
Farnesyl acetone	82.46	101.2	7.91E-5	0.1591	4.674	−0.2519
2′-Aminoacetophenone	93.41	100.8	0.00656	0.01404	10.74	0.008978
Acetophenone	**−2.388**	**90.19**	**0.1245**	**0.6696**	**0.07803**	**0.6292**
Phenylacetone	89.14	100.5	2.18E-4	0.0382	13.66	−0.06405
Propiophenone	24.83	91.18	0.05952	0.5433	0.1185	0.4394
Phenylpropanedione[†]	**9.045**	**90.58**	**0.09959**	**0.6167**	**0.09691**	**0.5636**
2-Phenylethanol	*	*	*	*	*	*
2-Acetylthiazole	**−65.09**	**97.91**	**1.258**	**0.7521**	**0.4172**	**0.7421**
Benzaldehyde	82.16	98.91	0.001011	0.1005	3.161	−0.02778

[a]*% of P. infestans sporangial germination; [†]Compounds considered efficient in both mycelial growth and sporangial germination inhibition; *Curve fitting did not converge. Grayed cells indicate S-containing compounds. Relevant non-linear regression parameters [Log(inhibitor) vs. Response] are derived from pharmacological assays. All test compounds were assayed at 1 mg, 100 μg, 10 μg, 1 μg, 100 ng per ml. In addition, DMDS, DMTS, and MMTS were tested at 10 ng per ml, n = 4. Curve fitting was performed with or without maximal constraints (top = 100% of P. infestans growth, bottom = 0% of P. infestans growth) to extrapolate IC$_{50}$ and relative IC$_{50}$ values for each compound, respectively. Bold indicates compounds displaying at least 30% inhibition, an IC$_{50}$ below 1 mg and $R^2 > 0.6$.*

as diphenylamine, were the only species to completely prevent the initiation of sporangia germination at 1 mg treatments. All other compounds displayed various degrees of germ tube elongation. Interestingly, the ketones 3-hexanone, 2-undecanone and, to a lesser extent, 2-decanone often led to decreased germ tube lengths and swellings or bursts of the growing germ tube tip, as well as ectopic initiation site (Supplementary Figure S4). This suggests that accumulation of these molecules specifically hinders the normal growth of the germ tube but not its initiation *per se*. We conclude that the sporangial germination process is widely sensitive to biogenic volatiles. Although the amounts employed in these experiments do probably not reflect the

bVOCs quantities produced in the rhizosphere or phyllosphere in addition to the poor water solubility of some test chemicals, it seems reasonable to postulate that several individual volatiles contribute synergistically to the activity of the whole blend of bVOCs.

Sulfur-containing and Simple Ketones VOCs are *Bona Fide P. infestans* Inhibitors

Out of the forty single volatiles we tested, two compound groups retained our attention for their potent differential activity on *P. infestans* (**Figure 3** and **Table 2**): the sulfur-containing compounds DMDS, DMTS and MMTS and the simple ketones 3-hexanone, 2-decanone, and 2-undecanone. The first compounds seem able to block *P. infestans* growth and development while the latter specifically hinders sporangial germination when directly applied to the sporangia. We therefore investigated further the mode of action of DMTS, MMTS, and 3-hexanone using *P. infestans* 208 m^2, a strain constitutively expressing the GFP fluorescent protein (Si-Ammour et al., 2003). With the aim to examine if the tested VOCs show sporicidal activities, we compared the GFP signal intensities of sporangial populations exposed to increasing amounts of volatiles for 24 h (**Figure 4A**). While control sporangia populations displayed a broad distribution of mean fluorescence signal intensities, ranging from close to background to highly fluorescent sporangia, both DMTS and MMTS treatments shifted the population fluorescence to low signals, presumably indicating that sporangia died during treatments. This hypothesis was further verified by treating ungerminated and germinated, GFP-expressing *Phytophthora* sporangia and cystospores with increasing amounts of these sulfur compounds. After 20 min of exposure with 1 mg MMTS, the GFP signal dramatically decreased in both spores and germ tubes (**Figure 4B**) and appeared practically abolished after 40 min without affecting the cellular structure while control treatments showed constant GFP signals over the same time frame. This suggests that MMTS directly blocked the spores' cellular activity. In addition, propidium iodide cell viability tests confirmed the death of treated cells (results not shown). Since cell wall-free zoospores are recognized as being highly sensitive to exogenous chemicals (Judelson and Blanco, 2005; Chen et al., 2012) we tested the zoosporicidal potential of MMTS. We subjected fresh, freely swimming zoospore preparations to serial dilutions of MMTS and assessed their motility under the binocular. Representative zoospore trajectories obtained via computer-assisted single particle tracking are shown in **Figure 4C**. Surprisingly, MMTS treatments displayed a significant decrease of zoospore swimming at concentrations as low as 1 fg.ml^{-1}, thus revealing a strong potency (data not shown). At highest concentrations (>1 μg.ml^{-1}) MMTS immediately blocked zoospore motility followed by cell lysis. Moreover, during the course of these experiments carried out in 24-well plates, the volatile diffusion of MMTS from the high concentration wells was sufficient to inhibit completely zoospore motility in distant control wells.

Conversely, exposure of GFP-expressing sporangia to 3-hexanone led to a population shift toward sporangia with higher fluorescent signals that did not correlate with sporicidal effects (**Figure 4A**). At highest amounts (>10 μg.ml^{-1}), 3-hexanone treatments triggered a reorganization of the inner sporangial GFP signal comparable to that of germinating sporangia although without germ tube production (**Figure 4D**). These observations, together with the abnormalities observed during sporangial germination described above (Supplementary Figure S4) seem to indicate that 3-hexanone is not sporicidal but rather directly interferes with the germination process of sporangia.

S-Containing VOCs Protect Leaf Disk from Sporangia-mediated Tissue Invasion

The ultimate goal of this work was to investigate whether biogenic volatiles produced by native potato-associated bacteria could be exploited to control late blight infection in planta. Consequently, we used our standardized laboratory leaf disk assay to assess the impact of pure VOCs on disease progression. Potato leaf disks inoculated with a sporangial suspension droplet were incubated for 8 days in presence or absence of 1 mg of DMDS, DMTS, or MMTS (**Figure 5**). At the end of the experiment, inoculated unexposed controls displayed strong symptoms with a dense mat of sporangiophores while treatments with the sulfur compounds reflected their respective inhibitory potential deduced from the experiments described above. At this amount, exposure to DMDS drastically reduced sporangiophore production and both DMTS and MMTS treatments prevented disease development. Regarding the facts that our leaf disk experimental design favors the direct sporangial germination in *P. infestans* infection, this suggested that both DMTS and MMTS treatments blocked this event. This assumption was further confirmed by microscopic observations (data not shown).

DISCUSSION

Late blight remains the most devastating potato disease worldwide and is commonly managed via recurrent applications of a wide range of systemic fungicides in conventional farming or of copper-based products in organic farming, respectively (Gachango et al., 2012; Olle et al., 2014). However, the selection pressure due to the increase in fungicide spraying frequencies in combination with the emergence of rapidly changing, recombinant pathogen populations first observed in the 1980s has brought the problem of resistance to the field (Zwankhuizen and Zadoks, 2002; Nowicki et al., 2012; Wang et al., 2012; Childers et al., 2015) and conventional late blight control has become tenuous. Likewise, the deleterious accumulation of copper and its toxicity for the soil ecosystems urgently calls for alternate, innovative solutions to fight *P. infestans* while preserving the environment (Dorn et al., 2007).

Many laboratories trying to pinpoint the molecules responsible for the observed activity of biogenic emissions of VOCs face the enormous complexity of the obtained compounds mixtures. In a previous work, we tested if the major
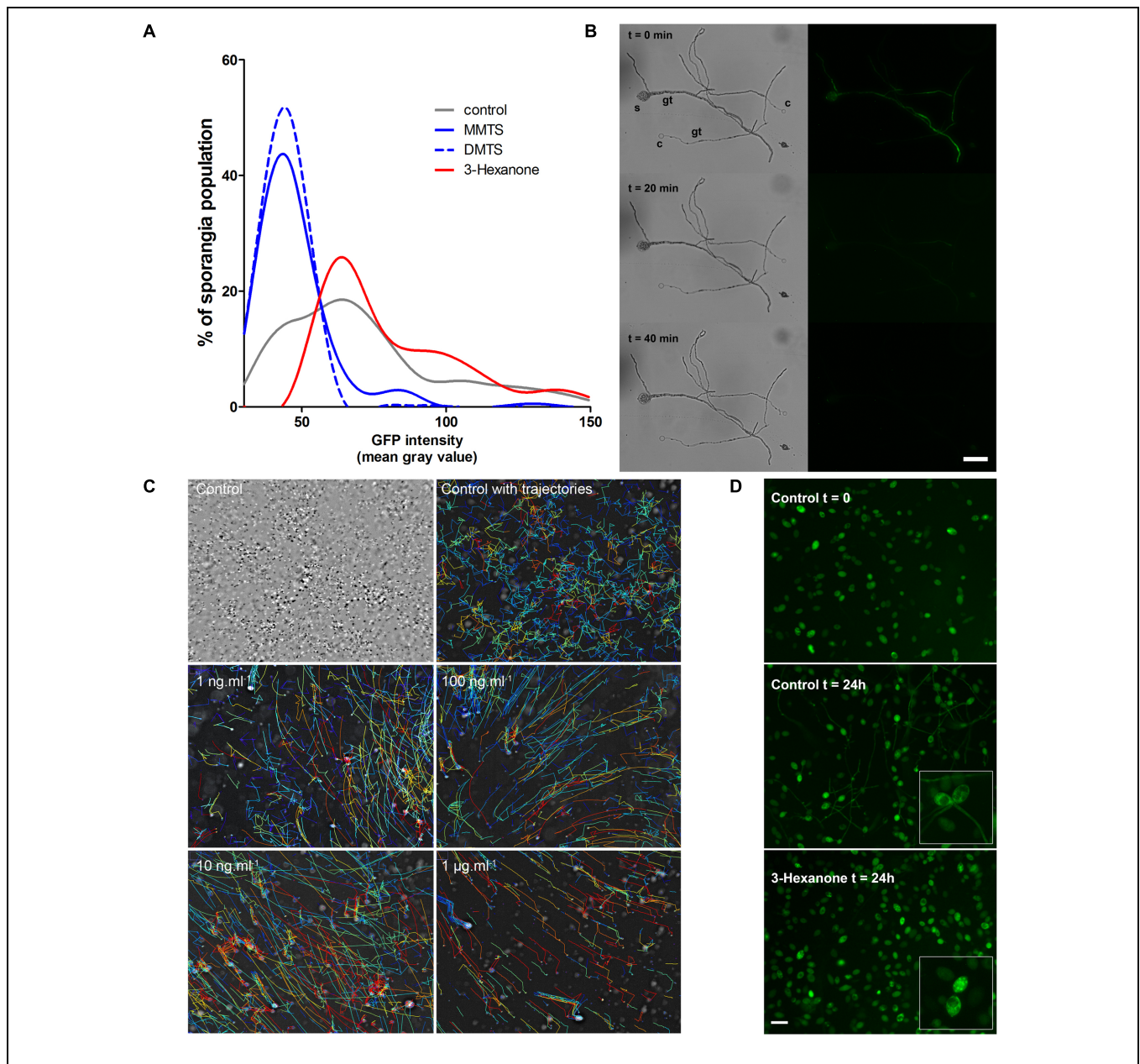
FIGURE 4 | (A) Distribution of *P. infestans* 208 m^2 sporangial population fluorescence intensity after exposure to selected single *Pseudomonas* VOCs. Representative curves shown here are from a single experiment using 10 µg treatments (n = 600–1500). Experiments were repeated three times with similar results. **(B)** 208 m^2 sporangium (s) and cystospores **(C)** show a rapid GFP signal decrease after MMTS treatment. Gt, germ tube. Bar = 50 µm. **(C)** Single particle tracking of zoospores upon MMTS treatments. Upper-left image shows an overlay of individual time lapse frames. Every second frame was color-inverted to expose the zoospore movements. Computed velocities of individual zoospores trajectories are shown in a thermal color gradient, red = high and blue = low velocity. Note the straightening of the trajectory lines under MMTS treatments. The experiment was repeated at least three times with similar results. **(D)** Representative micrographs of *P. infestans* 208 m^2 sporangia germinating in presence or absence of 3-hexanone. Inserts show higher magnification of the inner sporangia GFP signal. Bar = 50 µm.

component of the *Pseudomonas* volatilome, namely 1-undecene, had sufficient inhibitory power to hinder *P. infestans* growth and development (Hunziker et al., 2015). Although 1-undecene certainly contributes to the total activity of the whole volatile blend, the doses required to achieve significant *P. infestans* growth inhibition were very high. One would expect that the most potent chemical species should grant inhibitory effects

even at very low amounts. Bacteria producing large amounts of the volatile respiratory inhibitor HCN or ammonia were already demonstrated to hold such antimicrobial properties (Voisard et al., 1989; Rudrappa et al., 2008; Hunziker et al., 2015). However, *Pseudomonas* strains devoid of HCN or ammonia synthesis pathways have also been reported to impede the growth and development of several fungal or fungal-like species

FIGURE 5 | Representative micrographs of VOC-treated potato leaf disK infection assays. Note the complete absence of necrotic tissues or *P. infestans* sporangiophores in both DMTS and MMTS treatments. The experiment was repeated three times with similar results. Bar = 5 mm.

(Trivedi et al., 2008; Athukorala et al., 2010; Elkahoui et al., 2015; Hunziker et al., 2015; Sheoran et al., 2015), which has stimulated increasing interest to further expl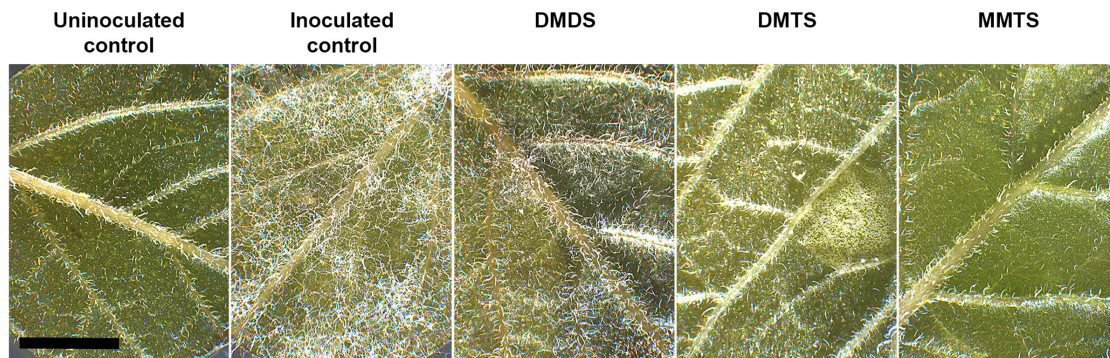ore the bacterial volatilomes. Indeed, the growing knowledge on the chemical diversity of VOCs produced by bacteria (Schulz and Dickschat, 2007; Bos et al., 2013; Kanchiswamy et al., 2015) shed light on the latent discovery of new molecules that could contribute to the development of sustainable crop management strategies. Hence, our deep although not yet exhaustive screening effort for the anti-oomycete activities of individual volatile chemicals produced by plant-associated *Pseudomonas* strains demonstrated that the VOCs-mediated inhibition of *P. infestans* observed with the whole natural blends is probably not exclusively caused by specific single chemical species, but is most likely a result of the joint and possibly synergistic activities of several compounds. Although the repertoire of *Pseudomonas* VOCs contains very harmful substances toward *Phytophthora*, such as DMTS or MMTS, single active ingredients of the complex mixture are unlikely to serve as a molecular marker for biocontrol strain selection. Supplementary quantification of the amounts and effects of individual volatile products on *P. infestans* and the inhibitory effects of entire bouquets of bacterial strains could further help to statistically determine the contribution of discrete compounds to the synergistic inhibitory action of a given volatilome. Such robust multivariate analysis was already performed to discriminate bacterial species and strains based on their VOCs profiles (Thorn et al., 2011).

It is generally admitted that bVOCs mainly originate from background catabolism such as degradation of fatty acids, proteolysis and glycolysis (Schulz and Dickschat, 2007; Kai et al., 2009; Kanchiswamy et al., 2015). It is then not surprising that most strains investigated in this study, although genetically diverse among the *Pseudomonas* group, present similar VOCs profiles (**Figure 1**, Supplementary Figure S2). Grown in the same conditions, our test strains exhibited slight variations in the amounts of produced compounds that point to no obvious relationship between the composition of the volatilome and its overall inhibitory activity. However, close scrutiny of the enzymatic activities leading to the dynamic production

of most potent volatile species could help understanding the collected experimental data. To that purpose, a comparative analysis of the plant-associated microbiome genomic data should be carried out to further detail the presence or absence of key genetic traits that grant efficient VOCs toolsets to individual bacterial strains or isolates. In particular, monitoring the expression of pivotal genes or proteins under different relevant growth conditions (Omasits et al., 2013) could provide useful differential expression information about the activity of selected pathways that lead to volatile production, as well as the dynamics of VOCs biogenesis and its occurrence *in natura*. Manipulations of the *Pseudomonas* critical VOCs-related genes would as well detail the individual contribution of single compounds, as demonstrated for plant-growth promoting, indole-producing rhizobacteria (Bailly et al., 2014). Finally, this knowledge, together with a continuous flow of reports of the identification of novel volatilome constituents could lead to the establishment of synthetic volatile combinations mimicking the natural blends. Such synthetic mixtures pave the way for their systematic pharmacological testing to investigate the putative synergistic effects of such complex signals. This approach has been successfully employed with VOCs originating from the fern endophytic fungus *Nodulisporium* sp., suppressing the growth of various plant fungal and oomycetes pathogens at low concentrations (Riyaz-Ul-Hassan et al., 2013).

Many studies, including our work, have reported the inhibition of a growing number of distinct plant pathogens through bacterial emissions in dual assays (Rengel and Marschner, 2005; Vespermann et al., 2007; Athukorala et al., 2010; Ting et al., 2011; Velazquez-Becerra et al., 2011; Effmert et al., 2012; Yuan et al., 2012; Groenhagen et al., 2013; Tenorio-Salgado et al., 2013; Wang et al., 2013; Contreras-Cornejo et al., 2014; Audrain et al., 2015; Hunziker et al., 2015), thus describing an experimental setup where bacteria grew on rich media in the physical presence of the target species. However, for technical reasons, the chemical identification of volatile compounds is generally performed in absence of the pathogen. Still, the possibility that bacteria respond to the target species should not be excluded and assessments of the expression levels of genes implicated in the production of VOCs in the presence

of the pathogen should be performed. Such experiments are currently under investigation in our laboratory. Furthermore, the experimental data demonstrating a clear role of VOCs in the direct biocontrol of plant pathogens outside of the controlled, *in vitro* laboratory setting is lacking to date. While the prospective sum of work has underlined the potential of volatiles in mediating benefits in plant health and fitness (Bailly and Weisskopf, 2012; Farag et al., 2013; Kanchiswamy et al., 2015), the concrete contribution of bVOCs to direct plant protection requires further study. As an example, the nutrient-rich rhizosphere hosts a vast microbial diversity and is assumed to offer enough metabolite variety to contribute to volatile production (Morgan et al., 2005; Rengel and Marschner, 2005; Bulgarelli et al., 2013). However, the cellular populations growing on the root surface are by far smaller than those used for chemoprofiling and one could expect that each individual microbial species adds to the complexity of the total volatilome expressed *in situ*. Blom et al. (2011a) clearly demonstrated that biogenic emissions primarily depend on the composition of feeding media, thus inferring that the volatiles profile of a given strain grown in Petri dishes would qualitatively and quantitatively differ from the profiles occurring in the natural plant-bacteria association. The same study also convincingly reported that bacteria have the ability to generate complex volatilomes even in very limited nutrient conditions (Blom et al., 2011a). Still, the experimental design that could precise the identity and amounts of VOCs produced on the root surface is yet to be established.

We report here, beyond the *P. infestans* mycelial growth inhibition, the negative impact of a dozen of *Pseudomonas* volatiles on the normal spore development, and clear sporicidal activity for four common components of the VOCs blend. Given the sporangia germ tube malformations triggered by some of the tested ketones and the general inhibitory potential of the compounds assayed against both sporangia and zoospores, it seems reasonable to assume that the volatiles from *Pseudomonas* strains that we isolated from the potato plant could participate in a supplementary defense line against *Phytophthora* infection. As discussed above, the prerequisites are in one hand that the synthesis of these active compounds occurs at local levels and in the other hand that sufficient amounts should be produced to reach inhibitory conditions. The mucilage surrounding the root cap, the porous nature of soil and the stomatal chamber space offer space-restricted niches where accumulation of VOCs can happen and where both bacteria and the pathogen can interact. The nascent concept of biofumigation (Matthiessen and Shackleton, 2005; Goates and Mercier, 2009; Morales-Rodriguez et al., 2012) envisages the application of natural bioactive compounds such as the plant-growth promoting, sulfur-containing DMDS (Meldau et al., 2013), already in use as suppressive soil fumigant in agriculture (Auger et al., 1989; Van Wambeke et al., 2009), via the enrichment of VOC-producing microorganisms to target pathogens. Therefore, the isolation, characterization, selection and stable reintroduction of native plant-associated bacteria into potato crops promises an efficient and sustainable strategy to manage late blight at low costs. Alternatively, the natural origin of potent inhibitors identified in the bacterial volatilome, like DMTS or MMTS, could also lead to different organic farming strategies as they can be readily extracted from *Cruciferae* and *Liliaceae* species (Nakamura et al., 1996; Kyung and Fleming, 1997).

AUTHOR CONTRIBUTIONS

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SUPPLEMENTARY MATERIAL

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Bacterial-Plant-Interactions: Approaches to Unravel the Biological Function of Bacterial Volatiles in the Rhizosphere

Marco Kai*, Uta Effmert and Birgit Piechulla

Department of Biochemistry, Institute of Biological Science, University of Rostock, Rostock, Germany

Rhizobacteria produce an enormous amount of volatile compounds, however, the function of these metabolites is scarcely understood. Investigations evaluating influences on plants performed in various laboratories using individually developed experimental setups revealed different and often contradictory results, e.g., ranging from a significant plant growth promotion to a dramatic suppression of plant development. In addition to these discrepancies, these test systems neglected properties and complexity of the rhizosphere. Therefore, to pursue further investigations of the role of bacterial volatiles in this underground habitat, the applied methods have to simulate its natural characteristics as much as possible. In this review, we will describe and discuss pros and cons of currently used bioassays, give insights into rhizosphere characteristics, and suggest improvements for test systems that would consider *in natura* conditions and would allow gaining further knowledge of the potential function and significance of rhizobacterial volatiles in plant life.

Keywords: bacterial volatiles, mVOC, plant growth promotion, plant growth inhibition, rhizobacteria

INTRODUCTION

Volatile metabolites are important infochemicals mediating indispensable communication processes in all kingdoms of life (Hare, 2011; Dweck et al., 2015). The chemical nature of these small, lipophilic molecules enables plants attracting their pollinators or repelling pathogens, and animals finding mating partners (Hare, 2011; Mithöfer and Boland, 2012; Knollhoff and Heckel, 2014; Dweck et al., 2015). Current research indicates that volatiles released by bacteria play also a major role in multifarious microbial interactions, since these microbes release a wide range of various different volatiles, of which quite a few are capable to manipulate physiological processes in other bacteria, as well as in fungi and plants (Kai et al., 2009; Wenke et al., 2010, 2012; Effmert et al., 2012; Bitas et al., 2013; Audrain et al., 2015; Schmidt et al., 2015a).

Bacteria are omnipresent. They successfully occupy ecological niches as well as colonization hotspots like the plant rhizosphere. Plants release up to 40% of their photosynthetic fixed carbon through the roots into the surrounding area (Barber and Martin, 1976; Lynch and Whipps, 1991; Marschner, 1995; Hütsch et al., 2002). Due to this so called rhizodeposition, they attract a tremendous diversity of microorganisms (Perry et al., 2007). Bacteria preferably colonize the root itself (rhizoplane) and the adjunct soil zone (rhizosphere; Lenc et al., 2011; Bulgarelli et al., 2013; Reinhold-Hurek et al., 2015) representing thereby a crucial link between the plant roots and the surrounding soil. They take advantage of a constant flow of organic plant-based substrates, but

in return promote plant growth by providing soluble inorganic nutrients and producing growth-promoting factors (Strzelczyk and Pokojska-Burdziej, 1984; Arshad and Frankenberger, 1988; O'Sullivan and O'Gara, 1992; van Rhijn and Vanderleyden, 1995; Spaink et al., 1998; Brimecombe et al., 2007; Nannipieri et al., 2007; Compant et al., 2010). The bacterial diversity and abundance and therefore the type of interactions with the plant root is shaped by the nature of rhizodeposits and soil properties. Rhizodepositions of course vary depending on species and growth stages of the plant, and environmental conditions (Bulgarelli et al., 2013). Soil properties are given e.g., by soil texture, chemical conditions, and moisture; many of them are strongly influenced by seasonal changes (Insam and Seewald, 2010; Bulgarelli et al., 2013). The interplay of all these factors causes a constant dynamic in the rhizo-ecosystem. The role of bacterial volatiles within this continuously changing community still remains mysterious.

Investigating these affairs in such a complex habitat is a challenge. Isolations of bacteria from the rhizosphere, their identifications (Berg et al., 2002), the determination of the volatiles produced (Kai et al., 2007, 2010; von Reuss et al., 2010), and particularly *in vitro* observations detecting the effects of rhizobacterial volatiles on plants (Ryu et al., 2003, 2004; Vespermann et al., 2007; Wenke et al., 2012) delivered first pieces of the puzzle. However, these data illustrate a dilemma. They have been mostly obtained from artificial and simplified bioassays and test systems, which do not or only partially reflect the complex conditions of the rhizosphere and therefore do not provide sustainable evidence whether microbial volatiles have a substantial impact on bacteria-plant-interactions in the rhizosphere *in natura*. In order to conceive the complexity of these interactions, additional experimental setups are needed to approach and simulate natural conditions and situations.

In this review, we will summarize currently used techniques and present the corresponding results. We will discuss the benefits, limitations, and pitfalls of these test systems and considering these facts, we would like to introduce ideas for improvements that might provide further insights into the volatile-based bacterial network within the rhizosphere and its implication for plant life.

DIFFERENT TEST SYSTEMS—CONTRASTING EFFECTS

The first documentation of bacterial volatile-mediated effects on plants was published by Cook and Stall (1969). It took more than 30 years until 2003 Ryu and coworkers seized the issue again and since then different test systems were used by different working groups. **Table 1** gives an overview of all experimental systems that have been published so far. These systems can be characterized as follows: (i) setups that used passive diffusion or a directed airflow in order to transport the volatiles to the plant, (ii) target organs were the aerial parts of the plant or the roots, (iii) test system operating with an open or closed loop, and (iv) bacteria growing on different nutrients and matrices. Various combinations of these experimental setups were used. Up to now, in more than

half of all existing studies the assays were operated with passive diffusion using divided Petri dishes (**Table 1**).

Passive Diffusion Systems
Closed Systems
The split Petri dish is a simple and the most favored system (**Figure 1A**). A barrier separates the dish into two or three compartments to ensure a physical separation of rhizobacteria and plants. The exchange of metabolites is facilitated solely via headspace. Primary target of volatiles is the aerial part of a plant. In order to prevent the escape of volatile metabolites, the Petri dishes were sealed with parafilm. This way, Ryu et al. (2003) could show that volatile compounds produced by plant growth promoting rhizobacteria dramatically increased the leaf surface area of *Arabidopsis thaliana* seedlings. The entire mixture of volatile metabolites as well as the fermentation products acetoin and 2R,3R-butanediol were shown to be responsible for this effect (Ryu et al., 2003). Both substances were found to trigger induced systemic resistance (Ryu et al., 2004). The data of Han et al. (2006) supported these results. An increase of the total leaf area of *A. thaliana* seedlings was also evident in experiments of Zhang et al. (2007), who additionally showed an influence of rhizobacterial volatiles on auxin homeostasis and cell expansion. Growth promotion was also observed in experiments with sealed split Petri dishes conducted by Banchio et al. (2009); Kai and Piechulla (2009); Ezquer et al. (2010); Kai and Piechulla (2010); Zou et al. (2010); Blom et al. (2011a,b); and Park et al. (2015). A variation of the sealed Petri dish setup is represented by systems that use a box-in-box strategy (**Figure 1C**). A little container like a glass vial or a Petri dish inoculated with bacteria was placed in a big sealed container hosting the plants. Xie et al. (2009); Banchio et al. (2009); and Ezquer et al. (2010) employed this method. In all experiments, plant growth promotion was observed.

Open Systems
Omitting the parafilm changed the Petri dish setup toward an open system, which had a distinct impact on the test conditions (**Figure 1A**). Volatile compounds do not accumulate inside the compartments, which interestingly caused a dramatic growth inhibition of *A. thaliana* (Vespermann et al., 2007; Kai et al., 2008; Wenke et al., 2012; Weise et al., 2013). Kai and Piechulla (2010) directly compared the sealed and unsealed Petri dish system using the moss *Physcomitrella patens*. They showed that in co-cultivation with *Serratia plymuthica*, the moss gained biomass in sealed systems, while in open systems it suffered a dramatic loss.

In additional experiments (**Supplementary Table 1**, Velázquez-Becerra et al., 2011; Orozco-Mosqueda et al., 2013; Bailly et al., 2014; Zamioudis et al., 2015), it unfortunately remained unclear whether an open or closed system had been used. However, it should been mentioned that the plant growth was promoted in these studies. Only Bailly et al. (2014) showed growth inhibition using *Pseudomonas* strains, which was attributed to bacterial HCN production.

Systems Targeting the Roots
In order to simulate natural conditions, Park et al. (2015) placed a *Pseudomonas fluorescens* culture in a plastic container

TABLE 1 | Methodical order of all experimental systems to study bacterial volatile-mediated effects on plants.

System	Effect	Bacteria	Plant	Medium	Compounds[a]	References
Petri dish bipartite (parafilm sealed)	**Plant growth promotion** (total leaf surface area)	Bacillus subtilis GB03, Bacillus amyloliquefaciens IN937a, Enterobacter cloacae JM22	Arabidopsis thaliana	TSA	acetoin, 2,3-butanediol	Ryu et al., 2003
	No visual effect	Bacillus pumilus T4, Bacillus pasteurii C-9, Serratia marcescens 90-166, Escherichia coli DH5α			–	Ryu et al., 2004
	Plant growth promotion (induction of systemic resistance)	Bacillus subtilis GB03, Bacillus amyloliquefaciens IN937a, Serratia marcescens 90-166, Bacillus pumilus T4		TSA	2,3-butanediol (released from GB03 and IN937a)	
	No induction of systemic resistance	Bacillus pasteurii C9, Enterobacter cloacae JM22, Pseudomonas fluorescens 89B61, Bacillus pumilus SE34			–	Han et al., 2006
	Plant growth promotion (induction of systemic resistance)	Pseudomonas chlororaphis O6		MS	2,3-butanediol	
	Plant growth promotion (regulation of auxin homeostasis and cell expansion)	Bacillus subtilis GB03		TSA	–	Zhang et al., 2007
	Plant growth promotion (shoot/root- fresh/dry- weight and length, leaf number)		Ocimum basilicum	MS	-	Banchio et al., 2009
	Plant growth promotion (dry weight)	Serratia plymuthica 4Rx13 (formely odorifera)	Physcomitrella patens	NB	CO_2	Kai and Piechulla, 2010
	Plant growth promotion (shoot fresh weight)	Bacillus megaterium XTBG34	Arabidopsis thaliana	TSA	2-pentylfuran	Zou et al., 2010
	Plant growth promotion (shoot fresh weight)	Bacillus cereus B-569, Burkholderia terricola LMG 20594, **Chromobacterium violaceum CV0**, Cupriavidus necator, Escherichia coli, Stenotrophomonas rhizophila	Arabidopsis thaliana	MR-VP	–	Blom et al., 2011a
		Burkholderia lata		LB		
		Serratia plymuthica		MS		
		Burkholderia anthina LMG 20980, Burkholderia caledonica LMG 19076, Burkholderia caribensis LMG 18531, Burkholderia caryophylli, Burkholderia cepacia, Burkholderia thailandensis, Burkholderia sordidicola, Burkholderia hospita, Serratia proteamaculans		MR-VP; LB		
		Burkholderia gladioli, Burkholderia graminis, Burkholderia phenazinium, **Burkholderia phytofirmans**, Burkholderia sacchari, Cellulomonas uda, Serratia entomophila, Serratia marcescens, Serratia plymuthica HRO-C48		MR-VP; MS		

(Continued)

TABLE 1 | Continued

System	Effect	Bacteria	Plant	Medium	Compounds[a]	References
		Burkholderia lata, Burkholderia glathei, **Pseudomonas aeruginosa, Pseudomonas chlororaphis, Pseudomonas putida**		MR-VP, Angle		
		Burkholderia andropogonis, Burkholderia glumae, Burkholderia xenovorans, Pandoraea norimbergensis		MR-VP, LB, MS		
		Burkholderia kururiensis, Burkholderia tropica		MR-VP, LB, Angle	–	
		Burkholderia fungorum, **Burkholderia phenoliruptrix, Pseudomonas fluorescens WCS 417r**		MR-VP, MS, Angle		
		Burkholderia pyrrocinia		MR-VP, LB, MS, Angle		
	Plant growth inhibition (shoot fresh weight)	**Burkholderia phenoliruptrix, Burkholderia phytofirmans,** Chromobacterium violaceum CV0, **Pseudomonas aeruginosa, Pseudomonas chlororaphis, Pseudomonas fluorescens WCS 417r, Pseudomonas putida**		LB		
		Limnobacter thiooxidans, **Serratia plymuthica IC14**		MR-VP, LB		
	Plant growth promotion (biomass, lateral root number)	Pseudomonas fluorescens SS101	Nicotiana tabacum	King's medium B	13-tetradecadien-1-ol, 2-butanone, and 2-methyl-n-1-tridecene	Park et al., 2015
Petri dish tripartite (parafilm sealed)	**Plant growth inhibition** (shoot fresh weight)	Pseudomonas fluorescens CHA0, Pseudomonas aeruginosa (strains PA01a, PA01b, TBCF10839, PA14, TB, PUPa3), Pseudomonas chlororaphis subsp. aureofaciens ATCC 13985, Serratia marcescens MG1, Serratia plymuthica IC14	Arabidopsis thaliana	LB	HCN	Blom et al., 2011b
Plastic container (parafilm sealed)	**Plant growth promotion** (accumulation of starch in plant leaves)	Bacillus subtilis 168, Escherichia coli BW25113, Pseudomonas syringae (1448A9, 49a/90, PKs), Salmonella enterica LT2, Agrobacterium tumefaciens (EHA105, GV2260)	Nicotiana tabacum, Solanum tuberosum, Zea mays, Horedum vulgare, Medicago sativa, Ocimum basilicum	M9 minimal medium + 50 mM glucose	–	Ezquer et al., 2010
	Plant growth promotion (shoot/root- fresh/dry- weight and length, leaf number)	Bacillus subtilis GB03	Ocimum basilicum	MS		Banchio et al., 2009
	Plant growth promotion (shoot fresh weight, dry weight, rosette number, silique number, increased photo efficiency)	Bacillus subtilis GB03	Arabidopsis thaliana	TSA		Xie et al., 2009

(Continued)

TABLE 1 | Continued

System	Effect	Bacteria	Plant	Medium	Compounds[a]	References
Petri dish bipartite (parafilm non-sealed)	**Plant growth inhibition** (shoot fresh weight determination)	*Pseudomonas fluorescens* L13-6-12, *Pseudomonas trivialis* 3Re2-7, *Serratia plymuthica* 4Rx13 (formely odorifera), *Serratia plymuthica* 3Re4-18, *Serratia plymuthica* HRO-C48, *Stenotrophomonas maltophilia* R3089, *Stenotrophomonas rhizophila* P69	*Arabidopsis thaliana*	NB	–	Vespermann et al., 2007
	Plant growth inhibition (induction of H_2O_2 production)	*Serratia plymuthica* HRO-C48, *Stenotrophomonas maltophilia* R3089				Wenke et al., 2012
	Plant growth promotion (shoot fresh weight determination)	*Serratia plymuthica* 4Rx13 (formerly odorifera)		NBG	–	Weise et al., 2013
	Plant growth inhibition (shoot fresh weight determination)			NB	ammonia	
	Plant growth inhibition (dry weight determination)		*Physcomitrella patens*		-	Kai and Piechulla, 2010
Pots with direct root inoculation	**Plant growth promotion** (induction of systemic tolerance to drought)	*Pseudomonas chlororaphis* O6	*Arabidopsis thaliana*	peat: vermiculite/perlite	2,3-butanediol	Cho et al., 2008
	Plant growth promotion (induction of systemic resistance)	*Bacillus subtilis* FB17		peat pellet	acetoin	Rudrappa et al., 2010
Petri dish placed underneath the soil at the bottom of a **plastic container**	**Plant growth promotion** (biomass, lateral root number determination)	*Pseudomonas fluorescens* SS101	*Nicotiana tabacum*	King's medium B	13-tetradecadien-1-ol, 2-butanone, and 2-methyl-n-1-tridecene	Park et al., 2015
Dynamic air stream targeting the aerial part	**Plant growth promotion** (shoot fresh weight determination)	*Serratia plymuthica* 4Rx13 (formerly odorifera)	*Arabidopsis thaliana*	NB	–	Kai et al., 2009
Dynamic air stream targeting the root						

[a] Identified compounds that were shown to affect plant growth. Labeled in **bold** are bacterial strains that exhibited both positive and negative effects depending on the respective growth medium.
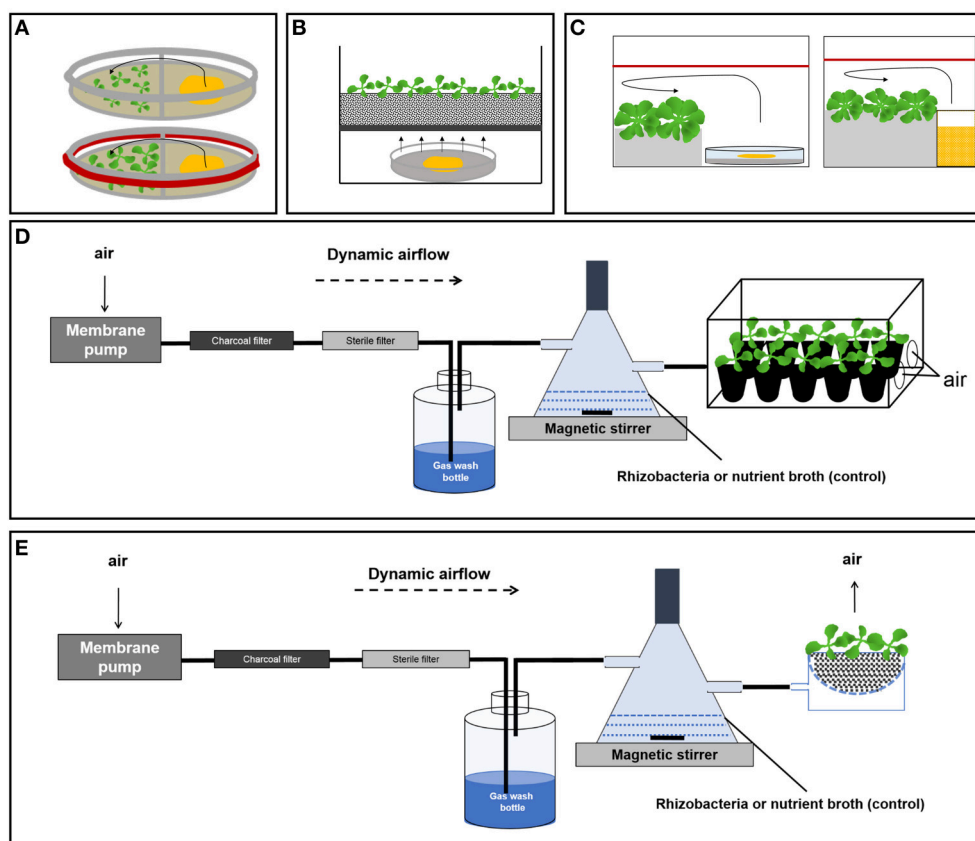
FIGURE 1 | Different bacterial volatile test systems. (A) Passive diffusion in the Petri dish system (upper panel: open system, lower panel: closed system).
(B) Passive diffusion targeting the roots. **(C)** Passive diffusion in container systems useful for older and bigger plants targeting the aerial plant parts (left: bacteria grown on Petri dishes, right: bacteria grown in liquid nutrient medium). **(D)** Dynamic air stream system targeting the aerial plant parts. **(E)** Dynamic air stream system targeting the roots.

underneath a defined soil compartment containing growing *A. thaliana* seedlings (**Figure 1B**). Bacterial volatiles passively spread and diffused into the soil. After 3 weeks of cultivation, the authors observed significant growth stimulation with an increase of fresh weight of *A. thaliana*.

Another approach was introduced by Cho et al. (2008) and Rudrappa et al. (2010). Roots of *A. thaliana* seedlings were inoculated with a suspension of *Pseudomonas chlororaphis* and *Bacillus subtilis*, respectively. This direct contact of bacteria with the root system did not exclude the influence of non-volatile bacterial metabolites, however, it was shown that the volatile metabolite 2R,3R-butanediol produced by *P. chlororaphis* triggered the induction of systemic tolerance to drought, and acetoin emitted by *B. subtilis* induced systemic resistance.

Dynamic Air Stream Systems

A dynamic streaming system was used by Cook and Stall (1969). A consistent flow of purified air passed over several bacteria containing agar plates and subsequently reached the aerial part of plants such as *Capsicum* sp., *Nicotiana* sp., *Lycopersicum* sp., and *Brassica* sp. The volatile mixtures

of *Xanthomonas vesicatoria*, *Xanthomonas campestris*, *Xanthomonas phaseoli*, *Erwinia carotovora*, *Erwinia amylovora*, *Pseudomonas cichorii*, *Pseudomonas tabaci*, and *P. fluorescens* induced necrosis in leaves of respective plants, whereby *X. vesicatoria* volatiles even killed *Capsicum annum*. Based on these results, it was assumed that volatiles might be associated with the hypersensitive response in plants (Cook and Stall, 1969).

Kai and Piechulla (2009) compared dynamic air stream systems that targeted (i) the aerial part as well as (ii) the roots of adult *A. thaliana* plants. They reported a considerable growth stimulation and increase in biomass. For system (i), they used a "mini" greenhouse for plant propagation (**Figure 1D**). Air enriched with volatiles of *S. plymuthica* grown in liquid culture was directed through the headspace of the growth container. For setting up system (ii), a glass bowl with a perforated base holding the plants was precisely positioned over a second glass bowl, which was designed to form a lower compartment equipped with an air inlet. Air enriched with volatile metabolites of *S. plymuthica* entered the lower compartment and escaped via the upper bowl through the soil thereby passing the roots of the plants (**Figure 1E**).

IN VITRO TEST SYSTEMS - PITFALLS AND LIMITATIONS

The Experimental System Matters

The overview of test systems (**Table 1**) illustrated a correlation between the setup and the outcome of the experiment. The most apparent difference was observed between closed and open compartment systems. This became most obvious considering the Petri dish systems. Studies using non-sealed plates revealed plant growth inhibition whereas almost all studies with sealed systems resulted in plant growth promotions. Solely, *Pseudomonas* strains were able to kill plants under both conditions. A split Petri dish setup is simple, inexpensive, easy to handle, allows a high throughput of samples, and assures a physical separation of producer and receiver. Besides these advantages, however, this test system suffers from some disadvantages. Sealing leads to an accumulation of metabolites even up to non-physiological/non-natural concentrations ultimately changing the micro-environment of the compartment and subsequently modifying the metabolism of the test organisms. Most prominent is the accumulation of CO_2 due to the bacterial metabolism (Kai and Piechulla, 2009) and the elevated level of humidity due to transpiration of plants (Tholl and Röse, 2006). The CO_2 content in the Petri dish can increase up to 10-fold compared to ambient concentrations; which most likely support plant growth in sealed Petri dishes (Kai and Piechulla, 2009). Non-sealed Petri dish systems avoid this accumulation, thus providing a completely different micro-environment.

Another aspect should be considered when the Petri dishes are used. The headspace is limited and therefore only young plant seedlings can be sampled. Their metabolism differs from that of adult plants (Jones et al., 2009). The model plant *A. thaliana* was often investigated, because of its small size, its short life cycle and the availability of myriads of mutants. For other test plants like *Nicotiana tabacum*, *Solanum tuberosum*, *Zea mays*, *Hordeum vulgare*, *Medicago sativa*, and *Ocimum basilicum* and for attempts to use adult plants, bigger growth containers were designed (box systems).

Common to both systems is the fact that the volatile metabolites are released into the headspace and subsequently primarily the aerial parts of the plants are exposed to bacterial volatiles (**Figures 1A–D**). The habitat of interest, however, is the part of the plant that grows underground. It may be argued that bacterial volatiles diffuse into the agar to target the roots, however, only rather hydrophilic volatiles permeate into the hydrophilic agar, while the lipophilic nature of most volatile metabolites obstructs a passage into the agar. Furthermore, compartmentalized systems, especially Petri dishes, do not meet different physiological demands of the test organisms. Despite the fact that the rhizosphere is a dark environment, bacteria as well as plant roots are exposed to light in the used experimental setups (**Figures 1A–E**).

Technical solutions that resolve some of these constraints allow a direct application of rhizobacterial volatiles to the roots (Cho et al., 2008; Kai and Piechulla, 2009; Rudrappa et al.,

2010; Park et al., 2015). However, persistent in all systems is the fact that the production of rhizobacterial volatile metabolites strongly depends on the nutrient source. Already, Cook and Stall (1969) observed a nutrient-dependent effect, since only bacteria grown on nutrient agar (NA) or Kings B medium (KBM) caused necrosis in leaf tissues. Blom et al. (2011a) comprehensively investigated the influence of bacteria grown on different media. Subsequently they observed altered and contrasting effects on plant growth. The deleterious effect was caused by HCN produced by *Pseudomonas* strains grown on protein rich media (LB agar; Blom et al., 2011b). The principle of this finding was also supported by Weise et al. (2013). *S. plymuthica* produced NH_3 only on protein enriched nutrient agar. This caused plant growth inhibition in open Petri dish systems whereas in sealed systems plant growth was promoted (Kai and Piechulla, 2009). Plants in unsealed systems were harmed by gaseous NH_3 itself, due to plant medium alkalization and/or NH_4^+ toxicity (Weise et al., 2013). In sealed dishes, the high CO_2 concentration might promote formation of acidic HCO_3^- thereby preventing alkalization and consequently growth inhibition.

These examples show that compartment systems and especially split Petri dishes develop very fragile and vulnerable micro-environments for both bacteria and plants. This has to be considered when interpreting the results. In summary, different test conditions hamper a direct comparison of the results obtained. However, every setup represents a valuable test system that contributed to the overall picture of bacteria-plant-interactions.

Soil Matters

While the adherence of bacteria to the rhizoplane seems to be supported by biofilm formation (Rudrappa et al., 2008; Reinhold-Hurek et al., 2015), the growth of bacteria and the fate of volatiles within the rhizosphere depend considerably on soil conditions (Effmert et al., 2012; Burns et al., 2015). In most experimental setups, bacteria were grown on artificial medium such as solid agar or liquid media. Although these media might be vaguely reminiscent of biofilm substrates or water filled pores, so far the influence of physicochemical properties of soil has been neglected in most experimental approaches. This involves the influence of soil on the bacterial life as well as the production and distribution of bacterial volatiles. Solely direct inoculations of bacteria (Cho et al., 2008; Rudrappa et al., 2010) and purging of volatile enriched air into the soil (Kai and Piechulla, 2009; Park et al., 2015) to some extent considered effects of the natural underground habitat.

Ecological Relevance of Rhizobacterial Volatiles—Quality and Quantity Matter

In many experimental setups, bacteria were inoculated into artificial media. These mostly nutrient rich conditions directed the metabolic activity of bacteria and thereby influenced the quality and quantity of the volatile metabolite synthesis. A nutrient enriched zone is in fact present on the root surface where root-derived organic compounds attract a diverse and

specialized bacterial community. Nutritional conditions in more distant areas from roots might be different resulting in an altered microbial community (Garbeva et al., 2014; Reinhold-Hurek et al., 2015). Regarding this aspect, *in vitro* concentrations and the quality of the mixture of bacterial volatiles should be critically examined.

Cell Numbers Matter (True Controls Matter)

An important aspect is whether the influence of bacteria on plant growth is a specific or a general phenomenon. Most studies present bacterial strains without any effect on plant growth as a corresponding negative control; e.g., the laboratory strain *E.coli* DH5α. However, due to different metabolic abilities to grow under the same nutritional conditions the growth rate and subsequently the concentration of volatile released differ between test and control strains. Images of split Petri dish setups of several studies illustrate these differences. Thus, volatiles produced by the control strain might be simply below the minimal affective concentration, e.g., different bacterial spot sizes implicating different bacterial growth rates (Ryu et al., 2003; Han et al., 2006; Kai et al., 2008; Zou et al., 2010; Blom et al., 2011a; Park et al., 2015). Bailly and Weisskopf (2012) already discussed this issue and assumed cell number dependent effects on plant growth. At a certain time point, slow and fast growing bacterial species appear in different growth phases thereby influencing the plant growth in different ways. Experiments performed by Blom et al. (2011a) confirmed this assumption. In addition, different cell densities might influence the regulation of the production of volatiles via quorum sensing (Kesarwani et al., 2011). As a consequence, the initial cell numbers of different bacterial species especially those of test and control strains should be adjusted as well as final cell numbers need to be determined and compared.

Interactions Matter

The physical separation of bacteria and plants represents an ambiguous aspect of setups that have been used so far. It allows for an exclusive investigation of effects of volatile-mediated interactions. Intra- and interspecific interactions, however, represent an intrinsic characteristic of the rhizosphere (Burns et al., 2015). The spectrum of bacterial volatiles will be influenced and even altered by bacterial and/or plant metabolites such as root exudates, infochemicals, or antibiotics, which do not have to be necessarily volatile. These metabolites might be continuously or only upon interaction produced and secreted. Interaction-induced allelochemical production and allelochemical-induced production of bacterial volatiles represent one of the most interesting aspects of rhizosphere investigations (Garbeva et al., 2011, 2014; Hol et al., 2015; Schulz-Bohm et al., 2015). The simulation of rhizodeposition might be realized by a defined nutrient composition of the bacterial medium (Blom et al., 2011a; Garbeva et al., 2014; Schulz-Bohm et al., 2015); the influence of allelochemicals on bacterial volatile production and the consequences for plant-bacterial interaction still awaits a substantial investigation.

CHARACTERISTICS OF THE HABITAT—KEY DEMANDS FOR NOVEL EXPERIMENTAL SETUPS

In vitro test systems used so far are indispensable to get first insights into volatile-mediated effects of rhizobacteria on plants. Nevertheless, refined systems and novel approaches are necessary to dig deeper into the precise mechanisms of volatile-based bacteria-plant-interactions. Adequate experimental setups should really mimic or simulate as closely as possible rhizosphere conditions. These conditions shape evolving microbial communities and consequently the quality and quantity of volatiles. At the same time, the rhizosphere represents the matrix that determines the fate of volatile metabolites and facilitates or limits their diffusion. Matrix properties include the soil properties, chemical conditions (pH, aeration), and environmental factors (temperature, water content, darkness; Voroney and Heck, 2015). Traits that particularly influence the microbial communities are the nutrient status (root exudates, trace elements) and the interplay within the microbial community/population. The following section will briefly highlight these characteristics that have to be considered in order to develop suitable novel experimental setups.

Soil Properties, Chemical Parameters, and Environmental Factors

Soil is an aggregation of inorganic and organic particles, whereby the inorganic material is glued together with the organic matter (Voroney and Heck, 2015). The particle size of the inorganic particles varies between 0.002 and 2 mm resulting in different soil components including clay (below 0.002 mm), silt (0.05–0.002 mm), and sand (2–0.05 mm). The proportion of the three components determines the soil texture. Aggregates of the soil minerals of different size and organic materials cluster together forming the soil structure. About 35% (mineral soils) up to 90% (organic soils) of a soil volume can be taken up by pore space. Hereby, soil pores with a diameter below 10 μm (micropores) are important for the aqueous environment of bacteria, while soil pores below 5 μm in diameter are not colonized by microorganisms most likely due to impaired diffusion of nutrients. However, the gaseous diffusion and more specifically the diffusion of volatiles into micropores is supposed to be slow because of the water content. In contrast, macropores (diameter > 10 μm) facilitate rapid air and volatile diffusion. Effects of volatiles mediated over short distances should involve micropores, whereas effects over long distances probably require macropores. Furthermore, size and shape of pores determine their water and/or air content. Water is the universal factor in the rhizosphere influencing soil aeration, moisture, osmotic pressure, and pH or nature and amount of soluble substances available to or affecting organisms. While for soluble compounds water represents a perfect medium of transportation, it hampers diffusion of volatile metabolites due to their lipophilic nature. Aeration, which is driven by diffusion between the atmosphere and soil, again, promotes the emanation of volatiles. Diffusion through air filled pores is 10,000 times better than through water

filled pores (Voroney and Heck, 2015). Closely connected to soil aeration is the availability of molecular oxygen (O_2). O_2 belongs to the most crucial factors for aerobic activity in soil. Due to the lower diffusion distance compared to the atmosphere, the partial pressure (pO_2) in the topsoil is higher compared to the deeper regions (Glinski and Stepniewski, 1985; Stepniewski and Stepniewska, 2009) and considering soil aggregates, the pO_2 diminishes from the outside to the center where even anoxic states are obtained (Sexstone et al., 1985; Zausig et al., 1993). The pO_2 in the rhizosphere, depends on respiration processes and the diffusive O_2 replenishment (Glinski and Stepniewski, 1985; Uteau et al., 2015). The respective oxygen status is the factor that clinches the switch from aerobic to anaerobic respiration in soil which fundamentally influences the metabolome and hence the volatile production. The fluctuation of temperature has to be considered as another fundamental factor of influence. Every organism has its own temperature optimum (Farrell and Rose, 1967). Bacteria, of course, can adapt to fluctuating temperatures, but this might be coupled with alterations of metabolism of the respective organisms, which may also lead to a different profile of volatiles. Furthermore, when temperatures reached threshold levels, for instance for mesophilic and thermophilic bacteria, the species diversity and abundance of communities shifted (Leven et al., 2007). Seasonally and/or diurnally changing temperatures should be therefore taken into account when natural conditions should be simulated (Voroney and Heck, 2015). Temperature, however, influences not only organisms. The decrease of temperature can cause an attenuated evaporation and diffusion of volatile molecules in the soil. The online screening of the soil/atmosphere exchange of volatiles conducted by Asensio et al. (2007) showed that the emission of some soil VOCs was enhanced due to increased soil temperatures.

Nutritional Conditions, Bacterial Growth, and Developmental Stages

Nutrient conditions within the rhizosphere are primarily regulated by the plants exuding excess photosynthetic products through the roots into the soil (Barber and Martin, 1976; Lynch and Whipps, 1991; Marschner, 1995; Hütsch et al., 2002). Thereby, the relative and absolute amounts of plant-derived nutrients in the rhizosphere vary with the plant species, plant age, and environmental conditions the plant has to cope with (e.g., soil properties, biotic, and abiotic stresses). Rhizodeposits include sugars, polysaccharides, amino acids, organic acids, fatty acids, and sterols (reviewed in Uren, 2007). Unfortunately, exudation of rhizodeposites was mostly studied *in vitro* on media other than soil, and therefore the *in vivo* exudation status of the rhizosphere and its change in time remains speculative (Uren, 2007). However, Bulgarelli et al. (2013) impressively showed that a variation of rhizodeposits caused an alteration of the plant root microbiome implicating a very dynamic production of bacterial volatiles. Besides this nutritional influence it can be assumed that due to bacterial growth and different developmental stages the bacterial metabolism and subsequently the formation of volatiles is changing. The production and effects of volatiles in dependence on bacterial developmental factors as well as growth stages, the formation of biofilms, generation of spores, or movement factors

like swimming and swarming has so far not been in focus of the current research.

Bacterial Interactions in the Rhizosphere

Bacterial interactions in the rhizosphere occur in three directions, (i) interaction of the bacteria with the plant (Bais et al., 2006; Rudrappa et al., 2008; Bednarek and Osbourn, 2009), (ii) the intra- and interspecific communication within the bacterial community (Ryan and Dow, 2008; Shank and Kolter, 2009; Garbeva et al., 2011; Tyk et al., 2014), and (iii) interaction with protozoa and metazoa (Matz and Kjelleberg, 2005; Ronn et al., 2012).

Plants release plenty of secondary metabolites, e.g., terpenes, flavonoids, glucosinolates, and phenylpropanoids into the rhizosphere (Dixon and Paiva, 1995; Rasmann et al., 2005; Van Dam et al., 2009; Bressan et al., 2009; Moore et al., 2014). Minor modifications in secondary metabolite level can have an important impact on soil microbial communities (Bressan et al., 2009). Flavonoids for instance are able to mimic quorum sensing (QS) molecules and thereby influencing the bacterial metabolism (Hassan and Mathesius, 2012). The production of 2-aminoacetophenone, a volatile metabolite produced by *Pseudomonas aeruginosa*, *Streptomyces* spp., and *Burkholderia ambifaria* is known to be QS regulated (Cox and Parker, 1979; Dickschat et al., 2005; Kesarwani et al., 2011; Groenhagen et al., 2013). Since it is predicted that many more volatiles are QS regulated, it is assumed that the interplay between plants and bacteria can change the pattern of volatile emission of bacteria. Thus, the QS system that controls basic processes of the bacterial life (e.g., biofilm formation and motility; Lowery et al., 2008) is likely to also affect the quality and quantity of volatiles. This is particularly important in highly competitive situations between different bacterial organisms that benefit from nutrient rich conditions in the rhizosphere. Moreover, bacteria evolved different strategies of antagonism including the release of antibiotics, lytic enzymes, siderophores, and toxins (Thomashow et al., 1990; O'Sullivan and O'Gara, 1992; Chernin et al., 1995; Pliego et al., 2008; and others). Driven by these manifold antagonistic properties and also due to the competition regarding water, nutrient and space, the bacteria react with the production of own weapons (Abrudan et al., 2015). For instance during interaction, *Streptomyces coelicolor* induced pigment production and hyphae formation in *B. subtilis* PY79 and simultaneously *B. subtilis* PY79 enhanced the production of cannibalism toxins in *S. coelicolor* (Watrous et al., 2012). In addition to antagonistic action also cooperation might affect emission of volatile metabolites. Metabolome profiling revealed that *B. megaterium* and *Ketogulonicigenium vulgare* cooperated by exchanging a number of metabolites (Zhou et al., 2011). This exchange increased the pool of own metabolites and hence different potential precursors of volatiles were available for the cooperating strains. It is therefore conceivable that the emission of volatiles might also alter due to bacteria-bacteria interaction. First data supporting this assumption were presented by Hol et al. (2015) and Schulz-Bohm et al. (2015). While Hol et al. (2015) showed that a non-random loss in bacteria communities reduced antifungal volatile production, Schulz-Bohm et al. (2015) could

show that both microbial interactions and shifts in microbial community composition had a strong effect on the volatile emission. Likewise, bacterial interactions with fungi have to be considered. Splivallo et al. (2015) showed impressively that fruiting body associated bacteria contributed to the smell of the truffle *Tuber borchii* by synthesizing thiophene from a currently unknown precursors produced by *T. borchii*. Furthermore, Schmidt et al. (2015b) showed that *Collimonas pratensis* and *S. plymuthica* PRI-2C showed significant changes in their motility when exposed to fungal volatiles coming to the conclusion that bacteria are able to sense and respond to fungal volatiles (Schmidt et al., 2015b).

FUTURE TEST SYSTEMS

Considering the complexity of the rhizosphere, it is eligible in a first step to use standardized conditions, such as sterilized sand inoculated with a single bacterial isolate or mixtures of bacterial species. Although plant growth on sand does not always reflect the most frequently used soil by plants, it has the advantage that variation in soil structure is limited compared to other soils. After these initial studies with sand, experimental setups should be approximated to more complex natural soil conditions. The second challenge is to match the diversity of the rhizobacterial community. Here the proposed strategy would be to start with one or a few bacterial species and gradually increase the number and variation of combinations to simulate the rhizosphere situation.

In order to investigate volatile compounds of root associated bacteria and to study their effects on plants, an experimental rhizosphere platform should be designed (**Figure 2B**). The core of the platform represents a root box equipped with an inlet and an outlet for air exchange (**Figure 2A**). The leakage of sand particles from the chamber is avoided by perforated barriers between the in- and outlet and the root box. The front plate of the box should be composed of transparent and inert Teflon® or glass material in order to observe root growth by e.g., eye or camera. On top of the box a small opening is the gateway for
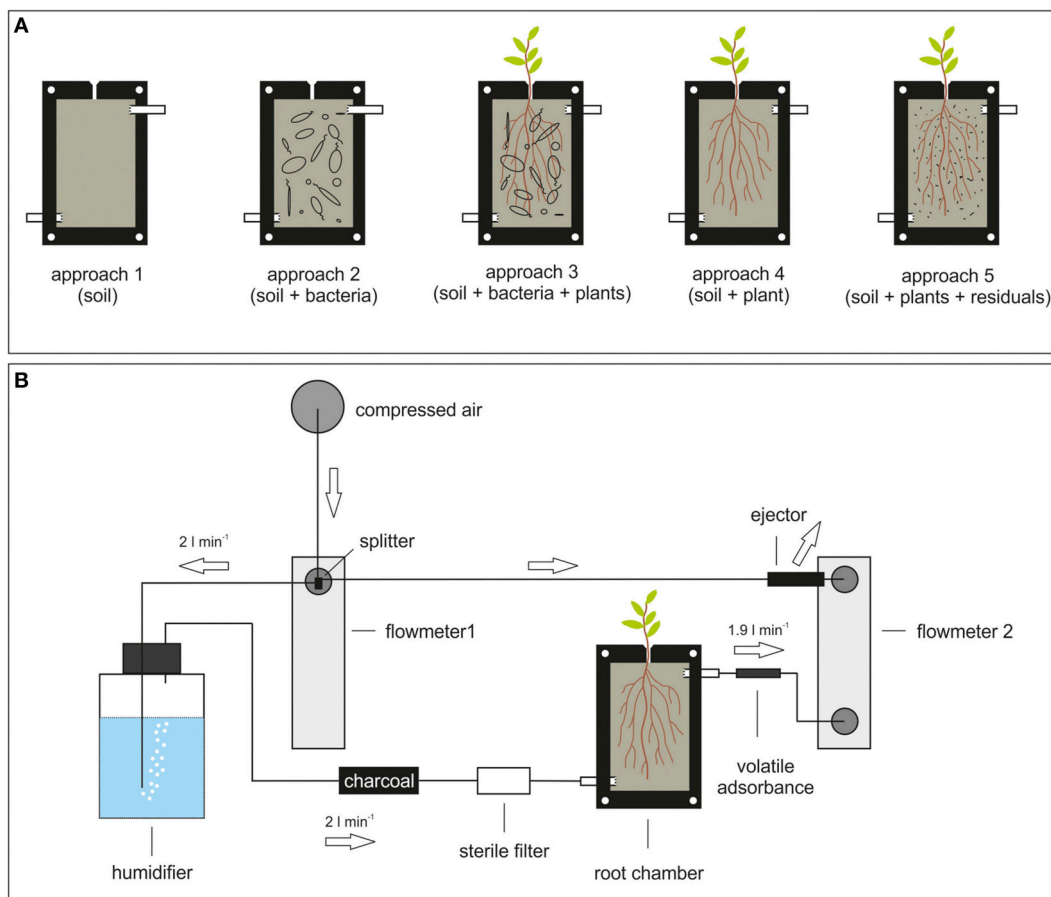
FIGURE 2 | Platform to analyze volatiles and volatile mediated effects. (A) Root chamber. Several approaches are exemplified: 1. soil only; 2. soil and bacteria; 3. soil, bacteria, and plants; 4. soil and plants; 5. soil and bacterial residuals. **(B)** Design of a volatile-collection system using the root chamber. Arrows indicate direction of airflow. The flow of 2 l/min is exemplary indicated, since it always depends on the kind of adsorbent used. The splitter is separating the incoming airflow. On one site the air is humidified (gas washing bottle), and purified (charcoal and sterile filter) before passing into the root chamber. On the other site the ejector reverses the airflow that the volatile enriched air is pulled out of the root chamber over an adsorbent trap.

plant roots expanding into the box. The space inside the box can be filled with the matrix of choice regarding texture and composition. Before starting the experiment, the box as well as the matrix will be sterilized by gamma- or UV radiation, or by autoclaving. Surface sterilized seeds would be placed at the top puncture of the root box and upon germination the roots will push their way through the hole and develop their root network into the matrix.

The advantage of this setup is the possibility of arbitrary co-cultivation and co-development of (various) bacterial species with the plant root. The correct assignment of emitted volatiles either to the plant root or bacterial species has to be performed by differential analysis of the several setups (soil only, bacteria only, plant only, bacteria, and plant and so on; **Figure 2A**). Beside the characterization of plant growth parameter (shoot- and root fresh weight, plant omics) under various different conditions, a continuous measurement of volatile emission of appropriate time intervals can be established. Thereby, it should be distinguished between volatiles that bind to soil particles or aggregates by using *in situ* polydimethylsiloxane micro-extraction (Eilers et al., 2015) and volatiles that do not bind to soil by passing air through the root box and further over an adsorbent trap (**Figure 2B**). The above mentioned differential analysis would provide information about plant growth in combination with data about the status of volatiles in the root system. A similar approach was introduced by Eilers et al. (2015) originally developed for Dandelion (*Taraxacum* sect. *ruderalia*) root volatiles. This system aimed to simply and inexpensively detect rhizosphere chemicals at experimentally less disturbed conditions. Therefore, it could be adapted in order to use it for bacteria-plant interaction. Nevertheless, since the assignment of volatiles to the producer in these systems is still difficult, there is a need to verify specificity of volatile-mediated effects by evaluating the obtained data in the compartmented test systems *in vitro*. These verifications must include the check for profiles of bacterial volatiles as well as the application of single compounds/mixtures of volatiles. Such a combinatory approach of the different test systems and

techniques will help to understand the volatile-mediated effects on plant growth.

CONCLUSIONS

Recent advances have enhanced our understanding that small volatile molecules emitted by bacteria can have dramatic effects on the growth and development of plants. These observations were mainly based on various different experimental setups, often revealing discrepancies between results. In addition, most test systems neglected properties and complexity of the rhizosphere. In order to go beyond the search for potential effects and to evaluate the significance of rhizobacterial volatiles *in situ*, setups that mimic the rhizosphere, that allow for a combination of various bacterial species and if desired other microorganisms, that assure for the usage of different soil matrices, and that enable *in situ*-volatile collections, the root box embedded into the so called rhizosphere platform could be considered to be a first step into this direction.

AUTHOR CONTRIBUTIONS

MK designed research; MK, UE, and BP wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.00108

Supplementary Table 1 | Investigations in which remained unclear whether an open or closed system had been used. [1]Identified compounds that were shown to affect plant growth.

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Airborne Bacterial Interactions: Functions Out of Thin Air?

Bianca Audrain, Sylvie Létoffé and Jean-Marc Ghigo*

Genetics of Biofilms Laboratory, Department of Microbiology, Institut Pasteur, Paris, France

Bacteria produce and release a large diversity of small molecules including organic and inorganic volatile compounds, hereafter referred to as bacterial volatile compounds (BVCs). Whereas BVCs were often only considered as wasted metabolic by-product sometimes perceived by animal olfactory systems, it is increasingly clear that they can also mediate cross-kingdom interactions with fungi, plants and animals. Recently, *in vitro* studies also reported the impact of BVCs on bacterial biology through modulation of antibiotic resistance, biofilm formation and virulence. Here, we review BVCs influence on bacterial adaptation to their environment and discuss the biological relevance of recently reported inter- and intra-species bacterial interactions mediated by BVCs.

Keywords: bacterial volatile compounds, metabolism, signaling, bacterial interactions, biofilm, antibiotic resistance

INTRODUCTION

While the contribution of diffusible soluble secondary metabolites in bacterial ability to communicate, compete or cooperate with neighboring microorganisms has been actively investigated, bacteria also produce and release a wide diversity of volatile compounds that can be readily detected in the bacterial headspace (Schulz and Dickschat, 2007). Nevertheless, the potential biological role(s) of organic and inorganic bacterial volatile compounds or gases (BVCs) was often overlooked. Recent studies, however, demonstrated that they could mediate a variety of interactions between bacteria and their environment. Indeed, several BVCs were shown to influence growth and differentiation in fungi, to induce systemic resistance against bacterial pathogens in plants or to affect behaviors in invertebrates (**Figure 1**; Gallagher and Manoil, 2001; Ryu et al., 2003; Kai et al., 2008, 2009; Niu et al., 2010; Effmert et al., 2012). In addition of their action on a wide range of eukaryotic organisms, several reports also revealed the potential impact of BVCs on bacteria themselves (Audrain et al., 2015). This review will present the current knowledge on BVCs influence on inter- and intra-species bacterial interactions and will discuss their biological relevance and the interest to further study this particular class of bacterial metabolites.

ROLE OF BVCs IN BACTERIAL COMPETITION

Bacteria often compete for space, nutrients or others resources through production of metabolic by-products providing them with an advantage over surrounding bacteria. Several BVCs display a direct negative effect, as it is the case for some volatile compounds emitted from rhizosphere bacteria *Bacillus, Pseudomonas, Serratia,* or *Streptomyces* affecting bacterial growth. For instance, dimethyl disulfide emitted from *Pseudomonas fluorescens* and *Serratia plymuthica* displays bacteriostatic effects against two plant bacterial pathogens, *Agrobacterium tumefaciens* and *A. vitis*

FIGURE 1 | Role of volatile compounds released by bacteria and their potential interests and applications. Graphical representation of the focus of the review on bacteria-bacteria interactions mediated by bacterial volatile compounds (BVCs). The figure also mentions BVCs impact on other organisms and illustrates the potential interests/applications of the study of BVCs as indicators of bacterial metabolism and pool of molecules with diagnostic or biocontrol potentials.

(Dandurishvili et al., 2011). Moreover, albaflavenone produced by *Streptomyces* sp. exhibits antibacterial activity against *Bacillus subtilis* (Gurtler et al., 1994).

Some soluble short-chain fatty acids (acetate, succinate, propionate, or isobutyrate) are also able to inhibit growth of several enteropathogens (*Salmonella enteritidis*, *S. typhimurium* and *Escherichia coli*) (Hinton, 1995), and also growth and sporulation of *Clostridium perfringens* (Wrigley, 2004). Although these experiments were performed using short-chain fatty acids in solution, these metabolites are produced by *Veillonella* species or *Bacteroides fragilis* (Hinton, 1995) and several other members of the intestinal microbiota (Effmert et al., 2012) suggesting that volatile short-chain fatty acids could also play a role in control of competing commensals and also enteropathogens in the intestinal tract.

Some BVCs are also able to modulate at a distance the production of antimicrobials. Indeed, volatile compounds produced by *Collimonas pratensis* increased production of secondary metabolites in *P. fluorescens* that showed antimicrobial activity against *Bacillus* sp (Garbeva et al., 2014). In *P. aeruginosa*, the production of molecules with antimicrobial activity such as pyocyanin seems also to be influenced by volatile compounds (Venkataraman et al., 2011, 2014). A recent study reported that 2,3-butanediol, produced by co-habitant fermenter bacteria such as *S. marcescens* enhances production of *P. aeruginosa*

pyocyanin exhibiting antimicrobial activity, which then could help *P. aeruginosa* to occupy a niche, especially in cystic fibrosis lungs (Venkataraman et al., 2014); 2,3-Butanediol and its volatile precursor 2,3-butanedione have thus been detected in airways of cystic fibrosis patients (Whiteson et al., 2014). All these study therefore suggest a potential direct and indirect role of BVCs in bacterial competition.

VOLATILE-DEPENDENT BACTERIAL RESPONSES TO THE ENVIRONMENT

Several studies described BVCs as potential airborne chemical cues modulating gene expression, membrane permeability or enzyme activation resulting in alteration of bacterial behaviors. For instance, *P. fluorescens* transcriptional response differs upon exposure to volatiles emitted by rhizospheric bacteria such as *C. pratensis* and *S. plymuthica*, including dimethyl disulfide and benzonitrile, which stimulate the growth of *P. fluorescens* (Garbeva et al., 2014). BVCs can therefore provide positive information about surrounding microorganisms or environment. Alternatively, aerial exposure to glyoxylic acid and 2,3-butanedione, both produced by *B. subtilis* reduces *Burkholderia glumae*, *P. aeruginosa*, *Paenibacillus polymyxa* and *E. coli* surface motility (Kim et al., 2013). In the

case of *E. coli*, this reduced motility correlates with the downregulation of 30 genes involved in chemotaxy and motility in *E. coli* (Kim et al., 2013). Several other BVCs such as 1-butanol, indole, 2-butanone or acetoin were also shown to influence *E. coli* and *P. aeruginosa* motility (Letoffe et al., 2014).

Bacterial volatile compounds cues also contribute to the development of bacterial community by influencing biofilm formation of Gram-negative and Gram-positive bacteria. Although still mechanistically unclear, volatile compounds such as indole, 1-butanol, 2-butanone, acetoin, ammonia, ethanol, hexadecane, glyoxylic acid, and trimethylamine display positive or negative influence on biofilm formation in one or several tested bacterial species (*B. subtilis, E. coli, P. aeruginosa,* and *Staphylococcus aureus)* (Letoffe et al., 2014). Recent studies also demonstrated that volatile acetic acid, a short-chain fatty acid, or ammonia can stimulate biofilm formation in *B. subtilis* and *S. aureus* (Nijland and Burgess, 2010; Letoffe et al., 2014; Chen et al., 2015). Whereas exposure to nitric oxide (NO) can positively affects biofilm formation of *Shewanella oneidensis*, *Azospirillum brasilense* or *Vibrio harveyi* (Henares et al., 2013; Barraud et al., 2014), it triggers biofilm dispersion in several Gram-negative and positive bacteria including *P. aeruginosa*, *E. coli, V. cholerae, B. licheniformis, S. marcescens, Fusobacterium nucleatum* (Barraud et al., 2009b), *S. woodyi* (Liu et al., 2012), *S. enterica* (Marvasi et al., 2014), and *Neisseria gonorrhoeae* (Potter et al., 2009). In *P. aeruginosa*, the dispersing role of NO could be correlated to degradation of cyclic-di-GMP, a bacterial small molecule playing a central role in the switch between biofilm and planktonic lifestyle (Barraud et al., 2009a; Liu et al., 2012).

The development of high cell density bacterial communities can also lead to the accumulation of organic and inorganic BVCs altering bacterial environment and triggering response to different stresses, including exposure to antibiotics (Heal and Parsons, 2002). For instance, ammonia emitted by bacterial population increases at a distance resistance to tetracycline and ampicillin, and decreases resistance to aminoglycosides in all tested Gram-negative and Gram-positive bacteria exposed to ammonia (Bernier et al., 2011). In *E. coli*, ammonia mode of action involved its import through the AmtB channel followed by an increase in polyamine synthesis leading to modulation of antibiotic resistance profiles (Bernier et al., 2011). Interestingly, at a distance alkalinization of bacterial growth medium (up to pH 8.5) upon exposure to volatile ammonia was reported and involved in the increased resistance to ampicillin of *S. marcescens* and *S. rubidaea* (Cepl et al., 2014). Similarly, volatile trimethylamine (TMA), produced by reduction of trimethylamine-oxide (TMAO) in TMAO-rich environments such as animal gut and tissues (Barrett and Kwan, 1985; Bos et al., 2013), can also modulate bacterial resistance to several classes of antibiotics through medium alkalinization that affects proton motive force and membrane permeability (Letoffe et al., 2014).

Another inorganic BVC produced by many bacteria, hydrogen sulfide (H₂S), confers multidrug resistance upon different pathogens (*B. anthracis, P. aeruginosa, S. aureus,* and *E. coli*)

under aerobic conditions via the mitigation of oxidative stress induced by antibiotic treatment upon suppression of DNA-damaging Fenton reaction (Gusarov et al., 2009). Exposure to volatile 2,3-butanedione and glyoxylic acid, both naturally produced by *B. subtilis* GB03, alter *E. coli* antibiotic resistance profiles, which could be correlated to the upregulation of *hipA*, encoding an anti-toxin module previously described as mediating persistence (Kim et al., 2013). Alteration of antibiotic resistance by BVCs can also occur at the level of persistence. Indeed, volatile 2-amino-acetophenone (2-AA) enhances antibiotic tolerance by increasing accumulation of persistent bacteria in *P. aeruginosa* and *B. thailandensis* but also in the non-2-AA producer *Acinetobacter baumanii* (Que et al., 2013), two pathogens isolated during co-infection with *P. aeruginosa*. Since 2-AA promotes persistence by altering bacterial translation, an highly conserved machinery, and it can affect both producing and non-producing bacteria, this suggests that volatile 2-AA could be involved in the ability of Gram-negative bacteria to tolerate antibiotic treatment in polymicrobial infections.

Finally, *P. putida* exposure to indole produced by *E. coli* induces an efflux pump leading to an increased antibiotic resistance (Molina-Santiago et al., 2014). However, although it is well established that soluble indole influences drug resistance in several Gram-negative bacteria (Hirakawa et al., 2005; Lee et al., 2008, 2009; Nikaido et al., 2008; Molina-Santiago et al., 2014), its role as a significant airborne signal affecting drug resistance still needs to be confirmed.

CONCLUDING REMARKS

BVCs, an Untapped Pool of Bioactive Compounds?

Beyond its fundamental ecological interest, a better understanding of BVC roles, biosynthesis pathways and mechanisms of action could provide new information on the extent of bacterial metabolic potential and lead to clinical or industrial applications (**Figure 1**). Indeed, several soil-associated bacteria were not only shown to have positive effects on plant resistance but also to control plant diseases by exhibiting antibacterial activity against plant pathogens (Berg, 2009; Pieterse et al., 2014). BVCs can also influence bacterial pathogenesis by altering the production of virulence factors (i.e., 2,3-butanediol increasing virulence factor production in *P. aeruginosa*) or by affecting host cell functions (i.e., colonic homeostasis, T- and B cell proliferation responses or cytokine production; Kurita-Ochiai et al., 1995; Smith et al., 2013; Venkataraman et al., 2014).

Considering bacterial potential for metabolic adaptation to available environmental resources, characterization of the volatile secondary metabolites produced in nature could provide leads for the development of diagnostic tool using BVC as potential biomarker in some pathological situations (Probert et al., 2009). However, most bacteria releasing complex blends of molecules, unraveling the chemical nature and roles of BVCs emitted in

mixed-species contexts will certainly constitute a major challenge of the field.

Laboratory Conditions vs. Nature: A True Biological Functions for BVCs?

In the studied described above, experimental set-up using physically separated source of volatile compounds and recipient bacteria unambiguously demonstrated that exposure to BVCs could have important biological functions. While some highly abundant BVCs are likely to play a role in intra- and inter-bacterial competition and cooperation phenomena, most, if not all studies were performed in laboratory conditions, using artificial media and controlled temperature, atmosphere and BVC concentrations of unknown physiological relevance. Moreover, although BVC-dependent interactions between bacteria (and also plants, fungi, nematodes) are potentially occurring in environments such as soil or mammalian intestines, the high solubility of BVCs in the liquids present in these environments raises the question of the true aerial nature of BVC-mediated impact on bacteria. Future work will therefore have to clarify the role played by BVCs in bacterial ability to adapt and/or respond to their environments by determining the physiological concentrations of relevant BVCs in diverse environments and to establish, preferentially *in vivo*, the importance of airborne bacterial interactions in microbial ecology.

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Volatiles in Inter-Specific Bacterial Interactions

Olaf Tyc[1,2]*, Hans Zweers[1], Wietse de Boer[1,2] and Paolina Garbeva[1]

[1] Department of Microbial Ecology, Netherlands Institute of Ecology, Wageningen, Netherlands, [2] Department of Soil Quality, Wageningen University and Research Centre, Wageningen, Netherlands

The importance of volatile organic compounds for functioning of microbes is receiving increased research attention. However, to date very little is known on how inter-specific bacterial interactions effect volatiles production as most studies have been focused on volatiles produced by monocultures of well-described bacterial genera. In this study we aimed to understand how inter-specific bacterial interactions affect the composition, production and activity of volatiles. Four phylogenetically different bacterial species namely: *Chryseobacterium*, *Dyella*, *Janthinobacterium,* and *Tsukamurella* were selected. Earlier results had shown that pairwise combinations of these bacteria induced antimicrobial activity in agar media whereas this was not the case for monocultures. In the current study, we examined if these observations were also reflected by the production of antimicrobial volatiles. Thus, the identity and antimicrobial activity of volatiles produced by the bacteria were determined in monoculture as well in pairwise combinations. Antimicrobial activity of the volatiles was assessed against fungal, oomycetal, and bacterial model organisms. Our results revealed that inter-specific bacterial interactions affected volatiles blend composition. Fungi and oomycetes showed high sensitivity to bacterial volatiles whereas the effect of volatiles on bacteria varied between no effects, growth inhibition to growth promotion depending on the volatile blend composition. In total 35 volatile compounds were detected most of which were sulfur-containing compounds. Two commonly produced sulfur-containing volatile compounds (dimethyl disulfide and dimethyl trisulfide) were tested for their effect on three target bacteria. Here, we display the importance of inter-specific interactions on bacterial volatiles production and their antimicrobial activities.

Keywords: volatolomics, soil bacteria, *Chryseobacterium*, *Dyella*, *Janthinobacterium*, *Tsukamurella*, inter-specific interactions, volatile activities

INTRODUCTION

Soil bacteria produce an astounding array of secondary metabolites. Gaseous secondary metabolites, commonly known as volatile organic compounds (VOCs) are small molecules (<300 Da) belonging to different chemical classes that can evaporate and diffuse easily through air- and water-filled pores (Schulz and Dickschat, 2007; Penuelas et al., 2014). These physiochemical properties make volatiles ideal metabolites for communication and antagonistic interactions between soil microorganisms living at a certain distance from each other. Indeed, recent studies indicate that soil microorganisms can employ volatile compounds as info-chemicals, growth stimulants, growth inhibitors, and inhibitors of quorum-sensing (Kai et al., 2009;

Chernin et al., 2011; Effmert et al., 2012; Kim et al., 2013). Furthermore, rhizosphere bacteria emit volatiles that can promote plant growth and elicit induced systemic resistance (ISR) and induced systemic tolerance (IST) in plants (Ryu et al., 2003, 2004). However, the role of volatiles in competitive interactions between soil bacteria is so far poorly understood.

In the past few years the research on volatiles emitted by bacteria received increased attention from a more applied point of view as these compounds have intriguing properties which are of great interest for agriculture (pathogen suppression), food preparation (aroma), and cosmetics industry (perfume odors; Krings and Berger, 1998; Wheatley, 2002; Beshkova et al., 2003; Schwab et al., 2008; Deetae et al., 2009; Effmert et al., 2012; Kanchiswamy et al., 2015).

Bacterial volatiles belong to different chemical classes like alkenes, alcohols, ketones, terpenes, benzenoids, pyrazines, acids, and esters. However, the composition of emitted volatiles (volatile blend composition) may vary with cultivation conditions, in particular with respect to the substrate composition of the growth media (Cleason, 2006; Blom et al., 2011; Groenhagen et al., 2013; Garbeva et al., 2014a). Other factors known to influence volatile production are microbial physiological state, oxygen availability, moisture, temperature and pH (Bjurman, 1999; Insam and Seewald, 2010; Romoli et al., 2014).

The technical developments that have been made in recent years in the field of mass spectrometry have led to the improvement of volatile compounds detection. The details of these developments have recently been summarized by Carter (2014). However, the main challenge in volatolomics is the ability to identify and quantify the entire set of emitted volatiles. The detected volatile blends are mostly quite complex and make the identification of biologically relevant volatiles a demanding and challenging task (Farag et al., 2012; Tait et al., 2014).

To date more than over 1000 microbial volatiles are reported and described in a special database for microbial VOCs called mVOC[1] (Lemfack et al., 2014). Nevertheless, this number is rather low compared to the high diversity of bacterial taxa in soil, suggesting a big underestimation of the actual real number of microbial volatiles (Kai et al., 2009; Lemfack et al., 2014). Moreover, most of the studies on microbial volatile detection have dealt with monocultures of already well-described bacterial genera. Thus, very little is known on how inter-specific interactions affect the volatile production. The investigation of volatiles production in more complex communities is of great interest since it could help to reveal the ecological role of these compounds. In the last years several independent studies reported that the production of secondary metabolites by soil bacteria can be influenced by interactions with microorganisms in their vicinity (Garbeva et al., 2011b; Traxler et al., 2013; Tyc et al., 2014). A high-throughput screening performed recently in our lab revealed that interactions between soil bacterial species have major effects in both directions: induction and suppression of antimicrobial activity (Tyc et al., 2014).

In this study we aimed to understand how inter-specific bacterial interactions affect the emission of volatiles and

their activity. For this we selected four strains belonging to different bacteria species that have been isolated from the soil bacterial community associated with sand sedge (*Carex arenaria* L.) namely *Chryseobacterium* sp. AD48, *Dyella* sp. AD56, *Janthinobacterium* sp. AD80, and *Tsukamurella* sp. AD106 (Tyc et al., 2014). In an earlier screening it was observed that these bacteria showed induced antimicrobial activity during interactions but not in monocultures. In the current study, it was examined if these observations were also reflected by the volatiles emission. To this end the effects of volatiles on growth of fungal, oomycetal, and bacterial model organisms produced by the bacteria in monocultures as well in pairwise combinations were tested. Our overall hypothesis is that the blend composition volatiles produced during interactions differs from that of monocultures and consequently has different effect on model target organisms.

MATERIALS AND METHODS

Bacteria and Culture Conditions

The bacterial isolates applied in this work were selected based on a previous observations of antimicrobial activity triggered by inter-specific interactions (Tyc et al., 2014). Four bacterial isolates were used: *Chryseobacterium* sp. AD48 (Class: *Flavobacteriia*) GenBank: KJ685263, *Dyella* sp. AD56 (Class: *Gammaproteobacteria*) GenBank: KJ685269, *Janthinobacterium* sp. AD80 (Class: *Betaproteobacteria*) GenBank: KJ685292, and *Tsukamurella* sp. AD106 (Class: *Actinobacteria*) GenBank: KJ685317. The bacterial isolates were pre-cultured from $-80°C$ glycerol stocks on 1/10th TSBA (5.0 g L^{-1} NaCl, 1.0 g L^{-1} KH$_2$PO$_4$; 3 g L^{-1} oxoid tryptic soy broth (TSBA); 20 g L^{-1} Merck Agar, pH 6.5; Garbeva and de Boer, 2009) and incubated for 3 days at $24°C$ before starting the experiments.

To test the effect of bacterial volatile compounds on bacterial growth and colony morphology three indicator bacteria were used: *Escherichia coli* WA321, *Staphylococcus aureus* 533R4 (Meyer and Schleifer, 1978; Tyc et al., 2014) and *Serratia marcescens* P87 (Garbeva et al., 2014b). All three indicator bacteria were pre-cultured from $-80°C$ glycerol stocks either on LBA media (LB-Medium Lennox, Carl Roth GmbH + Co. KG, Netherlands, art.no. X964.2, 20 g L^{-1} Merck Agar; *E. coli* WA321 and *S. aureus* 533R4; Sambrook and Russell, 2001) or on 1/10th TSBA (*S. marcescens* P87). The indicator organisms *E. coli* and *S. aureus* were incubated overnight at $37°C$ prior application, *S. marcescens* P87 was incubated at $24°C$ for 4 days prior usage. All bacterial isolates used in this study are listed in **Table 1**.

Cultures and Growth Conditions of Fungi and Oomycetes

The fungi *Rhizoctonia solani* AG2.2IIIB and *Fusarium culmorum* PV and the oomycete *Pythium ultimum* P17 were used in this study (Garbeva et al., 2014b). The fungi and oomycete were pre-cultured on 1/5th potato dextrose agar (PDA; 29 g L^{-1} Oxoid CM 139; Fiddaman and Rossall, 1993) and incubated at $24°C$ for 7 days prior usage. All fungal and oomycetal organisms are listed in **Table 1**.

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[1] http://bioinformatics.charite.de/mvoc/

Strain	Phylum/class	GenBank	Reference	Function
Volatile producing bacteria tested				
Chryseobacterium sp. AD48	Flavobacteriia	KJ685263	Tyc et al., 2014	Used for volatile analysis
Dyella sp. AD56	Y-proteobacteria	KJ685269	Tyc et al., 2014	
Janthinobacterium sp. AD80	β-proteobacteria	KJ685292	Tyc et al., 2014	
Tsukamurella sp. AD106	Actinobacteria	KJ685317	Tyc et al., 2014	
Fungal/oomycetal test organisms				
Rhizoctonia solani AG2.2IIIB	Basidiomycota	KT124637	Garbeva et al., 2011b	Eukaryotic model organisms for growth inhibition
Pythium ultimum P17	Oomycete	KT124638	Garbeva et al., 2014b	
Fusarium culmorum PV	Ascomycota	-	Garbeva et al., 2014b	
Bacterial test organisms				
Serratia marcescens P87	Y-proteobacteria	-	Garbeva et al., 2014b	Bacterial model organisms for growth inhibition and colony morphology changes
Escherichia coli WA321 DSMZ 4509	Y-proteobacteria	-	Tyc et al., 2014	
Staphylococcus aureus 533R4 Serovar 3 DSMZ 20231	Firmicutes	LN681573	Meyer and Schleifer, 1978	

Experimental Treatments

Ten different treatments were performed in triplicates. These treatments were: monoculture 1 (*Chryseobacterium* sp. AD48), monoculture 2 (*Tsukamurella* sp. AD106), monoculture 3 (*Dyella* sp. AD56), monoculture 4 (*Janthinobacterium* sp. AD80) and pairwise interaction of the isolates: interaction 1 (*Chryseobacterium* sp. AD48 + *Tsukamurella* AD106), interaction 2 (*Dyella* sp. AD56 + *Janthinobacterium* sp. AD80), Control 1 (glass Petri dish with TSBA media without inoculated bacteria, as background control in GC/MS measurement), Control 2 (two compartment Petri dish inoculated with model organisms without exposure to bacterial volatiles), Control 3 (top bottom Petri dish inoculated with fungal/oomycetal model organisms without exposure to bacterial volatile compounds). Control 4 (two compartment Petri dish inoculated with model organisms without exposure to the tested pure volatile compounds). The effect of the produced volatiles was tested on fungal, oomycetal, and bacterial growth via determination of hyphal biomass or growth inhibition assays. For the inoculation of the experiments a single colony of each test isolate was picked from a plate and inoculated in 20 mL 1/10th TSB (5.0 g L^{-1} NaCl, 1.0 g L^{-1} KH_2PO_4; 3 g L^{-1} TSBA) and incubated overnight at 24°C, 220 rpm. On the next day the OD_{600} of each isolate was measured on a GENESYS^{TM} 20 spectrophotometer (Thermoscientific, Netherlands, Cat# 4001-000) and a inoculation suspension for each treatment was prepared in 20 mL of 10 mM P-Buffer (pH 6.5) containing bacterial cells in a concentration of $\sim$1 × 10^5 CFU/mL.

Volatile Trapping

Next to the inhibition experiments, bacterial volatiles emitted in monocultures and pairwise combinations were trapped and analyzed. For trapping of VOCs emitted by bacteria a volume of 100 µl of inoculation suspension was spread on 1/10th TSBA (20 mL) in glass Petri dishes designed for headspace volatile trapping (Garbeva et al., 2014b). The Petri dishes were closed by a lid with an outlet connected to a steel trap containing 150 mg Tenax TA and 150 mg Carbopack B (Markes International, Ltd.,

Llantrisant, UK; Supplementary Figure S1). All treatments were inoculated in triplicate. The volatiles were collected after 48 and 72 h of incubation and the Tenax steel traps were stored at 4°C until GC-Q-TOF analysis.

GC-Q-TOF Analysis

The trapped VOCs were desorbed from the traps using an automated thermodesorption unit (Unity TD-100, Markes International, Ltd., Llantrisant, UK) at 210°C for 12 min (He flow 50 mL/min) and trapped on a cold trap at −10°C. The trapped volatiles were introduced into the GC-QTOF (model Agilent 7890B GC and the Agilent 7200A QTOF, Santa Clara, CA, USA) by heating the cold trap for 3 min to 280°C. Split ratio was set to 1:10, and the column used was a 30 mm × 0.25 mm ID RXI-5MS, film thickness 0.25 µm (Restek 13424-6850, Bellefonte, PA, USA). Temperature program used was as follows: 39°C for 2 min, from 39 to 95°C at 3.5°C/min, then to 165°C at 6°C/min, to 250°C at 15°C/min and finally to 300°C at 40°C/min, hold 20 min. The VOCs were detected by the MS operating at 70 eV in EI mode. Mass spectra were acquired in full-scan-mode (30–400 AMU, 4 scans/s). Mass-spectra's were extracted with MassHunter Qualitative Analysis Software V B.06.00 Build 6.0.633.0 (Agilent Technologies, Santa Clara, CA, USA) using the GC-Q-TOF qualitative analysis module. The obtained mass spectra's were exported as mzData files for further processing in MZmine V2.14.2. The files were imported to MZmine V2.14.2 (Copyright © 2005–2012 MZmine Development Team; Katajamaa et al., 2006; Pluskal et al., 2010) and compounds were identified via their mass spectra using deconvolution function (Local-Maximum algorithm) in combination with two mass-spectral-libraries: NIST 2014 V2.20 (National Institute of Standards and Technology, USA[2]) and Wiley 7th edition spectral libraries and by their linear retention indexes (LRIs). The LRI values were calculated using an alkane calibration mix before the measurements in combination with AMDIS 2.72 (National Institute of Standards and Technology, USA). The calculated

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[2]http://www.nist.gov

LRI were compared with those found in the NIST and in the in-house NIOO LRI database. After deconvolution and mass identification peak lists containing the mass features of each treatment (MZ-value/Retention time and the peak intensity) were created and exported as CSV files for statistical processing. The whole volatolomic workflow is shown in Supplementary Figure S2.

Bioassay for Testing the Effect of Bacterial Volatiles on Fungal and Oomycete Growth

To test the effect of the emitted bacterial volatiles on fungal/oomycete growth the hyphal extension and biomass were measured. The assays were performed in Petri dishes containing top and bottom growth areas (Supplementary Figure S3). At the bottom of the Petri dish, 100 µl of bacterial suspensions in 10 mM phosphate buffer (pH 6.5) containing $\sim 1 \times 10^5$ CFU/mL were spread on 20 mL 1/10th TSBA. At the lid of the Petri dish 12.5 mL of water-agar medium (WA; 20 g L^{-1} MERCK agar) was added and inoculated in the middle with a 6-mm-diameter PDA agar plug containing fungal (*R. solani*, *F. culmorum*) or oomycete (*P. ultimum*) hyphae. The plates were sealed with two layers of parafilm and incubated at 24°C for 5 days. In this way the tested fungi were exposed (without direct physical contact) to the volatiles produced by the bacteria in the bottom compartment. On the fifth day the extension of the hyphae was measured in 4 evenly spaced directions and compared to the hyphae extension in the control plates (fungi exposed to 1/10th TSBA growth medium without bacteria).

Determination of Fungal and Oomycetal Biomass

Fungal biomass was determined as described by Garbeva et al. (2014b). The whole growth area in the lids containing water agar and fungal hyphae was cut in ~ 2 cm^2 pieces and transferred to a glass beaker containing 100 mL of sterile demi-water (H$_2$O). The agar was melted for ~ 2.5 min in a microwave oven (temperature increased to about 100°C). The melted agar containing the hyphae was filtered over a tea strainer and the remaining hyphae were rinsed with about 150–200 mL of hot water (~ 80°C). The hyphae were picked with tweezers from the tea strainer and transferred to a micro centrifuge tube and stored at −20°C until analysis. For determination of fungal/oomycete biomass the frozen hyphae were transferred to a glass tube with lids with small holes and subjected to freeze-drying for 48 h (Labconco Freezone 12 with Labconco Clear Drying Chamber nr.7867000). The samples were stored in an exsiccator with dried silica gel for 3 h (Silica Gel Orange, 2–5 mm, indicator, Roth, art.nr.P077.2) prior weighing the dry biomass.

Bioassay for Testing the Effect of Bacterial Volatiles on Growth and Colony Morphology of Target Bacteria

The assays were performed in two-compartment Petri dishes (Greiner bio-one B.V., Alphen a/d Rijn, The Netherlands, Cat# 635102) containing two separated compartments

(Supplementary Figure S4). In such way the growth response of target bacteria to volatile producing bacteria could be determined without direct physical contacts. One compartment was supplemented with 12.5 mL TSBA and contained the volatile producing bacteria either in monoculture or in pairwise interactions. The second compartment contained the indicator bacteria and was supplemented either with 12.5 mL LBA (*E. coli* WA321, *S. aureus* 533R4) or with 12.5 mL TSBA (*S. marcescens* P87). The compartment for the volatile producing bacteria was inoculated with 100 µl bacterial suspensions master mix of monocultures or pairwise interactions prepared with 20 mL of 10 mM phosphate buffer (pH 6.5) containing $\sim 1 \times 10^5$ CFU/mL. The compartment for the indicator organisms was inoculated with four droplets (5 µL) of each indicator bacteria. The droplets of the indicator bacteria were placed in a distance of 2 cm to each other and contained 1×10^5, 1×10^4, 1×10^3, and 1×10^2 CFU/mL of either *E. coli* WA321, *S. aureus* 533R4, or *S. marcescens* P87 (Supplementary Figure S4). As controls the first compartment of the Petri dish was kept empty. After 4 days of incubation at 24°C the plates were examined and digital photographs were taken. The digital images were analyzed using the AXIO VISION v4.8 imaging Software (Carl Zeiss Imaging Solutions GmbH, Germany) for enumeration and surface-area determination (in pixel^2) of the bacterial colonies. All treatments were performed in triplicate.

Test of Pure Volatile Compounds on Bacterial Growth and Colony Morphology

The effect on growth, colony morphology and pigmentation by pure dimethyl disulfide (DMDS; CH$_3$S$_2$CH$_3$), dimethyl trisulfide (DMTS; CH$_3$S$_3$CH$_3$) and the mixture of both compounds was tested on *E. coli* WA321, *S. aureus* 533R4 and *S. marcescens* P87. The assays were performed in two-compartment Petri dishes (Greiner bio-one B.V., Alphen a/d Rijn, The Netherlands, Cat# 635102). Both compartments were supplemented with either 12.5 mL LBA (assay performed with *E. coli* WA321 and *S. aureus* 533R4) or with 12.5 mL TSBA (assay performed with *S. marcescens* P87). In one compartment a filter paper with a diameter of ~ 5.5 mm (Whatman$^{\text{TM}}$ filter paper Cat# 1003-150, 6 µm pore size) was placed on the agar surface in the middle of the compartment. Stock solutions with a concentration of 10, 1, and 0.1 µM of the pure volatile compounds (DMDS or DMTS) and the mixture of both compounds (DMDS + DMTS) were prepared by serial dilution of the pure compounds in Methanol (LiChrosolv$^{\circledR}$, Index-No: 603-001-00-X, Merck, Darmstadt, Germany). For the test a volume of 5 µl of each of the pure volatile stock solutions was added directly onto the filter paper resulting in a final concentration of 50, 5, and 0.5 µM, respectively. The other compartment was inoculated with the target bacteria *E. coli* WA321, *S. aureus* 533R4 or *S. marcescens* P87 by inoculating four spots in a distance of 2 cm from each other containing 1×10^5, 1×10^4, 1×10^3, and 1×10^2 CFU/mL (Supplementary Figure S4). As controls bacteria exposed to filter papers with no added volatile compounds

were applied. The Petri dishes were sealed with a double layer of parafilm and incubated for 4 days at 24°C. After incubation digital photographs were taken and the effect on colony growth, colony morphology and pigment production (prodigiosin) in *S. marcescens* P87 was examined. All digital images were analyzed using the AXIO VISION v4.8 imaging Software (Carl Zeiss Imaging Solutions GmbH, Germany) for enumeration and surface-area determination (in pixel^2) of the bacterial colonies. All treatments were performed in triplicate.

Statistical Analysis

Statistical analysis on volatolomic data was performed using the statistical analysis module of MetaboAnalyst V3.0, www.metaboanalyst.ca (Xia et al., 2012, 2015). Prior to statistical analysis data normalization was performed via log-transformation. To identify significant abundant mass features one-way-ANOVA with *post hoc* Tukey test (HSD-test) was performed between the data sets. To identify important mass features in the samples PLS-D analysis was performed. Mass features were considered to be statistical relevant if *p*-values were ≤ 0.05. Statistical relevant mass features were further used for the compound identification. Statistical analyses on fungal dry biomass and bacterial colony sizes were performed with IBM SPSS Statistics 23 (IBM, Somers, NY, USA) using one-way ANOVA and *post hoc* Tukey test between the data sets. The 5% level was taken as threshold for significance between control and volatile treatments.

Determination of HCN, NH$_3$ Emission, and pH Values in the Agar

All bacterial strains used in this study were tested for the emission of ammonia and HCN as well as for the ability to change the pH- value of the growth medium where the target organisms were inoculated. For these tests the bacteria were inoculated in two-compartment Petri dishes (start density ~1 × 10^5 CFU/mL) on 12.5 mL 1/10th TSBA. The second compartment was supplemented with 12.5 mL WA. After 4 days of growth the HCN and ammonia emission as well the pH-value of the target organism growth medium (WA) was determined. To test for the presence of Hydrocyanic acid the gaseous content of the Petri dish headspace was sucked through a Hydrocyanic acid test tube (Dräger Safety AG and CO. KGaA, Lübeck, Germany, order number: CH25701) using the Dräger accuro® gas detection pump (Dräger Safety AG and CO. KGaA, Lübeck, Germany). Presence of Hydrocyanic acid was determined by color change of the test tube (formation of a red reaction product; Supplementary Figure S5). The pH of the target organism growth medium (WA) exposed to bacterial volatiles was determined by slightly pressing a pH test-strip VWR PROLABO dosatest® (VWR international, Cat# 35309.606UK) for 30 s into the agar surface. The pH values were determined by color change of the test strip and compared to the color scale on the package (Supplementary Figure S6). The ammonia concentration was determined using the MQuant™ ammonium

test kit (Merck, Darmstadt, Germany, Cat# 110024) by placing a reaction activated test-strip on the lid of the Petri dish directly opposite to the bacterial culture and fixed with tape. The Petri-dish were closed and sealed with parafilm and incubated for 2 h at 24°C. After incubation the presence of ammonium was determined by color change of the test strip (Supplementary Figure S7).

RESULTS

Detected Headspace Volatile Compounds and GC/MS-Q-TOF Analysis

GC/MS-Q-TOF based volatolomic analysis revealed a total number of 35 compounds that were not detected in the non-inoculated controls (**Table 2**). 27 compounds were obtained from the monocultures of *Chryseobacterium* sp. AD48, 15 compounds were obtained from the monocultures of *Tsukamurella* sp. AD106 and 26 compounds were detected in the interactions between these two bacteria (**Table 2**; **Figure 1A**). For the combinations of *Dyella* sp. AD56 and *Janthinobacterium* sp. AD80 we obtained a total number of 18 compounds, whereas 16 compounds were detected in the monoculture of *Janthinobacterium* sp. AD80 and only 13 compounds in the monoculture of *Dyella* sp. AD56 (**Table 2**; **Figure 1B**). We were able to tentatively identify 19 VOCs belonging to seven different chemical classes including alcohols, amines, esters, indole, thiocyanates, thioesters, and sulfides. However, a vast number of the detected compounds (*n* = 16) could not be assigned with certainty to a VOC and remained unknown. The most prominent detected headspace VOCs were sulfur containing compounds (such as sulfordioxide, methyl thioacetate, dimethyl sulfoxide, etc.). Two sulfur compounds DMDS ($C_2H_6S_2$) and DMTS ($C_2H_6S_3$) were produced by all bacteria (except DMTS which was not detected for *Janthinobacterium* sp. AD80).

Effect of Inter-specific Interactions on Bacterial Volatile Blend Composition

Volatolomic analysis on monocultures and pairwise combinations of *Chryseobacterium* sp. AD48 with *Tsukamurella* sp. AD106 revealed that the volatile composition of the monocultures differed from that of the mixtures (**Figure 1A**; **Table 2**). Clear separations between controls, monocultures and pairwise combinations of *Chryseobacterium* sp. AD48 with *Tsukamurella* sp. AD106 were obtained in PCA score plots (**Figure 1A**). The volatile composition of the pairwise combinations resembled that of the monocultures of *Chryseobacterium* sp. AD48 (**Figure 1A**; **Table 2**). The indole produced by the monoculture of *Chryseobacterium* sp. AD48 was not detected in the interactions (**Table 2**).

The analysis on the volatiles emitted by monocultures and pairwise combinations of *Dyella* sp. AD56 and *Janthinobacterium* sp. AD80 revealed that the volatile profiles of the monocultures differed from that of the mixtures (**Figure 1B**; **Table 2**). Different PCA score plots

TABLE 2 | Tentatively identified volatile organic compounds emitted by four bacterial strains cultivated either in monoculture or in pairwise combination.

# Compound name/chemical class	RT*	ERI**	Chry	Tsuk	MIX Chry + Tsuk	Dye	Jant	MIX Jant + Dye
(1) Sulfurdioxide	2.58	521	x		x		x	x
(2) Cyclopentene	2.96	551	x		x	x	x	
(3) 2-Pentene	3.29	575				x		x
(4) Unknown compound 1	3.77	612	x	x	x	x	x	x
(5) Methyl isobutyrate	4.70	682				x		
(6) Methyl thioacetate	4.94	700	x		x		x	x
(7) Methyl thiocyanate	5.28	713		x			x	x
(8) 1-Butanol, 3-methyl-	5.69	728	x		x			
(9) Dimethyl disulfide	6.10	744	x	x	x	x	x	x
(10) Methyl isovalerate	6.86	769				x		
(11) S-methyl propanethioate	7.45	782	x		x		x	x
(12) 1,3 Dithiethane	7.64	786	x	x	x		x	x
(13) Dimethyl sulfoxide	8.46	806		x				
(14) 2,4-Dithiapentane	10.74	865	x	x	x		x	x
(15) Benzaldehyde	13.72	944	x	x	x		x	x
(16) Dimethyl trisulfide	14.33	960	x	x	x	x		x
(17) Unknown cycloalkane	16.86	1026	x	x	x	x	x	x
(18) Unknown branched alkene	17.39	1040	x	x	x	x	x	x
(19) Unknown sulfur containing compound	18.09	1058	x	x	x			
(20) 1,2,4-Trithiolane	19.30	1090	x	x	x		x	x
(21) Unknown compound 2	19.70	1101						
(22) Unknown compound 3	19.99	1110	x	x	x	x	x	x
(23) Unknown compound 4	20.63	1131	x		x			
(24) Dimethyl tetrasulfide	23.64	1227		x				
(25) Indole	25.82	1298	x					
(26) Butylhydroxytoluene	30.28	1540	x	x	x	x	x	x
(27) Unknown terpene like compound 1	32.84	1674	x		x			
(28) Unknown terpene like compound 2	33.46	1703	x		x			
(29) Unknown tetralin isomer	33.75	1710	x		x			
(30) Unknown aromatic isomer	34.22	1721	x		x			
(31) Unknown compound 5	34.34	1724	x		x			
(32) Unknown di-terpene	34.78	1734	x		x			
(33) Unknown terpene like compound 3	35.31	1746	x		x			
(34) Unknown compound 6	38.73	2101				x		x
(35) Unknown compound 7	42.04	2360	x		x			
Number of detected compounds (n)			**27**	**15**	**26**	**13**	**16**	**18**

# = Compound number, Chry = Chryseobacterium, Dye = Dyella, Jant = Janthinobacterium, Tsuk = Tsukamurella, MIX Chry + Tsuk = pairwise combination of Chryseobacterium + Tsukamurella. MIX Jant + Dye = pairwise combination of Dyella + Janthinobacterium.
X = detected.
RT* = Retention time, the RT value stated is the average.
ERI** = Experimental retention index value, the RI value stated is the average.

were obtained between controls, monocultures and pairwise combinations of *Dyella* sp. AD56 with *Janthinobacterium* sp. AD80 (**Figure 1B**). A higher number of volatile compounds were detected in the pairwise combinations of these two bacteria. However, the higher number of detected volatiles is most probably due to the combination of the volatile blends of these two bacterial isolates. We did not detect any novel or different volatile compounds which production was triggered during the pairwise interaction of these two bacteria. Interestingly the volatile compound cyclopentene produced by the monocultures of *Dyella* sp. AD56 and

Janthinobacterium sp. AD80 was not detected in the interactions (**Table 2**).

Effect of Bacterial Volatiles on Fungal and Oomycetal Growth

Volatiles produced by all treatments including monocultures and pairwise combinations of the selected bacteria revealed strong growth inhibition of the plant pathogenic fungi and oomycete. The dry biomass of fungi and oomycete exposed to bacterial volatiles was significantly reduced as compared to the controls without bacterial volatiles (**Table 3**; **Figures 2** and **3**).

FIGURE 1 | PCA 2D- plots of volatiles emitted by monocultures and pairwise combinations of bacteria including confidence intervals (in semi-transparent colors). (A) Monocultures and mixtures of *Tsukamurella* sp. AD106 and *Chryseobacterium* sp. AD48 and **(B)** monocultures and mixtures of *Dyella* sp. AD56 and *Janthinobacterium* sp. AD80.
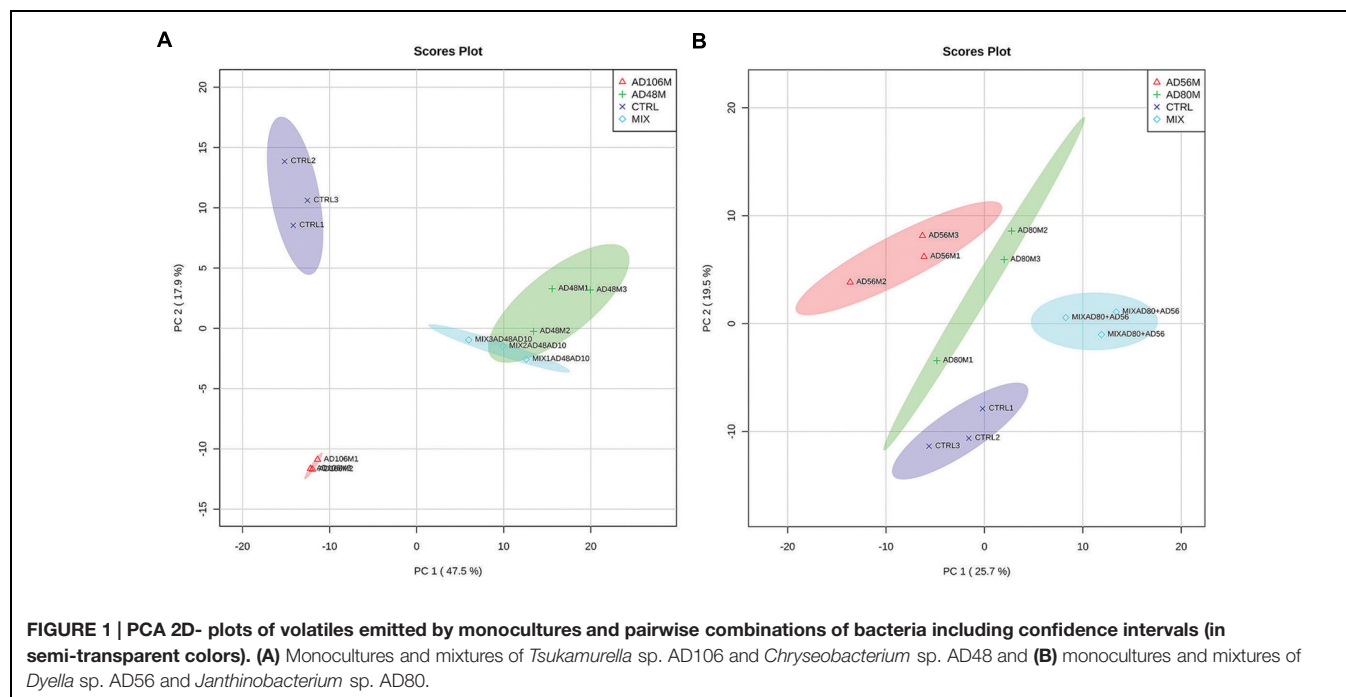
Effect of Bacterial Volatiles on the Growth and Behavior of Target Bacteria

Volatiles emitted by *Chryseobacterium* sp. AD48 and the mixture of *Chryseobacterium* sp. AD48 and *Tsukamurella* sp. AD106 inhibited the growth of *E. coli* WA321 significantly as compared to the control (**Figure 4A**). This observation is in agreement with the observed volatolomic profile (**Figure 1A**) which revealed that the volatolomic profile of the mixture is dominated by the volatiles produced by the monoculture of *Chryseobacterium* sp. AD48.

Besides growth inhibition we observed significant growth promotion of *S. aureus* 533R4 when exposed to volatiles emitted by the monocultures of *Dyella* sp. AD56 (**Figure 4B**).

Changes in colony morphology of *S. marcescens* P87 were observed when exposed to volatiles emitted by *Chryseobacterium* sp. AD48 and to volatiles emitted by the mixtures of *Dyella* sp. AD56 with *Janthinobacterium* sp. AD80. The *S. marcescens* P87 colonies were more circular and round shaped (Supplementary Figure S8). However, no significant effects of bacterial volatiles on the growth of the target bacteria were also observed (Supplementary Figure S9).

Effect of Pure Individual Volatile Compounds on the Growth and Colony Morphology of Target Bacteria

We applied a two-compartment Petri dish testing system (Supplementary Figure S4) in which the model organisms could grow without direct physical contacts to the tested pure volatile compounds. After 4 days of growth *S. marcescens* P87 colonies were small and showed a white phenotype when exposed to 50 μM of DMTS, indicating the lack of prodigiosin production

(**Figure 5A**). Furthermore we observed significant inhibition of growth of *S. marcescens* P87, *E. coli* WA321 and *S. aureus* 533R4 when exposed to 50 μM of DMTS (**Figures 5–7**).

Exposure to DMDS did not reveal any significant growth inhibiting or changes in colony morphology at all concentrations tested (500 nM, 5 and 50 μM). The mixture of DMDS and DMTS resulted in growth inhibition of *S. marcescens* P87 and *E. coli* WA321 at 50 μM concentration. However, the pigmentation in *S. marcescens* P87 was not affected by the mixture of these compounds. The two lowest applied concentrations 5 and 0.5 μM of DMTS and DMDS and

TABLE 3 | Effect of bacterial volatiles on fungal and oomycetal biomass production (mg/dry weight of fungal/oomycetal biomass).

Treatment	F. culmorum	P. ultimum	R. solani
Monocultures			
Chryseobacterium sp. AD48	1.63±0.25*	0.83±0.28*	1.67±0.75*
Dyella sp. AD56	1.03±0.55*	1.47±0.47*	1.1±0.71*
Janthinobacterium sp. AD80	1.05±0.77*	0.9±0.44*	1.1±0.44*
Tsukamurella sp. AD106	2.3±0.69*	1.47±0.12*	2.67±0.47*
Interactions			
Chryseobacterium sp. AD48 + *Tsukamurella* sp. AD106	1.73±0.4*	1.47±0.25*	2.53±0.37*
Janthinobacterium sp. AD80 + *Dyella* sp. AD56	1.3±1.27*	0.97±0.40*	1.23±0.15*
Controls	5.97±2.13	4.42±0.88	5.47±1.23

Data represent mean and standard deviation of three replicates.
Asterisk indicates significant differences between the treatments and the respective control (one-way ANOVA, post hoc Tukey test p < 0.05).
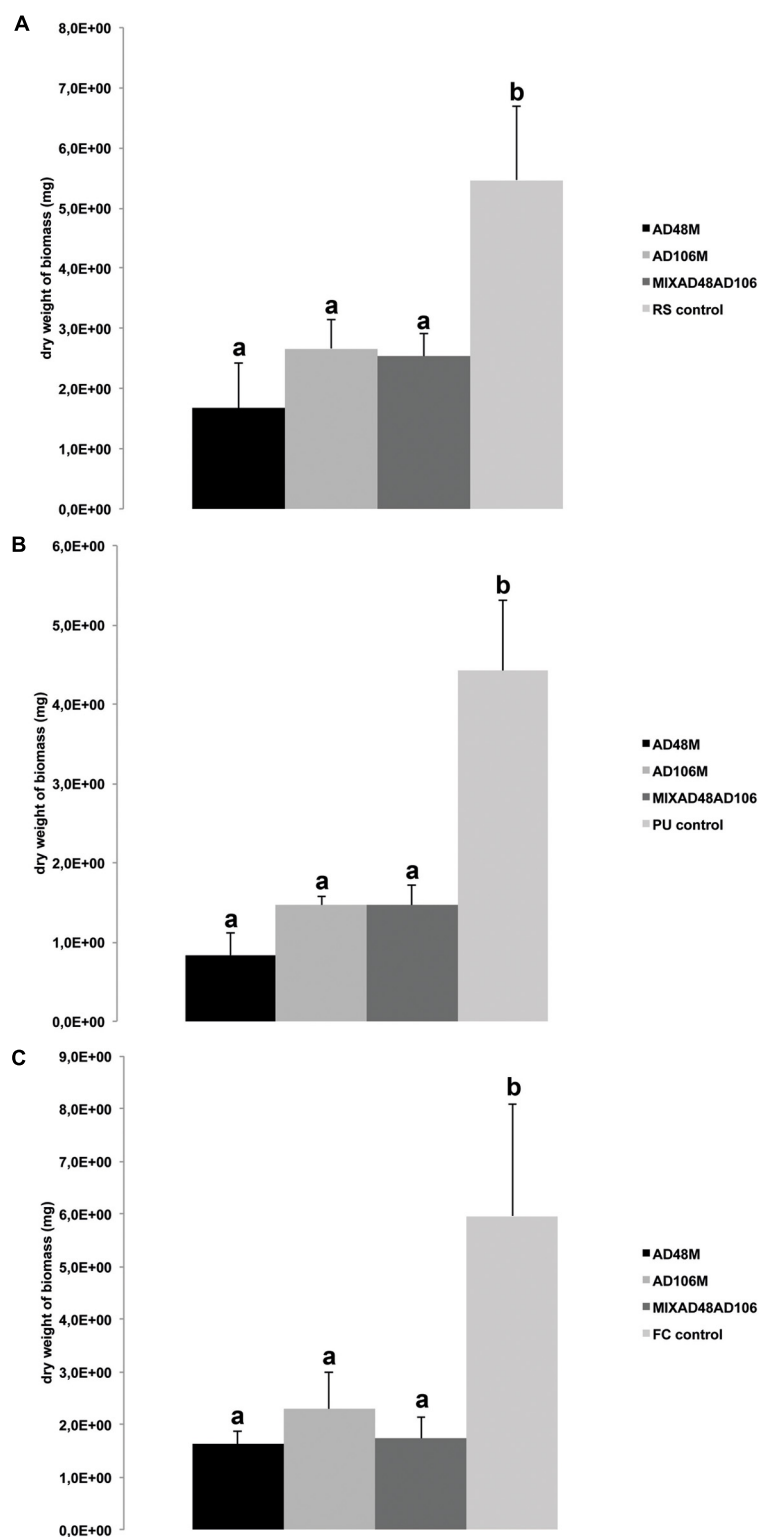
FIGURE 2 | Effect of volatiles produced by monocultures and mixtures of *Tsukamurella* sp. AD106 and *Chryseobacterium* sp. AD48 on growth of eukaryotic plant-pathogens. Bars represent the average values for fungal and oomycetal biomass dry weight and error bars represent standard deviation of the mean. **(A)** Dry weight of *Rhizoctonia solani;* **(B)** dry weight of *Pythium ultimum*; **(C)** dry weight of *Fusarium culmorum*. Significant differences between treatments and the control are indicated by different letters (one-way ANOVA, *post hoc* Tukey test $p < 0.05$).
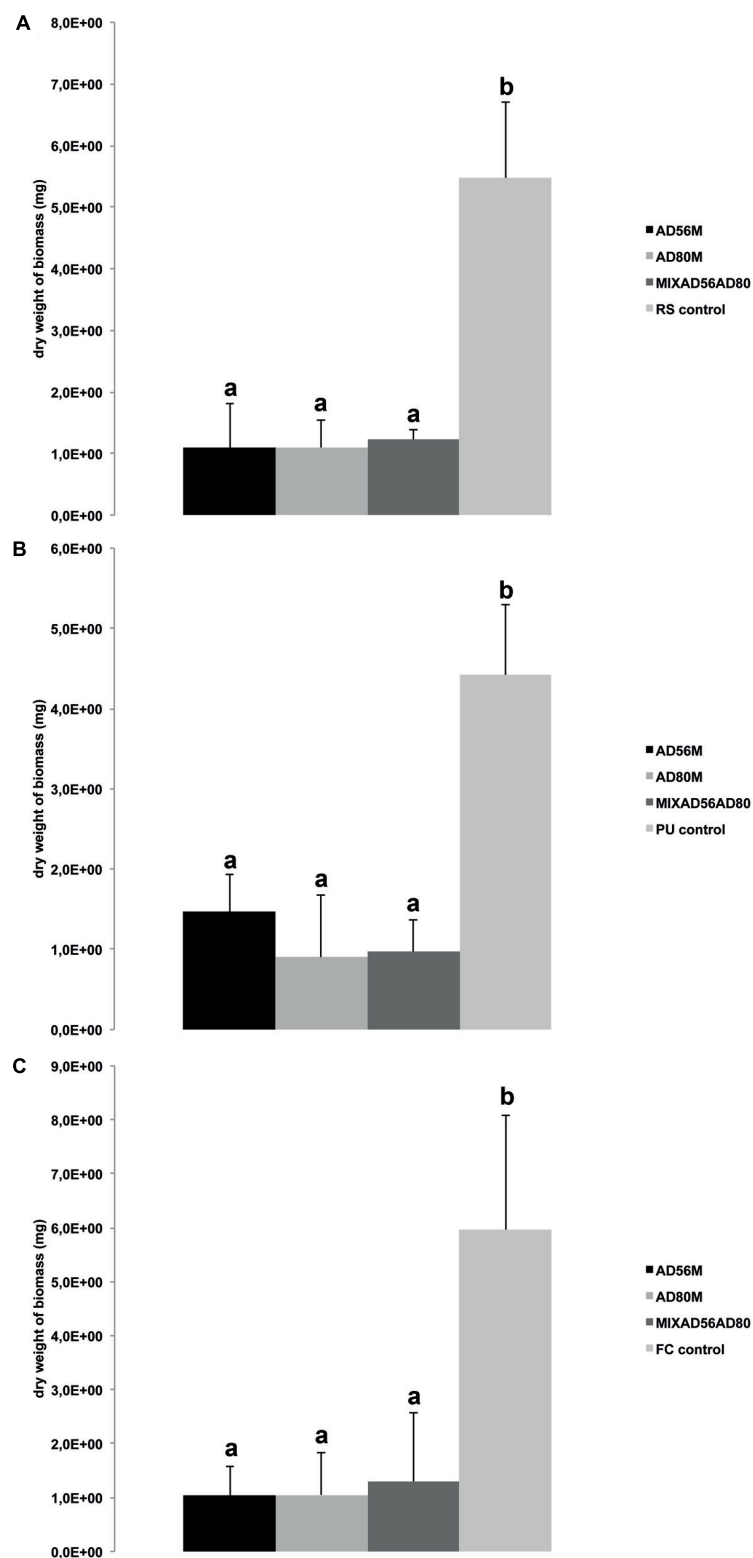
FIGURE 3 | Effect of volatiles produced by monocultures and mixtures of volatile emitting *Dyella* sp. AD56 and *Janthinobacterium* sp. AD80 on growth of eukaryotic plant-pathogens. Bars represent the average values for fungal and oomycetal biomass dry weight and error bars represent standard deviation of the mean. **(A)** Dry weight of *R. solani*; **(B)** dry weight of *P. ultimum*; **(C)** dry weight of *F. culmorum*. Significant differences between treatments and the control are indicated by different letters (one-way ANOVA, *post hoc* Tukey test $p < 0.05$).
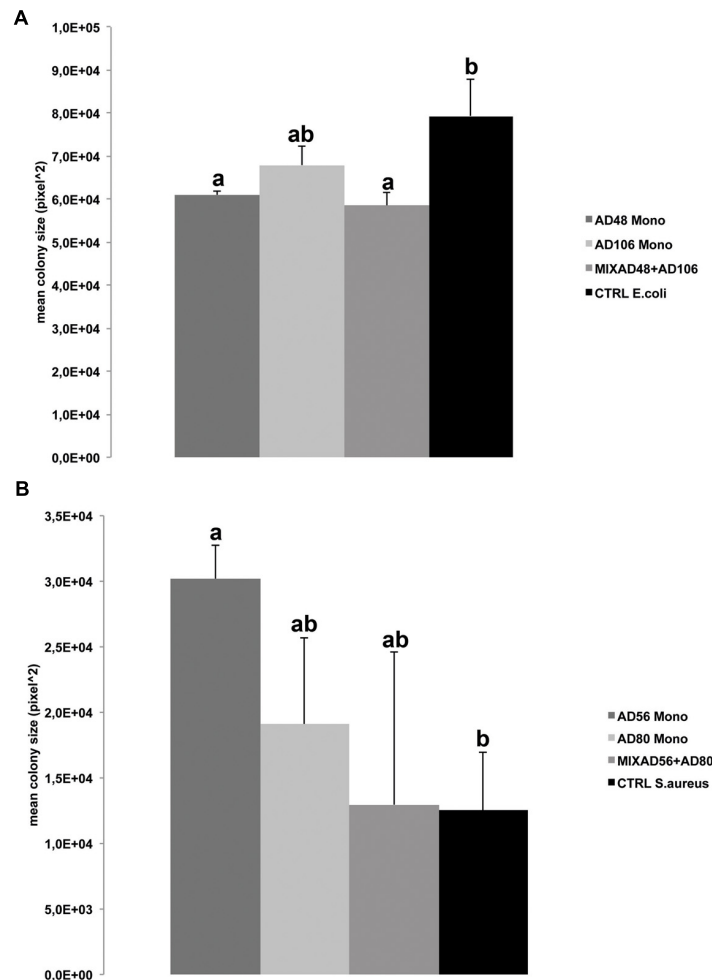
FIGURE 4 | Effect of volatiles produced by monocultures and pair-wise combinations of the four selected rhizosphere bacterial strains on average colony size of the target bacteria. (A) Mean colony size of *Escherichia coli* WA321 exposed to volatile compounds of *Chryseobacterium* sp. AD48 and *Tsukamurella* sp. AD106 and the mixture of both bacteria. **(B)** Mean colony sizes of *Staphylococcus aureus* 533R4 exposed to volatile compounds of *Dyella* sp. AD56, *Janthinobacterium* sp. AD80 and the mixture of both bacteria. Significant differences between treatments and the control are indicated by different letters (one-way ANOVA, *post hoc* Tukey test $p < 0.05$). Data represented are the mean of three replicates, error bars represent standard deviation of the mean.

the mixture of both compounds did not reveal any effect on colony morphology or growth of the tested bacteria (**Figures 5–7**).

DISCUSSION

Bacteria coexist with many different species in a heterogeneous and challenging soil environment (Gans et al., 2005). In this environment inter-specific interactions between microorganisms are ongoing and are a key factor for their spatial distribution (Keller and Surette, 2006). To cope with the competitive conditions, bacteria developed different survival strategies such as the production of secondary metabolites with inhibitory capacity (Hibbing et al., 2010; Cornforth and Foster, 2013). Most of the studies on bacterial secondary metabolites so far were focused on non-volatile compounds (Korpi et al., 1998; Foster

and Bell, 2012). However, bacteria do also release complex blends of VOCs. Yet, the effect of inter-specific interactions on volatiles production and composition is still unknown (Garbeva et al., 2014a).

Here, we compared the volatile blends emitted by four phylogenetically different soil-bacteria either grown in monocultures or in pairwise combinations. Our results revealed that the blend of volatiles emitted during pairwise combinations differed from the volatile blends of the respective monocultures. Yet, the volatile blend of the mixtures mostly included volatiles compounds produced by monocultures, although some compounds produced by the monocultures were not detected in mixtures. For example dimethyl sulfoxide produced by *Tsukamurella* sp. AD106 was not detected in the mixture with *Chryseobacterium* sp. AD48. Another interesting example is indole which was produced by the monocultures of *Chryseobacterium* sp. AD48 but was not detected in the
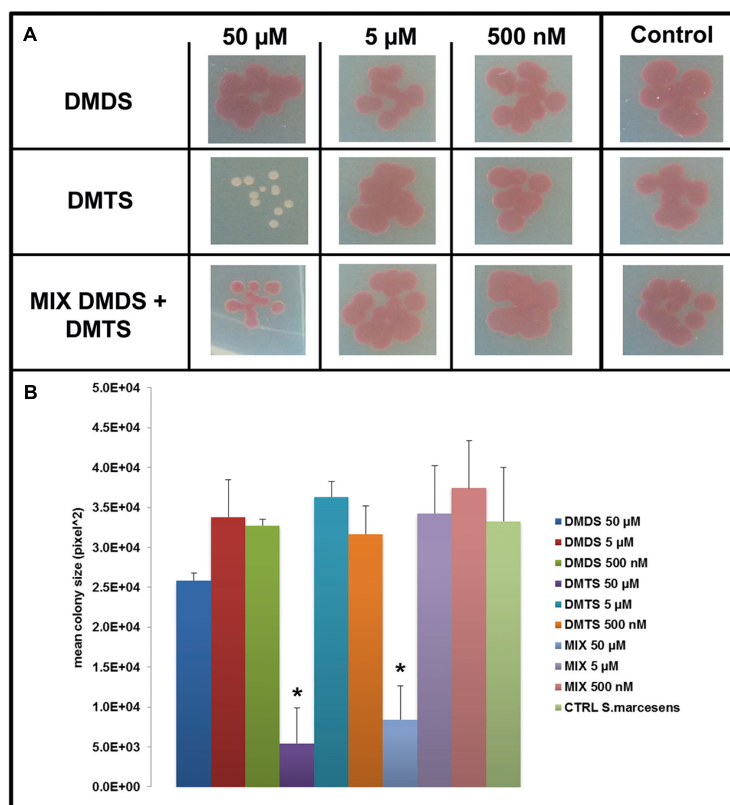
FIGURE 5 | Effect of dimethyl disulfide (DMDS), dimethyl trisulfide (DMTS), and the mixture of both volatile compounds (DMDS + DMTS) on colony development of *S. marcescens*. (A) Colony morphology and growth of *S. marcescens* P87 after 4 days of incubation. The pure volatile compounds were applied in a concentration ranging from 500 nM to 50 μM. Control *S. marcescens* P87 grown without exposure to the compounds. **(B)** Mean colony sizes of *S. marcescens* P87 exposed to volatile compounds of DMDS, DMTS, and the mixture of both volatile compounds (DMDS + DMTS). Asterisk indicates significant differences between the treatments and the control (one-way ANOVA, *post hoc* Tukey test $p < 0.05$). Data represented are the mean of three replicates, error bars represent standard deviation of the mean.

presence of *Tsukamurella* sp. AD106. Indole is a very well-studied compound and has been reported to be produced by about 85 different bacterial species including *Chryseobacterium* sp. (Yamaguchi and Yokoe, 2000; Lee and Lee, 2010). Indole and its derivatives [quinolones and (*S*)-3-hydroxytridecan-4-one] are involved in intercellular and multispecies signaling controlling diverse bacterial physiological properties like sporulation, plasmid stability, biofilm formation, drug resistance and virulence (Wang et al., 2001; Di Martino et al., 2003; Diggle et al., 2006; Nikaido et al., 2008; Lee et al., 2009; Lee and Lee, 2010). In addition, indole has been shown to have inhibitory activities on fungal growth (*Aspergillus niger*) and plant growth stimulating properties (*Arabidopsis thaliana*; Kamath and Vaidyanathan, 1990; Blom et al., 2011). In general indole is known to be a stable compound in the producing bacteria, however, many non-indole producing bacteria are able to modify and to degrade indole (Shimada et al., 2013; Lee et al., 2015). The fact that indole was not detected during the interaction of *Chryseobacterium* sp. AD48 with *Tsukamurella* sp. AD106 suggests that the production of such signaling compounds in nature depends strongly on the inter-specific interactions. Similar result was observed for the compound

cyclopentene produced by the monocultures of *Dyella* sp. AD56 and *Janthinobacterium* sp. AD80 but not produced during the interaction of these two bacteria. With the volatolomic methods applied in this study we were able to detect 35 compounds from which 19 were tentatively identified. This discrepancy between numbers of detected and identified compounds shows that the identification of bacterial volatiles is yet a challenging and time demanding task, even with the use of sophisticated programs and software for metabolomics data analysis. Hence, the produced volatile blends are very complex and consist of a mixture of many unknown and difficult to identify compounds (Tait et al., 2014). Most of the VOCs that were tentatively identified within this study (∼58%) contained sulfur (e.g., methyl thiocyanate, DMDS, DMTS, dimethyl tetrasulfide, etc.). The high abundance of sulfur containing volatiles in this study can be related to the cultivation of the tested bacteria on 1/10th TSBA growth media. Several studies indicated that the composition of the volatile blend greatly depends on the growth media composition and the growth conditions (Schulz et al., 2004; Schulz and Dickschat, 2007; Blom et al., 2011; Garbeva et al., 2014b). The high amount of dimethyl di- and trisulfide detected in both monocultures and interactions
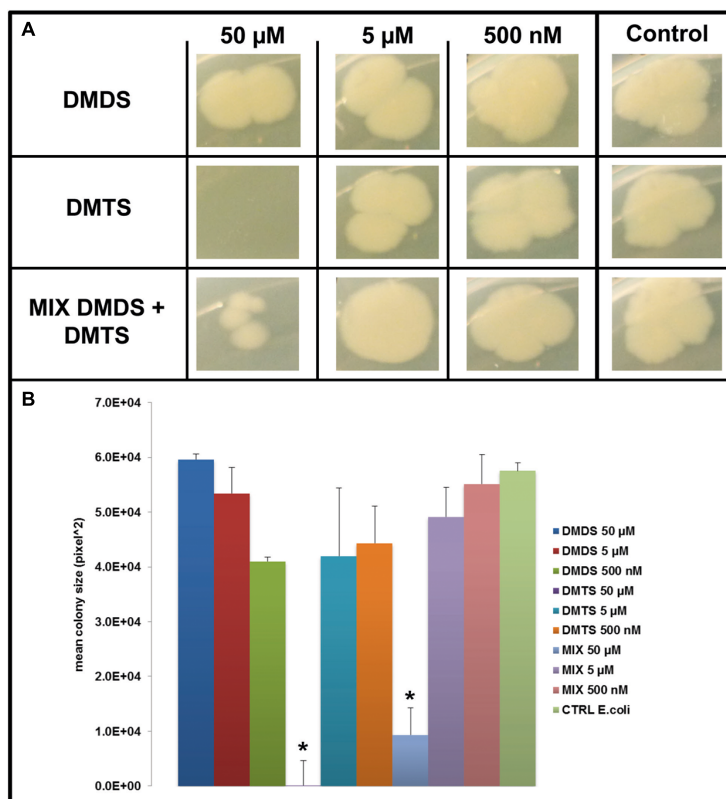
FIGURE 6 | Effect of DMDS, DMTS, and the mixture of both volatile compounds (DMDS + DMTS). (A) Colony morphology and growth of *E. coli* WA321 after 4 days of incubation. The pure volatile compounds were applied in a concentration ranging from 500 nM to 50 µM. Control *E. coli* WA321 grown without exposure to the compounds. **(B)** Mean colony sizes of *E. coli* WA321 exposed to volatile compounds of DMDS, DMTS, and the mixture of both volatile compounds (DMDS + DMTS). Asterisk indicates significant differences between the treatments and the control (one-way ANOVA, *post hoc* Tukey test $p < 0.05$). Data represented are the mean of three replicates, error bars represent standard deviation of the mean.

indicate that these compounds are commonly produced. Many studies have shown that bacterial volatiles play a major role in soil fungistasis (Zou et al., 2007; Garbeva et al., 2011a, 2014b; van Agtmaal et al., 2015). Indeed our results revealed that the fungal and oomycete tested organism are sensitive to bacterial volatiles and were inhibited significantly by all monocultures and pairwise combinations. The observed fungal and oomycetal growth inhibition is most probably related to sulfur containing volatiles. Sulfur containing volatiles like dimethyl di- and trisulfide have been shown to effect fungi and are able to inhibit the growth of different plant pathogenic fungi (Kai et al., 2009; Li et al., 2010; Huang et al., 2012; Wang et al., 2013; Garbeva et al., 2014b; Kanchiswamy et al., 2015).

While many study tested the effect of bacterial volatiles on various fungi, little is known so far on the effect of bacterial volatiles on other bacteria. In this study *E. coli* WA321 was inhibited by the volatiles emitted by *Chryseobacterium* sp. AD48 and the mixture of *Chryseobacterium* sp. AD48 with *Tsukamurella* sp. AD106. The observed growth promotion of *S. aureus* 533R4 was caused by the volatiles emitted by *Dyella* sp. AD56. However, this growth promotion was not observed by the volatiles emitted during the interaction of *Dyella* sp. AD56 with *Janthinobacterium* sp. AD80 correlating with a shift

in volatile blend composition. Interestingly volatiles emitted by the monocultures of *Chryseobacterium* sp. AD48 and the mixture of *Dyella* sp. AD56 with *Janthinobacterium* sp. AD80 induced changes in colony morphology of *S. marcescens* P87. Our previous high-throughput screening for production of non-volatile antimicrobial compounds revealed that all four bacteria used here, showed induced antibacterial activity during pairwise interactions as compared to monocultures (Tyc et al., 2014). This was not observed in the present study, as we didn't observed novel produced volatile compounds during the pairwise interactions. Therefore, it's questionable if volatiles solely play an important role as a competitive strategy between bacteria. However, it is possible that volatiles have synergistic or additive effect to other non-volatile antibacterial compounds (Schmidt et al., 2015). Many bacteria are known to emit inorganic volatiles like CO_2, NH_3, HCN, which also have biological activities and can have an additive effect (Effmert et al., 2012). However, such compounds were not detected in this study as significant volatile compounds.

Here, we tested two commonly produced bacterial volatile compounds for their effect on the target bacteria. The experiments with pure DMTS revealed strong growth inhibition on all tested bacterial model organisms, when applied in a
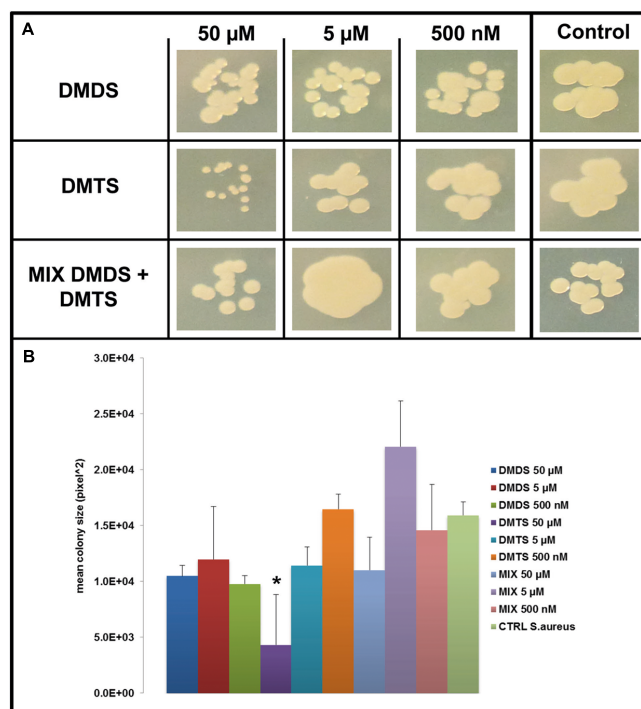
FIGURE 7 | Effect of DMDS, DMTS, and the mixture of both volatile compounds (DMDS + DMTS). (A) Colony morphology and growth of *S. aureus* 533R4 after 4 days of incubation. The pure volatile compounds were applied in a concentration ranging from 500 nM to 50 µM. Control *S. aureus* 533R4 grown without exposure to the compounds. **(B)** Mean colony sizes of *S. aureus* 533R4 exposed to volatile compounds of DMDS, DMTS, and the mixture of both volatile compounds (DMDS + DMTS). Asterisk indicates significant differences between the treatments and the control (one-way ANOVA, *post hoc* Tukey test $p < 0.05$). Data represented are the mean of three replicates, error bars represent standard deviation of the mean.

concentration of 50 µM. Bacterial growth suppression was already reported for DMDS emitted by *Pseudomonas* strains against the crown-gall diseases causing *Agrobacterium* sp. (Dandurishvili et al., 2011; Popova et al., 2014). Dimethyl trisulfide effected colony morphology and pigmentation in *S. marcescens* P87 when applied in a concentration of 50 µM. Volatiles exposed colonies showed reduced growth and white coloration indicating the lack of prodigiosin production. It is plausible that this observation is related to the inhibition of quorum-sensing as previously reported by Morohoshi et al. (2007), Chernin et al. (2011). However, the effective concentration of 50 µM DMTS is most probably very high and far away from the concentrations in which those volatile compounds are produced in nature (Groenhagen et al., 2013) as we did not observed this effect in the experiments where *S. marcescens* P87 was exposed to the volatile blend produced by bacteria. The biological relevant concentration of volatile compounds remains to be determined in future studies.

CONCLUSION

This work revealed that inter-specific bacterial interactions affect volatile blend composition. This observed change is most probably related to the combination of volatile compounds produced by each isolate rather than triggering the production of

novel volatiles as the volatile blend was composed of the mixture of the respective interacting bacteria. Furthermore, the loss of production of certain compounds during pairwise interaction suggests that the production of volatile signaling compounds (e.g. indole) in nature is influenced by inter-specific interactions. While fungi and oomycetes showed to be very sensitive to bacterial volatiles the effect of volatiles on bacteria varied greatly between no effects, growth inhibition to growth promotion depending on the volatile blend composition.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb. 2015.01412

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Legacy effects of anaerobic soil disinfestation on soil bacterial community composition and production of pathogen-suppressing volatiles

Maaike van Agtmaal[1], Gera J. van Os[2], W.H. Gera Hol[3], Maria P.J. Hundscheid[1], Willemien T. Runia[4], Cornelis A. Hordijk[1] and Wietse de Boer[1,5]*

[1] Department of Microbial Ecology, Netherlands Institute of Ecology, NIOO-KNAW, Wageningen, Netherlands, [2] Applied Plant Research, Flowerbulbs, Nursery Stock and Fruit, Wageningen University and Research Centre, Lisse, Netherlands, [3] Department of Terrestrial Ecology, Netherlands Institute of Ecology, NIOO-KNAW, Wageningen, Netherlands, [4] Applied Plant Research, Subdivision Arable Farming, Multifunctional Agriculture and Field Production of Vegetables, Wageningen University and Research Centre, Lelystad, Netherlands, [5] Department of Soil Quality, Wageningen University and Research Centre, Wageningen, Netherlands

There is increasing evidence that microbial volatiles (VOCs) play an important role in natural suppression of soil-borne diseases, but little is known on the factors that influence production of suppressing VOCs. In the current study we examined whether a stress-induced change in soil microbial community composition would affect the production by soils of VOCs suppressing the plant-pathogenic oomycete *Pythium*. Using pyrosequencing of 16S ribosomal gene fragments we compared the composition of bacterial communities in sandy soils that had been exposed to anaerobic disinfestation (AD), a treatment used to kill harmful soil organisms, with the composition in untreated soils. Three months after the AD treatment had been finished, there was still a clear legacy effect of the former anaerobic stress on bacterial community composition with a strong increase in relative abundance of the phylum *Bacteroidetes* and a significant decrease of the phyla *Acidobacteria, Planctomycetes, Nitrospirae, Chloroflexi,* and *Chlorobi*. This change in bacterial community composition coincided with loss of production of *Pythium* suppressing soil volatiles (VOCs) and of suppression of *Pythium* impacts on Hyacinth root development. One year later, the composition of the bacterial community in the AD soils was reflecting that of the untreated soils. In addition, both production of *Pythium*-suppressing VOCs and suppression of *Pythium* in Hyacinth bioassays had returned to the levels of the untreated soil. GC/MS analysis identified several VOCs, among which compounds known to be antifungal, that were produced in the untreated soils but not in the AD soils. These compounds were again produced 15 months after the AD treatment. Our data indicate that soils exposed to a drastic stress can temporarily lose pathogen suppressive characteristics and that both loss and return of these suppressive

characteristics coincides with shifts in the soil bacterial community composition. Our data are supporting the suggested importance of microbial VOCs in the natural buffer of soils against diseases caused by soil-borne pathogens.

Introduction

In the light of sustainable agriculture and the call for reduction of pesticide use, insights in the mechanisms of natural suppression of soil-borne pathogens are essential. Therefore, understanding the interactions of plant pathogens with other members of soil microbial communities is needed to develop strategies for effective and consistent control (Chaparro et al., 2012). In general, depletion of carbon sources by indigenous microbes hampers the pre-infective growth of soil-borne pathogens resulting in lower infection rates (Hoitink and Boehm, 1999). This competition-related mechanism of pathogen control is also known as "general disease suppression" (Hoitink and Boehm, 1999). Disease suppression is closely related to soil fungistasis, the restricted ability of fungal propagules to germinate or grow in most soils (Dobbs and Hinson, 1953). As for general suppression, it has been hypothesized to be caused by microbial withdrawal of nutrients from soil or even from fungal propagules (Lockwood, 1977). However, besides substrate competition, also inhibitory compounds, released by microbes, have been indicated to contribute to fungistasis (Romine and Baker, 1972; De Boer et al., 2003). This implies that not only the carbon-withdrawing activity of the total soil microbial community is involved in fungistasis but also the secondary metabolite production of certain groups within the soil microbial community. Based on this, Garbeva et al. (2011) argued that the composition of soil microbial communities is more important in fungistasis than previously has been appreciated.

Most soil-borne pathogens are poor competitors and have limited saprotrophic capacities in their pre-infective stages. Therefore, they are sensitive to general disease suppression. Among them is the oomycete genus *Pythium,* which includes many plant pathogenic species (Boehm and Hoitink, 1992). They infect roots of seedlings generally resulting in damping-off and, consequently, reduced yield in a broad range of crops (Martin and Loper, 1999). In flower bulb crops, several species of *Pythium* cause severe root rot, leading to considerable losses in bulb yield (Van Os et al., 1998). Infection can occur by zoospores and is initiated by a chemotactic response to compounds exuded by roots. Yet, *Pythium* is considered to be a poor competitor for these root exudates and, therefore, natural control of *Pythium* infection is attributed to high competitive pressure exerted by other exudate-consuming soil microbes (Chen et al., 1988; Van Os and van Ginkel, 2001). Hence, the current view on the cause of natural buffering of soils against *Pythium* infection is mainly pointing at resource competition rather than at interference competition (involvement of inhibitory secondary metabolites).

Antimicrobial volatile organic compounds (VOCs), emitted by soil microbes, may be an important factor in causing fungistasis facilitated by their ability to diffuse through the porous soil matrix (Wheatley, 2002; Garbeva et al., 2011; Effmert et al., 2012). The potential role of VOCs in suppression of soil-borne plant pathogenic organisms was already reviewed in Stotzky et al. (1976) but regained interest recently (Garbeva et al., 2011; Effmert et al., 2012; Weisskopf, 2013). Production of antifungal volatiles has been shown for a broad range of bacterial phyla: it has been estimated that 30–60% of the soil bacterial species can produce fungistatic volatiles (Wheatley, 2002; Zou et al., 2007). Further support for the role of volatiles in fungistasis came from an extensive inventory by Chuankun et al. (2004), who observed a significant positive correlation between fungistatic activity (inhibition of spore germination) and production of VOCs by 146 soils. The inhibition of pathogen growth by bacterial VOCs has been shown in several studies (McCain, 1966; Alström, 2001; Wheatley, 2002; Kai et al., 2007, 2009; Zou et al., 2007; Effmert et al., 2012) indicating the potential of microbial volatiles in disease reduction. Inhibition of *Pythium* mycelial growth by bacterial volatiles has been shown, albeit under *in vitro* conditions and not in soils (Garbeva et al., 2014a; Hol et al., 2015). Hence, possible involvement of volatiles in natural soil suppression of *Pythium* is unknown.

Agricultural management practices may influence the composition of soil microbial communities and, therefore, also the production of pathogen-suppressing secondary metabolites. Different management practices are in use to reduce pathogen pressure. Anaerobic soil disinfestation (AD) uses crop residues and airtight covering of the soil with plastic foil to stimulate the development of anaerobic microbes producing toxic substances that eliminate harmful nematodes and fungi (Blok et al., 2000). Although AD is used as an environmentally-friendly alternative for chemical disinfestation it is expected to have a tremendous effect on microbial community composition and functioning as aerobic soil microbes face a period of anaerobiosis. Little is known on the possible legacy that AD may have on the composition and functioning of soil microbial communities after the treatment has been finished and cultivation of new crops is started. It has been shown that stress-induced shifts in soil microbial community composition can cause a drastic reduction of fungistasis (De Boer et al., 2003). Hence, there is a potential risk that AD and other disinfestation treatments have similar effects on the pathogen-suppressing activities of soil microbial communities. The current study was aimed to address possible legacy effects of AD of sandy bulb soils on bacterial community composition and soil suppressive characteristics, with special emphasis on the production of pathogen-suppressing volatiles. To this end measurements were done at the start of the flower bulb season (planting of bulbs in autumn) in the year that AD had been applied (3 months after AD) and 1 year later. The

oomycete *Pythium intermedium,* a notorious pathogen of flower bulbs*,* was used to test soil suppressiveness as *Pythium* species are opportunistic pathogens that can rapidly cause problems under conditions where general suppressiveness has been reduced (Postma et al., 2000). Simultaneously the production of *Pythium*-suppressing volatiles by AD-treated and control soils were tested and compared with results of bioassays (root development of Hyacinth bulbs in the presence of *P. intermedium*) to determine the role of volatiles in natural disease suppression. Bacterial community composition was determined using 454 sequencing of 16S rDNA fragments.

The tightly linked series of analyses and experiments lend strong support to the importance of bacterial community composition and–volatile production in natural suppression.

Materials and Methods

Soil Treatments and Sampling

Experiments were performed with soil samples from the experimental fields of Applied Plant Research (Wageningen UR) in Lisse, The Netherlands (coordinates: N 52.25. 52; E 4.54. 77). At this location the alluvial sandy soil has a low organic matter content ranging between 1.0 and 1.5%, which is representative of the soil type used for cultivation of flower bulbs along the dunes of the coastal area of the North Sea. In 2010, a field trial was initiated to examine the effect of soil organic matter content and management practices on disease suppression against several soil-borne pathogens. From the current experiment, plots of four soil treatments (**Table 1**) with four replicates per treatment (60 m^2 per replicate) were included. In May 2010, organic matter (OM) content was elevated by incorporating a mixture of peat (95%) and cattle manure (5%) (504 tons ha^{-1}, 0–30 cm deep), resulting in an increase of the soil OM content from 1.2 to 3.0%. In August 2011, anaerobic soil disinfestation (AD) was applied to a subset of the plots according to the method of Blok et al. (2000) using "Herbie 7025" (Van Overbeek et al., 2014), a defined protein-rich vegetal by-product of food processing industry (Thatchtec B.V., Wageningen, The Netherlands). Herbie was applied 24 tons ha^{-1}, was incorporated 0–30 cm deep and anaerobic conditions were created by watering followed by airtight covering of the soil with plastic for 6 weeks. Three and fifteen months after removal of the plastic cover (November 2011 and November 2012 respectively), soil samples were taken from each field plot (22 kg per plot, randomly collected from 0 to 20 cm depth) and kept at 4°C until use. In between sampling dates, *Gladiolus* was cultivated on all field plots (April-November 2012).

Bioassay for Assessment of Root Rot

From each of the 16 field plots, soil samples were artificially infested with a three-week-old oatmeal culture (1% v/v) of *Pythium intermedium* (isolate P52, Applied Plant Research Flowerbulbs, Nursery Stock and Fruit, Lisse). Non-infested and pasteurized soils (2 h at ≥ 70°C) were used as controls. Soil moisture content was adjusted to 20% (w/w). Five bulbs from *Hyacinthus orientalis* cultivar "Pink Pearl" were planted in pots (3 L) and incubated during 8 weeks at 9°C in the dark in climate cells (Hyacinth bulbs are infected during the belowground root growing phase of the bulb). Pots were sealed with plastic foil to maintain soil moisture but allow oxygen diffusion. Impact of *Pythium* on Hyacinth root development was assessed by measuring root weight and by rating root-rot disease symptoms. At the end of the growing period, bulbs were removed from the soil and roots were washed with tap water. Root-rot ratings of infested treatments were related to the healthy root systems of non-infested control treatments. Roots were visually examined for root rot severity according to Van Os et al. (1998) using an arbitrary disease index ranging from 0 to 5, where 0 = no root rot, 1 = 1–20%, 2 = 21–40%, 3 = 41–60%, 4 = 61–80%, and 5 = >80% root rot, i.e., relative loss of healthy root mass induced by infection, compared to the corresponding healthy root systems. Roots were scored for each plant individually and a mean root rot index for each pot was calculated. After the scoring of the disease index, roots were removed from the bulbs and excess water was removed by blotting the roots on filter paper and total fresh root weight per pot (5 bulbs) was determined. Means of four pots per soil treatment were used in statistical analysis. Separate bioassays were performed for both years.

In Vitro Tests for Production of *Pythium*-Suppressing Volatiles from Soil

An experimental set-up was designed to enable exposure of *Pythium intermedium* to volatiles produced by the soils, without direct contact between *Pythium* and soil. Soil samples equal to 20 g dry weight [20% (w/w) soil moisture content] were spread evenly on the bottom of 90 mm Petri dishes and incubated for 1 week at 10°C. A 4 mm layer of water yeast agar (WYA, 20 g agar, 1 g KH_2O_4, 0.1 g $(NH_4)_2SO_4$, 0.1 g yeast extract (Difco) L^{-1} pH 6.5) was poured in lids of Petri dishes. Agar plugs of 6 mm potato dextrose agar (PDA 19,5 g L^{-1} (Oxoid, Basingstoke, UK) with CMN agar 7,5 g L^{-1} (Boom, Meppel, The Netherlands) colonized by *P. intermedium* (incubated 5–10 days at 20°C) were transferred to WYA plates and kept at 10°C. After 48 h, a WYA agar disk (Ø 6 mm) containing *Pythium* mycelium

TABLE 1 | Overview of soil treatments, soil properties, application-, and sampling dates.

Code	Treatment	Organic matter/pH	Date of application	Plots	Sampling dates
U	Untreated	1.2% pH 7.0	–	4	Nov. 2011, 2012
P	Peat	3.0% pH 7.1	May 2010	4	Nov. 2011, 2012
AD	Disinfested	1.2% pH 6.9	Aug. 2011	4	Nov. 2011, 2012
ADP	Disinfested + Peat	3.0% pH 7.0	Aug. 2011, May 2010	4	Nov. 2011, 2012

was placed in the center of the lid. The mycelium-containing lid was carefully placed on top of the bottom compartment containing soil and sealed using Parafilm (Figure S1). Plates were incubated for 10 days at 10°C. Petri dishes without soil and with gamma-radiated soil (untreated 2012) (>25 kGray, Isotron, Ede, the Netherlands) were used as controls for conditions without microbially produced volatiles. Before the start of the experiment the gamma-radiated soil was left for 4 days in a sterile flow cabinet to remove all residual volatiles. Mycelial biomass determination was done according to the method of Garbeva et al. (2014b) with some modifications. Briefly, *Pythium* mycelia were harvested by melting the colonized agar from the lids of the Petri-dishes in a beaker glass with water in a microwave oven (c. 100°C), followed by sieving with a tea strainer and three washing steps with water (c. 90°C) in order to remove agar residues. For measurements of dry biomass weight, mycelia were frozen at −20°C and freeze-dried during 24 h. Pictures of *Pythium* hyphae were taken before harvest 1 cm from the edge of the plate with a stereo microscope (Olympus, SZX12, Tokyo, Japan) connected to a AxioCam MRC5 camera (Zeiss, Jena, Germany) under a 90x magnification.

Trapping and GC/MS Analysis of Microbial Volatiles

For collection and analysis of released volatiles from soil the method of Garbeva et al. (2014b) was used with some modifications. Soil from two plots per treatment was randomly selected for GC/MS analysis. Briefly, soil samples were plated in special designed glass petri dishes, with an exit to which a steel trap could be connected with as trapping material 150 mg Tenax TA and 150 mg Carbopack B (Markes International Ltd., Llantrisant, UK) which could fix VOCs released from the soil. VOCs were collected after 168 hours of incubation at 10°C. Then, traps were removed, sealed and stored at 4°C until further analysis. Volatiles were desorbed from the traps using an automated thermodesorption unit (model Unity, Markes International Ltd., Llantrisant, UK) at 200°C for 12 min (He flow 30 ml/min). Each trap was heated for 3 min up to 270°C to introduce the volatiles into the GC/MS (model Trace, ThermoFinnigan, Austin, TX, USA). Split ratio was set to 1:4, and the used column was a 30 × 0.32 mm ID RTX-5 Silms, film thickness 0.33 μm (Restek, Bellefonte, PA, USA). The used temperature program was: from 40°C to 95°C at 3°C min^{-1}, then to 165°C at 2°C min^{-1}, and to 250°C at 15°C min^{-1}. The VOCs were detected by the MS operating at 70 eV in EI mode. Mass spectra were acquired in full scan mode (33–300 AMU, 0.4 scans s^{-1}). Compounds were identified by their mass spectra using deconvolution software (AMDIS) in combination with NIST 2008 (National Institute of Standards and Technology, USA,), Wiley 7th edition spectral libraries and by their linear retention indices (lri). The lri values were compared with those found in the NIST and the NIOO lri database. Candidate compounds possibly related to volatile inhibition of *Pythium* growth were identified by screening for volatiles that were absent in disinfested soils (AD and ADP) in 2011 and present 1 year later (2012) and in non-disinfested soils (U and P) 2011 and 2012.

Pyrosequencing of Soil Bacterial Communities

DNA was extracted directly after sampling from three randomly selected plots per treatment using Mobio 96 well Powersoil® extraction kit according to the manual. Amplicons for barcoded 16S pyrosequencing were generated using PCR reactions (5 min 95°C followed by 25 cycles 95°C 30 s, 53°C 60 s, 72°C 60 s + 1 s per cycle finishing with 10 min 72°C and 10°C soak) performed in triplicate for each sample using the primerset 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACVSGGGTATCTAAT-3′) (Caporaso et al., 2011). The 515F primer included the Roche 454-pyrosequencing adapter and a GT linker, while 806R included the Roche 454- sequencing adapter, a 12-bp barcode (unique to each sample), and a GG linker. PCR products were cleaned (Qiagen Pcr purification kit) pooled and were sequenced (Macrogen Inc. Company, South Korea) on a Roche 454 automated sequencer and GS FLX system using titanium chemistry (454 Life Sciences, Branford, CT, USA). The obtained 454 sequences were filtered and analyzed using QIIME (Caporaso et al., 2010) in the Galaxy interface. Sequences were denoised (DENOISER, Reeder and Knight, 2010) and chimeras were removed using UCHIME (Edgar et al., 2011) followed by trimming of low quality reads (<200 bp, quality score 20). The remaining high quality sequences were clustered into operational taxonomic units (OTU's) using UCLUST (Edgar, 2010) with a minimal sequence identity cut off of 97% using the most abundant unique sequence as cluster representative. Sequences were deposited in the European Nucleotide Archive under accession number PRJEB6155 (http://www.ebi.ac.uk/ena/data/view/PRJEB6155).

Data Analysis

In the bioassay, mean disease indices per pot were converted to percentages. The assumption of normality was tested with Shapiro–Wilk statistics and Levene's test was used to confirm homogeneity of variances. An analysis of variances, a Three-Way ANOVA, was performed to test the effects of soil treatment, *Pythium* addition, organic matter level and their interactions on root weight or percentage root rot.

To test the effects of VOC produced in soil on *Pythium* biomass the average hyphal weight per Petri dish per soil treatment was determined in 2011 ($n = 16$) and 2012 ($n = 8$). Data were calculated as percentage of the growth of the control. Normality was tested with Shapiro-Wilk test and homogeneity of variances was assessed with Levene's test. A Two-Way ANOVA was performed to determine differences between soil treatments.

A Three-Way ANOVA was performed on the data from the pyrosequencing analysis in order to test the effects of peat addition, soil treatment and sampling year and their interactions on number of reads. Pyrosequencing data were rarefied to the lowest number of obtained reads, 2047 reads per sample. Per phylum all soil treatments in the two seasons were tested for a change in relative abundance, based on the number of reads per phylum. The average number of reads per phylum per soil treatment was calculated and tested with One-Way ANOVA. The average number of OTUs per treatment was used to express OTU richness. Although the OTU richness data did not meet the assumption of normality in the analysis these data were

also analyzed with a One-Way ANOVA to determine differences between treatments. Statistical analyses were done in R3.0.2 and PAST (Hammer et al., 2001).

Results

Impact of *Pythium* on Hyacinth Root Rot (Bioassays)

Management practices strongly affected root biomass and root rot severity. Addition of *Pythium* to the soils showed an overall effect of the pathogen: the root weight was significantly reduced in all soils in the consecutive years. However, the magnitude of the effect of *Pythium* on root biomass reduction was different depending on the management regime the soil had received (**Figures 1A,B**, **2C**). *Pythium*-induced root biomass reduction was most strong in recently (2011) anaerobic disinfested (AD) soils. This was also indicated by the significant interaction between soil disinfestation and *Pythium* addition in 2011 (**Table 2**). In contrast to 2011, the effect of *Pythium* addition to the soil in 2012 was independent of the former AD treatment and did not show differences between the differently managed
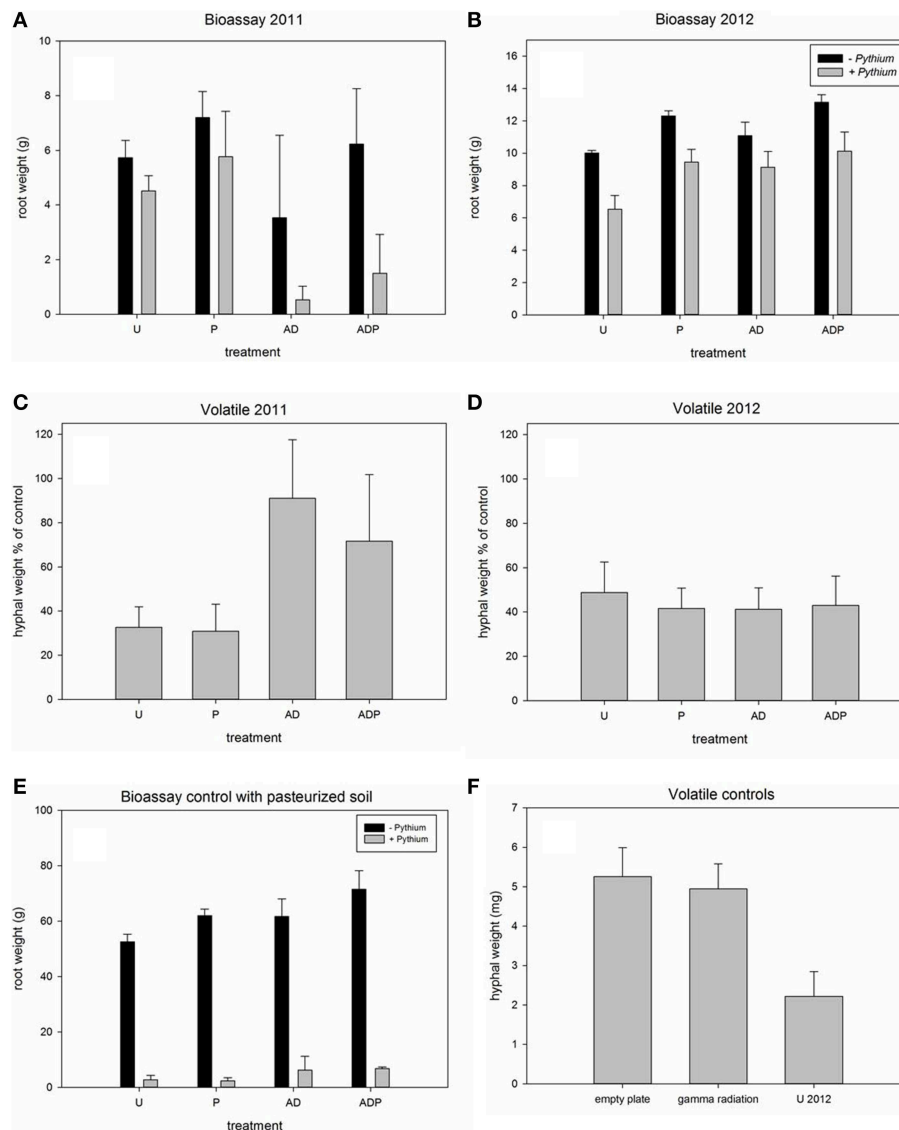
FIGURE 1 | Root biomass of Hyacinth bulbs in soils with and without addition of *Pythium intermedium* and production of hyphal biomass by *P. intermedium* during exposure to soil volatiles. (A,B) Average weight of roots extending from Hyacinth bulbs grown in differently managed soils (*U*, untreated; *P*, peat addition; *AD*, anaerobic disinfestation) with and without *Pythium* addition. **(C,D)** Average hyphal weight of *P. intermedium* hyphae that had been exposed to volatiles produced by differently managed soils. *Pythium* biomass is presented as percentage of the empty plate control. **(E,F)**, control experiments: **(E)**, Bulb root weight in pasteurized soils with and without addition of P. intermedium; **(F)**, Average hyphal weight of empty plates, gamma irradiated soil and the untreated soil in 2012. Significant results of main treatment effects and interactions are presented in **Table 2**, marked in bold. Error bars represent standard deviation.
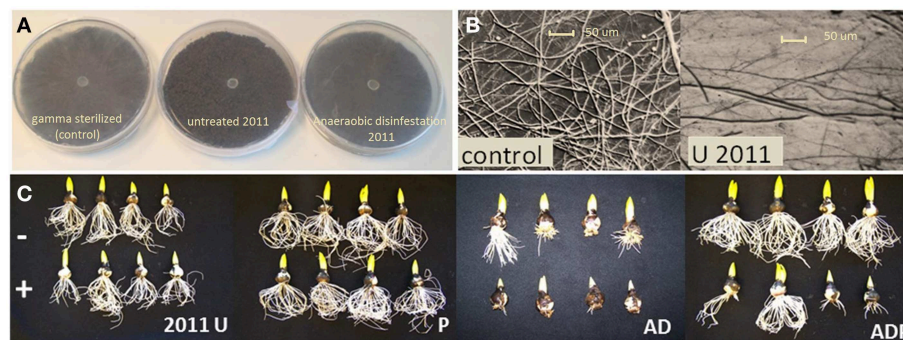
FIGURE 2 | *Pythium* volatile exposure assays and Hyacinth bioassays. (A) Differences in *Pythium* hyphal density upon volatile exposure sterilized soil (control) and untreated and disinfested soil in 2011. **(B)** Detailed pictures taken from agar plates after volatile exposure. **(C)** Results of Hyacinth bioassays in soil from differently managed fields without (−) and with (+) *Pythium intermedium* addition. U, untreated; P, peat addition; AD, anaerobic disinfestation.

TABLE 2 | Analysis of variance for root biomass and disease indexes of hyacinth bulbs in the soil bioassays and hyphal biomass in the volatile exposure assays.

|	Df	F 2011	p 2011	Df	F 2012	p 2012
ROOT WEIGHT						
Anaerobic soil disinfestation	1	26.24	**3.1E–05**	1	22.77	**7.4E–05**
Peat addition	1	8.23	**8.5E–03**	1	57.63	**7.9E–08**
Pythium addition	1	21.64	**1.0E–04**	1	107.4	**2.4E–10**
Disinfestation: peat amendment	1	0.18	0.68	1	3.92	0.06
Disinfestation: *Pythium* addition	1	5.19	**0.03**	1	1.60	0.22
Peat amendment: *Pythium* addition	1	0.76	0.39	1	0.18	0.68
Disinfestation: peat: *Pythium* addition	1	0.45	0.51	1	2.42	0.13
VOLATILE ASSAY						
Anaerobic soil disinfestation	1	85	**4.0E–13**	1	0.58	0.45
Peat addition	1	4	0.05	1	0.43	0.52
Disinfestation: peat amendment	1	3	0.11	1	1.17	0.29
DISEASE INDEX						
Anaerobic soil disinfestation	1	54.62	**1.3E–07**	1	2.74	0.11
Peat addition	1	4.52	**0.04**	1	17.38	**3.4E–04**
Pythium addition	1	5.81	**0.02**	1	125.09	**5.3E–11**
Disinfestation: peat amendment	1	5.07	**0.03**	1	1.27	0.27
Disinfestation: *Pythium* addition	1	5.51	**0.03**	1	1.93	0.18
Peat amendment: *Pythium* addition	1	0.20	0.66	1	6.00	**0.02**
Disinfestation: peat: *Pythium* addition	1	0.91	0.35	1	0.22	0.65

*Bold numbers indicate significant main effects and significant interactions. Df, degrees of freedom; F, F-value; p, p-value. This table supplies the results of statistical analysis of the data shown in **Figure 1**.*

soils (**Figure 1B**). In all pasteurized soil samples, inoculation with *Pythium* resulted in a severe loss of root biomass, average root weight was reduced by >60% (**Figure 1E**).

Root biomass in the bioassays was not only affected by *Pythium* but also by the different management practices as became apparent from the control bioassays, i.e., the pots without *Pythium* addition. Both peat amendment and soil disinfestation significantly affected root weight in both years of sampling (see **Figures 1A,B**, **2C**; **Table 2**). Addition of organic matter significantly increased the root weight in both years; the root

biomass was significantly higher in peat-amended soils (2011, 21% for P and 43% for ADP; 2012 19% for P and 16% for ADP) than in the comparable soils without peat addition. Soils that had received a recent AD treatment had a significantly reduced root weight, 39% (AD) and 14% (ADP), as compared to the untreated (U) and peat-amended soil (P) respectively. One year later this effect was reversed. In 2012, plants in formerly anaerobic disinfested soils had a higher root biomass, 10% and 7% more, compared to the non-disinfested soils with the same organic matter level. Similar to the effects of *Pythium*

on root weight, anaerobic disinfestation enhanced the effects of *Pythium* on root rot symptoms in 2011 (Figure S2A; **Table 2**). This interaction was no longer apparent in 2012 (Figure S2B; **Table 2**). Even without addition of *Pythium*, an increase of root rot symptoms was found for recently disinfested soils (Figure S2A; **Table 2**). Peat addition reduced the severity of root-rot symptoms significantly (Figure S2B, **Table 2**). The infective ability of the applied *Pythium* inoculum was confirmed, as a strong increase of root rot symptoms was seen in pasteurized soil (**Figure 1E**).

Emission of *Pythium*-Inhibiting Volatiles by Soils and Soil Microbes

Exposure of *Pythium* to volatiles released from the soils resulted in a strong reduction of *Pythium* biomass production (**Figures 1C,D**, **2A,B**). There were, however, differences between treatments and sampling years. Compared to the empty plate control, both the untreated soil and soil with peat amendment gave a 3-fold reduction in mycelial biomass in 2011 ($p < 0.0001$) (**Figures 1C,D**; **Table 2**). In contrast, exposure of *Pythium* to volatiles released from the anaerobic disinfested soils did not (AD) or only slightly (ADP) result in reduction of *Pythium* biomass or hyphal density (**Figures 1C,D**, **2A,B**; **Table 2**). In 2012, this lack of volatile suppression in disinfested soils was no longer apparent as volatiles from all soils significantly reduced *Pythium* growth by at least 50% compared to soils (**Figures 1D**, **2A,B**). The impact of soil-derived volatiles on *Pythium* growth was not significantly affected by peat addition. Volatile-suppression of *Pythium* growth was not seen when exposed to gamma-radiated soils (**Figure 1F**), indicating that no growth-reducing volatiles were produced in soil without microflora.

Trapping and GC/MS Analysis of Bacterial Volatiles

GC/MS analysis identified >700 different volatile compounds that were released from the soil of which 15 compounds were found to be absent in the anaerobic disinfested soil in 2011 (**Table 3**), mostly ketones. Some of these compounds, namely 2-octanone, 2-undecanone and 2-nonanone, are known to be inhibitors of eukaryotic pathogenic soil organisms (**Table 3**). Besides ketones the 15 potential suppressive compounds included glycol ethers, alkanes, a fatty acid and two yet unidentified compounds with retention indices of 1692 and 1743. One year later, the 15 volatile compounds were again released by the previously disinfested soils.

Pyrosequencing of Soil Bacterial Communities

Four hundred fifty four Pyrosequencing identified over 3000 different OTUs from 31 bacterial and 2 archaeal phyla (**Figure 3A**). There was no soil treatment effect on the number of reads obtained per sample, average numbers of reads were not different between soil treatments or sampling years (**Figure 3B**). In 2011, anaerobic soil disinfestation had resulted in reduction of OTU richness. At higher organic matter level the reduction was significantly less (**Figure 3A**). In 2012 no differences in OTU richness were seen between soil treatments. Most abundant

TABLE 3 | Volatile organic compounds of which the production appeared to be negatively affected by the anaerobic disinfestation treatment in 2011.

2011	2012							
	U	P	AD	ADP	U	P	AD	ADP
2-octanone[a]	−	+	−	−	+	+	+	+
2-nonanone[b,c]	+	+	−	−	+	+	+	+
2-undecanone[c,d]	+	−	−	−	−	+	+	+
2-hexanone	−	+	−	−	+	−	+	+
2-tetradecanone	+	−	−	−	−	+	−	+
2,5-hexanedione	−	+	−	−	+	−	+	−
1-octen-3-one	+	+	−	−	+	−	−	+
1-butoxy-2-propanol	+	+	−	−	+	+	+	+
2-butoxyethanol	−	+	−	−	+	+	+	+
hexadecane	+	+	−	−	+	+	+	+
1-nonanol	+	+	−	−	+	+	+	−
nonylcyclohexane	−	+	−	−	+	+	+	+
heptanoic acid	−	+	−	−	+	+	+	+
unknown 1692a*	+	+	−	−	+	+	+	+
unknown 1743a**	+	+	−	−	+	+	+	+

Bold compounds have been previously identified as potential fungus suppressing compounds. Chemical names are according IUPAC.
*Unknown 1692a Lri: 1692; EI: 88 (100), 121 (75), 174 (10).
**Unknown 1743a Lri: 1743; EI: 104(100), 78(11), 208(3).
[a]Zou et al. (2007); inhibiting concentration not given.
[b]Chen et al. (2008); inhibiting concentrations not given.
[c]Weisskopf (2013), inhibiting concentrations not given.
[d]Fernando et al. (2005), inhibiting concentration not given.

(36–63%) in all samples were OTUs assigned to *Proteobacteria.* Relative abundances of classes within the *Proteobacteria,* i.e., *Alpha- Beta- Gamma-* and *Deltaproteobacteria,* did not change significantly between different soil treatments (Figure S3). In 2011, six phyla showed significant differences between plots with and without anaerobic soil disinfestation (**Figures 3C–H**). At the start of the bulb planting season for spring flowering bulbs anaerobic soil disinfestation had still clear effects on the bacterial community composition. Relative abundances of OTUs assigned to *Acidobacteria, Chloroflexi, Nitrospirae, Chlorobi,* and *Planctomycetes* were significantly lower in the disinfested soils, whereas relative abundance of OTUs assigned to *Bacteroidetes* was higher compared to untreated soils. In 2012, 15 months after the disinfestation treatment, the relative abundance of these phyla was restored to the same levels as occurred in untreated soils for *Acidobacteria, Chlorobi* and *Planctomycetes* ($p < 0.05$), with the same tendency for *Chloroflexi* and *Nitrospirae* ($p < 0.1$) (**Figure 3**, Table S1).

Discussion

Volatile organic compounds form an important part of the underground chemical communication network between plants, fungi and bacteria (Ryu et al., 2003; Vespermann et al., 2007; Insam and Seewald, 2010; Effmert et al., 2012; Bitas et al., 2013; Fiers et al., 2013). They can have different roles in the soil including plant growth promotion and signaling (Vespermann
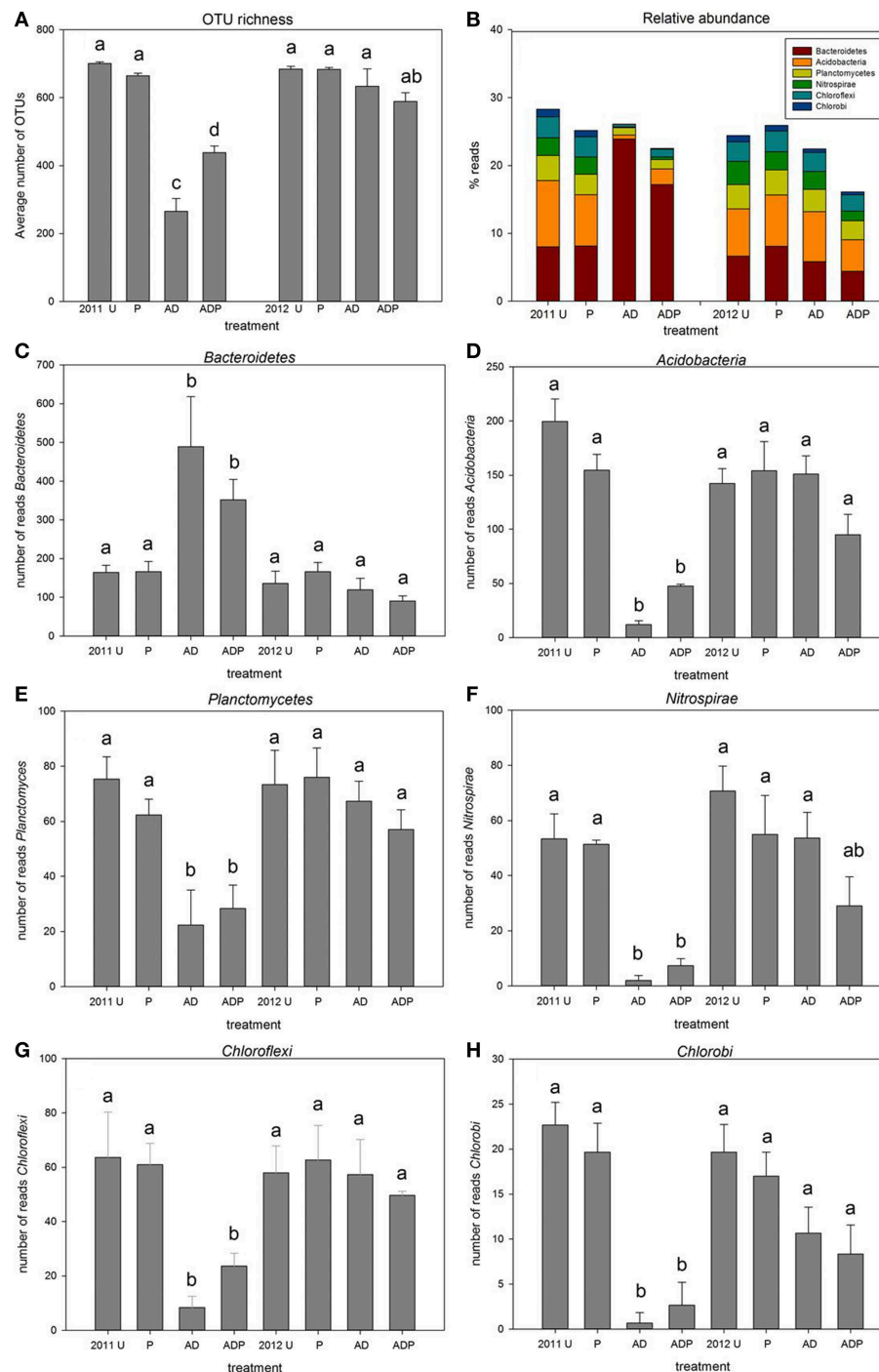
FIGURE 3 | OTU richness and average relative abundance of selected bacterial phyla. (A) average number of OTUs in differently managed soil (*n* = 3, error bars represent stdev, *U*, untreated; *P*, peat addition; *AD*, disinfestation), **(B)** relative abundance **(C–H)**, average relative abundance of phyla that differ significantly between the disinfested soils in 2011 (AD and ADP) and all other treatments in 2011 and 2012.

et al., 2007). There are also indications that VOCs produced by soil micro-organisms can have an important contribution to the restriction of growth and germination of pathogenic fungi (fungistasis) that occurs in most soils (Garbeva et al., 2011).

However, despite the ability of several soil microbial VOCs to reduce pathogenic growth; little attention has been paid to the role of these VOCs in suppression of plant diseases caused by soil-borne pathogens.

Our results provide an indication about the involvement of VOCs in natural disease suppression of a soil-borne pathogen. Our study revealed interesting co-incidences of severe *Pythium*-induced root weight loss, absence of production of *Pythium* suppressing soil volatiles and shifts in bacterial community composition shortly after an anaerobic disinfestation treatment. One year later these effects of the disinfestation treatment had largely disappeared. The coinciding dynamics of root biomass and production of suppressing volatiles suggests that microbial volatiles can have an important contribution in the natural control of *Pythium intermedium*. Furthermore, our results point at the importance of microbial community composition as disinfestation-induced shifts in community composition which coincided with the loss in suppressiveness by volatiles.

Induced changes in microbial community composition can yield important information on the functioning of the original soil microbial communities (Griffiths and Philippot, 2013). Management practices can alter the abundance of microbial groups that are thought to be involved in disease suppression (Garbeva et al., 2004; Mazzola, 2004; Chaparro et al., 2012; Sipilä et al., 2012). In the current investigation we observed that anaerobic soil disinfestation had a dramatic effect on soil microbial diversity and community composition. This is not surprising as the microbes in the well-drained sandy soils were confronted with a long period of oxygen-depletion resulting in a shift from aerobic metabolism to predominant anaerobic metabolism. The impact of this period of anaerobiosis was still clearly visible in the bacterial community composition at the time that flower bulbs are usually planted i.e., 3 months after the soil disinfestation treatment had been ended (removal of cover plastic). Most striking was the high abundance of *Bactereroidetes*, a phylum that has been shown to strongly respond to fluctuating redox conditions (DeAngelis et al., 2010).

In contrast, the phyla *Acidobacteria, Planctomycetes, Nitrospirae, Chloroflexi*, and *Chlorobi* decreased significantly after disinfestation. So far, these groups have rarely been studied in the context of disease suppression, due to limitations to obtain cultivable representatives. Strongest reduction in relative abundance of OTUs after soil disinfestation was seen for *Acidobacteria*. A recent study showed that *Acidobacteria* were one of the groups that were most sensitive to a strongly disturbing soil treatment (fumigation) (Domínguez-Mendoza et al., 2014). Earlier studies have shown that changes in land use, fertilization and management caused shifts in relative abundance of *Acidobacteria* and its different subgroups (Jones et al., 2009; Barnard et al., 2013; Navarrete et al., 2013). However, the impact of such shifts in abundance of *Acidobacteria* on disease suppressiveness has not been examined. Yet, next to our results a study by Hunter et al. (2006) provides indications for a possible role of *Acidobacteria* in disease suppressiveness. In that study it was observed that *Acidobacteria* were present in peat suppressive to *P. sylvaticum* whereas they were absent in peats that were conducive for *P. sylvaticum* damping-off. No other documentation is available on antagonistic roles of *Acidobacteria*, nor on production of antimicrobial VOCs. Our data suggest a potential role in the production of suppressing volatiles by *Acidobacteria*. However, the actual role of *Acidobacteria* in

volatile production and disease suppression, as well as that of the other phyla that showed similar dynamics upon disinfestations remains to be established.

Remarkably, the main classes of *Proteobacteria*, which contain many known potential biocontrol bacteria (Weller et al., 2002; Haas and Defago, 2005) did not change significantly as a result of the disinfestation treatment (Figure S3). However, this does not necessarily imply that *Proteobacteria* did not contribute to *Pythium* disease suppression. Mendes et al. (2011) compared a soil suppressive against the plant pathogen *Rhizoctonia solani* with another, similar soil that was conducive for disease caused by this fungus and found similar abundances for all classes of *Proteobacteria* in both soils. However, at species level, e.g., within the genus *Pseudomonas*, differences were observed with higher abundance of antibiotic-producing species in the suppressive soil.

Comparison of the volatiles produced by differently treated soils revealed potential *Pythium*-inhibiting compounds, mostly methyl ketones. These VOCs were present in the untreated and peat-amended soils that exhibited a high level of natural suppression against *Pythium*, but absent in the recently disinfested soils that were susceptible to infection of bulbs by *Pythium*. Among these VOCs, there were compounds like 2-octanone, 2-nonanone and 2-undecanone that were previously found to be suppressive against soil fungi and nematodes (Chen et al., 1988; Alström, 2001; Wheatley, 2002; Gu et al., 2007; Kai et al., 2007; Effmert et al., 2012). Thus these findings support the potential suppressive role of the VOCs identified in this study. However, the antimicrobial role of the other potential suppressive compounds identified in this study remains to be assessed. Besides the compounds highlighted in this study (**Table 3**) it is possible as well that a mixture of different compounds (Tunc et al., 2007; Veras et al., 2012), compounds that were not detected with the chosen method and/or concentration dependent effects (Wheatley, 2002) are responsible for the suppression against *Pythium*. The biological and ecological relevance of concentration effects and volatile mixture compositions remains to be studied for the natural soil habitat.

Anaerobic soil disinfestation is applied to kill a broad range of pathogens (Blok et al., 2000). The demonstrated reduction of disease suppression shows that such a drastic treatment of the soil has the risk of a (partial) elimination of the natural suppressive microflora. After one growing season, 15 months after application, disease suppression against *Pythium* was restored to the level of the non-disinfested plots. Similar loss of suppressing activity of the indigenous microflora has been found with pathogen-eliminating measures like flooding (8 weeks) and chemical soil disinfestation with cis-dichloropropene or methylisothiocyanate (Van Os et al., 1999). Postma et al. (2000) observed enhanced *Pythium* outbreaks in cucumber grown on rockwool after sterilization of the rockwool and recolonisation by a microbial community which lacked the suppressive properties of the original community. Hence, (temporal) changes in the suppressive community, by reducing the competition pressure or elimination of useful microbes, can enhance disease outbreaks of opportunistic pathogens such as *Pythium* (Van Os et al.,

1999; Van Os and van Ginkel, 2001). Our study included two consecutive years of bulb planting to determine the longer term effect of the management treatments on *Pythium* suppression. Fifteen months after the disinfestation treatment, the bacterial community composition resembled the composition of the non-disinfested soils. This is in agreement with the results of Mowlick et al. (2013) who found a restoration of the original microbial community composition in the biological disinfestation treatment after plant growth. Anaerobic disinfestation had an impact on the taxonomic composition of the soil microbial community but also on an important function, namely disease suppression. In our study, after the cultivation of the summer crop *Gladiolus* the suppression of *Pythium* in both volatile assay and bioassay also returned to the level of that in non-disinfested soils. This recovery of the natural suppression against *Pythium* indicates resilience of the soil to re-establish this essential ecosystem function after a strong disturbance (Griffiths and Philippot, 2013).

In the year of application (2011), the anaerobic soil disinfestation had a negative effect on Hyacinth root development even without addition of *Pythium*. This may be the result of phytotoxic effects of compounds that have been produced during anaerobic decomposition and were still present. It is known that decomposition of crop residues during the period of oxygen depletion can produce phytotoxic compounds (Bonanomi et al., 2007b). Since this reduced root growth was only significant in the disinfested soil without peat addition, the increased organic matter levels in peat-amended soils may have absorbed possible phytotoxic compounds.

Organic amendments do influence soil physical-chemical properties as well as soil microbial activity and composition (Hoper and Alabouvette, 1996; Bonanomi et al., 2007a, 2010). Therefore, we expected to find an effect of peat addition on volatile suppression. The addition of peat increased root weight and reduced root rot symptoms in 2012 as compared to the untreated soil. However, the volatile suppression of peat-amended soils was not different from that of the unamended soils. This is in line with the microbial community composition which was not strongly affected by peat amendment but does imply that other mechanisms of disease suppression, besides volatiles, contribute to disease suppression after peat addition. It is clear that organic amendments and disease control measures can have long-term effects on both the soil microflora and on disease suppression, although the effects of the amendments might depend on the nature and maturity of the organic additions (Hoitink and Boehm, 1999; Termorshuizen et al., 2006). In

order to get more understanding of time-related changes it is necessary to monitor these soil characteristics during longer periods of time. Since flower bulb production, and more general arable agriculture, are not only seriously threatened by *Pythium*, but also by several other soil-borne fungi and nematodes, is would be recommendable to extend these studies to include also other pathogens like *Rhizoctonia solani*, *Pratylenchus penetrans* or *Meloidogyne hapla*. In conclusion, our study indicates that the production of suppressing volatiles by soil microbes may be an important factor in the natural suppression of root-infection by *Pythium*. More general, this indicates that microbial volatiles may be an essential part of the natural buffering of soils against soil-borne diseases, the so-called general disease suppression. This would open new perspectives and insights for the control of soil-borne pathogens. Volatile-inhibition tests as well as the presence of certain VOCs and microbial groups could be an indicator of the susceptibility of a given soil to soil-borne pathogens. Obviously, more research is needed to find support for this. In depth studies are needed to further assess the role of volatiles in disease suppression and should also consider the dynamics of production of VOCs in soils, as well as the conditions that affect the sensitivity of the pathogens to VOCs.

Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00701

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A fragrant neighborhood: volatile mediated bacterial interactions in soil

Kristin Schulz-Bohm[1]*, Hans Zweers[1], Wietse de Boer[1,2] and Paolina Garbeva[1]

[1] Department of Microbial Ecology, Netherlands Institute of Ecology (NIOO-KNAW), Wageningen, Netherlands, [2] Department of Soil Quality, Wageningen University, Wageningen, Netherlands

There is increasing evidence that volatile organic compounds (VOCs) play essential roles in communication and competition between soil microorganisms. Here we assessed volatile-mediated interactions of a synthetic microbial community in a model system that mimics the natural conditions in the heterogeneous soil environment along the rhizosphere. Phylogenetic different soil bacterial isolates (*Burkholderia sp.*, *Dyella sp.*, *Janthinobacterium sp.*, *Pseudomonas sp.*, and *Paenibacillus sp.*) were inoculated as mixtures or monoculture in organic-poor, sandy soil containing artificial root exudates (ARE) and the volatile profile and growth were analyzed. Additionally, a two-compartment system was used to test if volatiles produced by inter-specific interactions in the rhizosphere can stimulate the activity of starving bacteria in the surrounding, nutrient-depleted soil. The obtained results revealed that both microbial interactions and shifts in microbial community composition had a strong effect on the volatile emission. Interestingly, the presence of a slow-growing, low abundant *Paenibacillus* strain significantly affected the volatile production by the other abundant members of the bacterial community as well as the growth of the interacting strains. Furthermore, volatiles released by mixtures of root-exudates consuming bacteria stimulated the activity and growth of starved bacteria. Besides growth stimulation, also an inhibition in growth was observed for starving bacteria exposed to microbial volatiles. The current work suggests that volatiles produced during microbial interactions in the rhizosphere have a significant long distance effect on microorganisms in the surrounding, nutrient-depleted soil.

Keywords: volatiles, inter-specific interactions, rhizosphere, synthetic microbial communities, low-abundant bacteria, soil microcosms

INTRODUCTION

Microorganisms produce a great variety of secondary metabolites including antibiotics, toxins, pigments, and others. Interestingly, small molecular mass metabolites such as volatile organic compounds (VOCs) were for a long time overlooked. Research of the last decades demonstrated that bacteria produce a large set of VOCs (Kai et al., 2009; Insam and Seewald, 2010; Effmert et al., 2012; Bitas et al., 2013; Peñuelas et al., 2014). However, the knowledge about the biological and ecological function of those volatiles is still limited. Similar to well-studied plant VOCs, it can be assumed that VOCs released by bacteria perform diverse and crucial functions (Bitas et al., 2013). Recent studies revealed that bacterial volatiles can inhibit the growth of fungi or bacteria (Wrigley, 2004; Kai et al., 2007; Vespermann et al., 2007; Zou et al., 2007; Weise et al., 2012; Garbeva et al., 2014b) and in some cases they can even function as growth-promoting agent (Wheatley, 2002; Horii and Ishii, 2006; Garbeva et al., 2014a). Additionally, volatiles emitted by bacteria can influence the

metabolism of other surrounding bacteria (Kai et al., 2009; Garbeva et al., 2014a). Most studies, however, are performed *in vitro* on semi-solid media using nutrient rich conditions and may not represent the natural conditions in the microbial environment. Culture conditions including nutrient availability (Kai et al., 2009; Insam and Seewald, 2010) and the type of incubation medium substantially affect the spectrum of released VOCs (Weise et al., 2012). Thus, bacteria produce a different set of volatiles when incubated in soil as compared to incubations on agar plates (Garbeva et al., 2014b).

Soil is a complex, nutrient-poor and highly heterogeneous environment consisting of both water- and air-filled pores (Young et al., 2008). Due to their physical properties such as low molecular weight (<300 D), lipophilicity, high vapor pressure and low boiling points (Effmert et al., 2012; Bitas et al., 2013; Lemfack et al., 2014), VOCs can diffuse through water- and gas-filled pores in soil and rhizosphere environments. Therefore, VOCs can act over a wider range of scale and may play essential roles in the communication and competition between physically separated microorganisms in soil (Kai et al., 2009; Effmert et al., 2012; Garbeva et al., 2014a). Soil microbes occur generally in multi-species communities. However, our current knowledge on the ecological role of microbial volatiles is based mostly on studies focusing on volatiles released by (non-interacting) monocultures. During inter-specific interactions, the production of various secondary metabolites can be triggered (e.g., Garbeva and De Boer, 2009; Traxler et al., 2013) and these secondary metabolites can act differently as compared to metabolites released by species in monoculture (Garbeva et al., 2014a; Tyc et al., 2014).

In the present study, we aim to reveal new insights into the ecological role of VOCs in microbial interactions by assessing multi-species interactions of a synthetic microbial community in a soil model system that reflects the natural conditions in the heterogeneous soil environment along the rhizosphere. The main research questions to address were: (1) what is the role of microbial interactions and shifts in microbial community composition on volatile emission in the rhizosphere and (2) can volatiles produced during bacterial interactions in the rhizosphere stimulate the activity of starving soil microbes in the surrounding environment?

Overall, our results demonstrated that both microbial interactions and shifts in microbial community composition had a significant effect on the volatile emission and that the presence of slow growing, non-abundant bacterial species influenced the volatile production of the bacterial community. Furthermore, our results revealed that volatiles released by microbial interactions in the rhizosphere have a long distance effect on the surrounding non-active microbial community in the nutrient-depleted soil.

MATERIALS AND METHODS

Bacterial Model Strains and Growth Media
Bacterial strains (**Table 1**) were previously isolated from the rhizosphere of sand sedge (*Carex arenaria*) in different sandy dune soil sites (De Ridder-Duine et al., 2005) and characterized by 16S rRNA gene sequencing (Tyc et al., 2014). All bacterial isolates were pre-cultured from frozen glycerol stocks on 0.1 strength tryptone soya broth (TSB) agar (De Boer et al., 2007). Overnight cultures in 0.1 TSB medium were prepared prior to each microcosm experiment.

Rhizospheric Soil Microcosms
Sandy soil of low carbon-content (Figure S1) and low amount of mineral nitrogen (0.2 mg/kg nitrite and nitrate) and phosphate (1.1 mg/kg) was collected from an old river dune site near the village Bergharen (51°10′N, 05°40′E) in the Netherlands. The soil was dried, sieved (ø 2 mm), and gamma-sterilized by Synergy Health Ede B.V. (Netherlands). Before each microcosm experiment, the gamma-sterilized soil was acclimatized for 5 days under sterile conditions. Artificial Root Exudates (ARE) stock solution (7.5 mg carbon per ml, C/N 20.6) containing 18.4 mM glucose, 18.4 mM fructose, 9.2 mM sucrose, 9.2 mM citric acid, 4.6 mM fumaric acid, 20.5 mM lactic acid, 6.9 mM malic acid, 13.8 mM succinic acid, 2.0 mM cysteine, 6.1 mM L-alanine, 2.0 mM L-argenine, 3.6 mM L-glutamic acid, 6.1 mM L-serine, and 10 mM KH_2PO_4 was freshly prepared or stored at $-20°C$. The composition of the ARE stock solution was based on previously published ARE-mixes (Griffiths et al., 1999; Baudoin et al., 2003; Garbeva et al., 2014b) and adapted to root-exudate composition found for the sedge *Schoenus unispiculatus* (Shane et al., 2006) belonging to the same family Cyperaceae as *C. arenaria*. Liquid inoculums consisting of bacteria (10^8 cells ml^{-1} per strain), ARE stock solution (pH 6.5), and phosphate buffer (10 mM KH_2PO_4, pH 6.5) were prepared. The inoculums were thoroughly mixed with the soil to establish rhizospheric soil microcosms containing 136 µg carbon per g soil and 1×10^5 CFU per g soil for each strain. The initial carbon concentration in the soil microcosms was adjusted to estimated daily carbon inputs by roots of 100–1500 µg carbon per g rhizosphere soil (Trofymow et al., 1987; Cheng et al., 1996). Control microcosms consisted of sterile soil, phosphate buffer and ARE stock solution without bacterial inoculation. The moisture content was 5.7% (w/w). Microcosms were incubated at 20°C.

Bioassay for the Selection of Bacterial Strains
Three soil microcosms for each bacterial strain were set up in 25 ml gas tight glass bottles as described above. The head space in the bottles was filled with atmospheric gas. All microcosms were incubated for 7 days at 20°C and the bottles were flushed with fresh air every 2 days. Gas samples were taken before and after flushing with fresh air and the CO_2 production was measured with an Ultra GC gas chromatograph (Interscience, The Netherlands) equipped with a flame ionization detector (FID) and a Rt-QBond (30 m, 0.32 mm, ID; Restek, USA) capillary column. Carrier gas was helium at a flow rate of 5 ml min^{-1}. Injector, oven and detector temperatures were 150, 50, and 350°C, respectively. CO_2 concentrations in the headspace were calculated based on external gas standards (Westfalen AG, Germany) and the ideal gas law.

Soil samples were regularly taken to monitor the bacterial growth. For this 1 g of soil was mixed with 9 ml phosphate buffer

Bacterial strain (Accesion number)	Origin	Phylum (family)
Achromobacter spp. 58-38 (KC888968)	River dune near Bergharen, Gelderland (De Boer et al., 2015)	Beta-Proteobacteria (*Alcaligenaceae*)
Bosea sp. AD132 (KJ685339)	Coastal outer dunes of Midsland, Terschelling (De Ridder-Duine et al., 2005)	Alpha-Proteobacteria (*Bradyrhizobiaceae*)
Burkholderia sp. AD024 (KJ685239)	Dune grassland near Ouddorp, Zeeland (De Ridder-Duine et al., 2005)	Beta-Proteobacteria (*Burkholderiaceae*)
Dyella sp. AD056 (KJ685269)	Drift sand near Loon op Zand, Brabant (De Ridder-Duine et al., 2005)	Gamma-Proteobacteria (*Xanthomonadaceae*)
Janthinobacterium sp. AD080 (KJ685292)	Coastal outer dunes of Midsland, Terschelling (De Ridder-Duine et al., 2005)	Beta-Proteobacteria (*Oxalobacteraceae*)
Microbacterium sp. AD141 (KJ685346)	Costal outer dunes of EastTerschelling (De Ridder-Duine et al., 2005)	Actinobacteria (*Microbacteriaceae*)
Paenibacillus sp. AD087 (KJ685299)	Pine plantation near Loon op Zand, Brabant (De Ridder-Duine et al., 2005)	Firmicutes (Paenibacillaceae)
Pedobacter sp. V48 (NZ_AWRU00000000)	Coastel dune site Terschelling (De Boer et al., 1998)	Bacteroidetes (*Sphingobacteria*)
Pseudomonas sp. AD021 (DQ778036)	Coastel inner dunes of Midsland, Terschelling (De Ridder-Duine et al., 2005)	Gamma-Proteobacteria (*Pseudomonadaceae*)
Rhizobium sp. 45-29 (KC888976)	River dune near Bergharen, Gelderland (De Boer et al., 2015)	Alpha-Proteobacteria (*Rhizobiaceae*)

All strains are from organic poor, sandy dune soils in the Netherlands.

(pH 6.5) in 20 ml Greiner tubes. The tubes were shaken on a rotary shaker at 180 rpm for 30 min at 20°C and serial dilutions were plated on 0.1 TSB agar. All plates were incubated at 20°C and colony forming units (CFU) were counted after 3 days.

Collection of Bacterial Volatiles and Monitoring of Bacterial Growth in Rhizospheric Soil Microcosms

For the collection of bacterial VOCs, rhizospheric soil microcosms were established as indicated above in glass Petri-dishes with an exit at the top to which a steel trap filled with 150 mg Tenax TA and 150 mg Carbopack B (Markes International Ltd., Llantrisant, UK) could be fixed. In the rhizospheric soil microcosms, the bacterial strains *Burkholderia* sp. AD024, *Dyella* sp. AD056, *Janthinobacterium* sp. AD080, *Pseudomonas* sp. AD021, and *Paenibacillus* sp. AD087 were either incubated as monocultures or as mixtures of four (without *Paenibacillus sp.* AD087) or five strains. The growth of the strains was monitored by quantification of bacterial 16S rRNA genes from extracted DNA of soil samples (three per microcosm) taken at the beginning of the experiment and after 96 h incubation. Soil samples were stored at −80°C until nucleic acid extraction (see below). VOCs produced by the monocultures, bacterial mixtures and control (see above) were trapped for 24 h after 3 days of incubation. Traps were removed, capped and stored at 4°C until analysis.

VOCs Analysis

VOCs were desorbed from the traps using an automated thermodesorption unit (model UnityTD-100, Markes International Ltd., UK) at 210°C for 12 min (He flow 50 ml/min). The desorbed VOCs were subsequently collected on a cold trap at −10°C and introduced into the GC-QTOF (model Agilent 7890B GC and the Agilent 7200A QTOF, USA) by heating the cold trap for 3 min to 280°C. Split ratio was set to 1:201, and the column used was a 30 × 0.25 mm ID RXI-5MS with as film thickness of 0.25 μm (Restek 13424-6850, Bellefonte, PA, USA). The temperature program was as follows: 2 min at 39°C, 3,5°C/min to 95°C, 6°C/min to 165°C, 15°C/min to 250°C and finally 40°C/min to 300°C which was hold for 20 min.

VOCs were detected by the MS operating at 70 eV in EI mode. Mass spectra were acquired in full scan mode (30–400 AMU, 4 spectras/s). GC/MS data were collected and converted to mzData file using the Chemstation B.06.00 (Agilent Technologies, USA). Data were further processed with MZmine 2.14.2 (Pluskal et al., 2010) with the tools mass detection (centroid mode, noise level = 1000), chromatogram builder (Min time span = 0.04 min, Min height = 1.8E04–2.5E04, m/z tolerance of 1 m/z or 5 ppm), and chromatogram deconvolution (local minimum search, chromatographic threshold = 40%, Min in RT range = 0.1 min, Min relative height = 2.0%, Min absolute height = 1.5E04, Min ratio of peak top/edge = 2, peak duration = 0.0–0.7 min). Detected and deconvoluted peaks were identified by their mass spectra using NIST MS Search and NIST 2014 (National Institute of Standards and Technology, USA, http://www.nist.gov) and aligned by RANSAC aligner (mz tolerance = 1 m/z or 5 ppm, RT tolerance = 0.1, RT tolerance after correction = 0.05, RANSAC iteration = 10000, Min number of points =60%, threshold value = 0.1). Processed data were exported for further statistical analysis (see below). The identification of detected compounds was further evaluated by using the software AMDIS 2.72. The retention indexes were calculated for each compound and compared with those found in NIST 2014 and in house databases. Detected compounds were named as identified compounds if the Match and Reverse-Match factor of the listed compound in the NIST and in house NIOO-library was higher than 800, the spectra of the detected compound matches the one of the listed compound, and the difference between the retention index calculated for the detected compound and of the listed compound (for a semi-standard non-polar column) was not bigger than four. The Match and Reverse match factor refer to the similarity of the mass spectrum of the sample with the library spectrum of the Hit (Watson and Sparkman, 2013). Some identified compounds were also verified by co-injection of pure compounds (**Table 2**).

Bioassay to Test the Effect of Microbial VOCs on Nutrient-limited Bacteria in Soil

Soil microcosms containing the bacterial strains *Burkholderia* sp. AD024, *Dyella* sp. AD056, *Janthinobacterium* sp. AD080,

TABLE 2 | Volatile organic compounds produced by a bacterial mixture of five strains (*Burkholderia sp.* AD024, *Dyella sp.* AD056, *Janthinobacterium sp.* AD080, *Pseudomonas sp.* AD021, and *Paenibacillus sp.* AD087), referred to as 5-Mix, or four strains excluding *Paenibacillus sp.* AD087, referred to as 4-Mix.

Compound	Class	RI
COMPOUNDS DETECTED FOR 4-MIX AND 5-MIX		
2-Pentanone[a,c]	Ketone	683
3-Pentanone[a,c]	Ketone	695
Unknown[c]	n.s.	709
*Dimethyl disulfide[b,c,e]	Organosulfur	744
2-Octanol[a,c]	Alcohol	996
L-Fenchone[a,c]	Monoterpene, Ketone	1088
Unknown[d]	n.s.	1091
Camphor	Terpenoid	1144
Unknown	n.s.	1316
2,6-Bis(1,1-dimethylethyl)-2,5-Cyclohexadiene-1,4-dione (DBQ)	Aromate/Phenol	1461
Butylated Hydroxytoluene[a,b,c,d,e]	Aromate	1501
Unknown[c]	n.s.	1515
2,2,4-Trimethyl-1,3-pentanediol diisobutyrate[a,e]	Ester	1588
Unknown[b,c,d]	n.s.	1718
1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester[a,b]	Ester	1874
COMPOUNDS DETECTED FOR 4-MIX		
Methoxy-acetaldehyde[b,d]	Aldehyde	559
Cyclopentene[a,b]	Alkene	570
tert-Butanol[a,b,c,e]	Alcohol	575
Acetic acid	Organic acid	593
2-methyl-2-propen-1-ol[a,b,c]	Alcohol	605
Ethyl benzene	Aromate	857
3-Heptanol[b,c,e]	Alcohol	896
n-Hexadecanoic acid	Organic acid	1964
COMPOUNDS DETECTED FOR 5-MIX		
Sulfur dioxide[a]	Organosulfur	548
1,3,5-Trifluorobenzene[a,b]	Aromate	624
Propanoic acid, 2,2-dimethyl-, methyl ester[b]	Ester	720
2-Hexanone[c,d]	Ketone	790
2-Heptanone[c]	Ketone	888
Unknown	n.s.	1020
Methyl 2-ethylhexanoate	Ester	1039
Endo-borneol[a,b,d]	Terpene	1167
*2,5-Bis(1-methylethy)-pyrazine	Aromate	1185
Unknown	n.s.	1200
Unknown	n.s.	1231
Unknown[a,c]	n.s.	1244
Unknown	n.s.	1283
Unknown	n.s.	1343

(Continued)

TABLE 2 | Continued

Compound	Class	RI
Unknown[a,c]	n.s.	1690
*Docosane[b]	Alkene	2200

Bacteria were incubated for 4 days in soil supplied by ARE.
[a]*Volatiles also detected for monocultures of Burkholderia sp. AD024;*
[b]*Volatiles also detected for monocultures of Dyella sp. AD056;*
[c]*Volatiles also detected for monocultures of Janthinobacterium sp. AD080;*
[d]*Volatiles also detected for monocultures of Paenibacillus sp. AD087;*
[e]*Volatiles also detected for monocultures of Pseudomonas sp. AD021;*
RI Linear retention Index of a 30 × 0.25 mm ID RXI-5MS column;
n.s., not specified;
** Verified by co-injection of pure compound*

Pseudomonas sp. AD021, and *Paenibacillus* sp. AD087 were set-up in two-compartment Petri-dishes (**Figure 2** and Figure S3). Per Petri-dish, one nutrient-limited compartment was established containing 20 g gamma-sterilized soil mixed with 1×10^5 CFU per g soil of each strain and phosphate buffer (10 mM KH_2PO_4, pH 6.5). The second compartment was either filled with a mix of 20 g soil, phosphate buffer and ARE stock solution (138 µg carbon per g soil), referred to as control, or a mix of 20 g soil, the five bacterial strains (1×10^5 CFU per g soil of each strain), phosphate buffer and ARE stock solution, referred to as Treatment 1 or Treatment 2 (**Figure 3A** and Figure S3A). In case of Treatment 2, bacteria in the nutrient-limited compartment were pre-incubated for 2 days before the bacteria-ARE-soil-mix was added to the second compartment. It can be assumed that during the pre-incubation available carbon sources in the soil should be fully consumed so that bacteria were strongly nutrient limited before they were exposed to microbial volatiles of the second compartment. The composition of the ARE containing compartment is equivalent to the rhizospheric microcosms described above. The moisture content of all compartments was 5.8% (w/w). All samples were set-up in triplicates and incubated at 20°C for 6 days. To monitor bacterial activity and potential growth, about 1.5 g soil were taken two times per compartment at the start of the experiment and after 2, 4, and 6 days of incubation. For nucleic acid extraction, 0.5 g of the collected soil was immediately treated with a salmon sperm DNA solution and freeze-dried at −80°C. Leftover soil collected was stored as backup at −80°C.

Nucleic Acid Extraction

A solution (∼pH 8) of 10 mg per ml low molecular weight salmon sperm DNA (Sigma-Aldrich, The Netherlands) was prepared with DNase- and RNase-free water (Qiagen, The Netherlands). According to Paulin et al. (2013), 0.5 g of soil per sample was mixed with 0.5 ml of salmon sperm DNA solution in Lysing Matrix E tubes (MP Biomedicals, The Netherlands) and freeze-dried overnight at −80°C. DNA and RNA were co-extracted using the modified protocol described by Griffiths et al. (2000). Briefly, 500 µl of cetyl-trimethyl ammonium bromide (CTAB) buffer (10% CTAB in 0.7 M NaCl mixed with 1 volume 240 mM phosphate buffer pH 8.0), 50 µl of 2% N-lauroyl sarcosine, 50 µl

of 2% sodium dodecyl sulfate, and 400 µl of phenol-chloroform-isoamyl alcohol (25:24:1, Sigma-Aldrich, The Netherlands) were added to the freeze-dried soil. A subsequent bead beating lysis for 30 s at 5.5 ms^{-1} was performed three-times followed by 7 min centrifugation at 14,000 rpm and 4°C. The resulting aqueous supernatant was mixed with 1 volume (vol.) of chloroform-isoamyl alcohol (24:1, Sigma-Aldrich, The Netherlands) and centrifuged for 5 min at 14,000 rpm and 4°C. After a second phenol extraction with 1 vol. chloroform-isoamyl alcohol the aqueous supernatant was thoroughly mixed with 2 vol. of 20% polyethylene glycol 6000 (AppliChem, Germany) in 1.6 M NaCl, 0.1 vol of 100 mM MgCl$_2$, and 1.5 µl glycogen (20 mg ml^{-1}; Thermo Fisher Scientific, The Netherlands). Nucleic acids were precipitated for 2 h at 4°C, followed by 40 min centrifugation at 14,000 rpm and 4°C. The pellet was washed with 70% ethanol, air-dried and eluted in 37–65 µl DNase- and RNase-free water. Nucleic acid extracts were stored at −80°C or subsequently used for quantification of bacterial 16S rRNA genes (see below).

DNase Treatment and Reverse Transcription

Nucleic acid extracts were treated according to the manufacturer's protocol wi th 1–2 U DNase I (Thermo Fisher Scientific, The Netherlands) and incubated for 45 min at 37°C. RNA was purified by an overnight precipitation at −20°C with 0.1 vol. ammonium acetate (2.5 mM, Sigma-Aldrich), 2.5 vol. 100% ethanol, and 1.3 µl glycogen, followed by centrifugation for 40 min at 13,000 rpm and 4°C. The RNA pellet was washed twice with 70% ethanol and eluted in 27 µl DNase- and RNase-free water. RNA was stored at −80°C or subsequently used for reverse transcription with SuperScript$^{®}$ III First-Strand Synthesis System (Invitrogen, The Netherlands). The reverse transcription was performed according to the manufacturer's instruction. Resulting cDNA was precipitated overnight with 0.1 vol. 2 M NaCl, 2.5 vol. 100% ethanol, and 0.7 µl glycogen at −20°C, followed by 40 min centrifugation at 13,000 rpm and 4°C. After washing with 70% ethanol, the cDNA pellet was eluted in 25 µl DNase- and RNase-free water. cDNA was stored at −20°C or immediately used for the quantification of bacterial 16S rRNA.

Quantitative PCR (qPCR) of Bacterial 16S rRNA Genes and Transcripts

All qPCRs were performed with a Rotor-Gene Q cycler (Qiagen, The Netherlands) whereas each template DNA or cDNA was quantified in triplicates. The 20 µl reaction mixture consisted of 1-fold SensiFAST™ SYBR$^{®}$ No-ROX Kit (Bioline GmbH, The Netherlands), BSA (0.5 µg µl^{-1}), 375–500 nM forward and reverse primers (Table S1), and 5 µl of diluted template DNA or cDNA of 2–6 ng µl^{-1}. Negative controls consisting of DNase- and RNase-free water instead of template DNA were included in every qPCR run. Conditions for the primer sets targeting 16S rRNA gene of *Burkholderia*, *Dyella*, and *Paenibacillus* (Table S1) were as follows: 5 min initial denaturation at 95°C, 37 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 64°C, elongation for 30 s at 72°C, and fluorescence signal

detection for 15 s at 82°C. The 37th PCR cycle was followed by a melting curve analysis from 64 to 95°C with increments of 1°C. QPCR conditions for the primer sets targeting 16S rRNA gene of *Janthinobacterium* and *Pseudomonas* were the following: 5 min at 95°C, ensued by 37 cycles of denaturation for 30 s at 95°C, annealing for 20 s at 62°C, elongation for 20 s at 72°C, fluorescence signal detection for 15 s at 82°C, and a melting curve analysis from 62°C to 95°C immediately after the last qPCR cycle. Agarose gel electrophoresis of qPCR products displayed single bands of expected size (Table S1). Gene copy numbers were calculated according to a standard curve which was set up by serially diluting M13uni/rev PCR products of a pGEM-T vector containing a 16S rRNA gene fragment of the respected target organism (Zaprasis et al., 2010).

Statistical Analysis

All experiments were performed in triplicates. The statistical analysis of processed GC-MS data consistent with detected mass features per sample was conducted with MetaboAnalyst 3.0 (http://www.metaboanalyst.ca/MetaboAnalyst, Xia et al., 2015). Prior One-way ANOVA and multivariate analysis (PCA), data were filtered (interquantile range), and normalized (log transformation and auto scaling).

The statistical analysis on qPCR data as well as of CFU counts of *Pseudomonas sp*. AD021 growing as monoculture in soil microcosms supplied with or without ARE was performed with R 3.1.1 (http://www.r-project.org/) using ANOVA followed by Tukey's HSD test (De Mendiburu, 2014). To obtain normality of errors, data were log-transformed. Significance of enhanced CO$_2$ production by *Pseudomonas sp*. AD021 growing in soil supplied with ARE was assessed by student's *t*-test. Differences revealed by statistical tests were considered significant for $P < 0.05$.

RESULTS

Bacterial Growth and Volatile Production in Rhizospheric Soil Microcosms Containing Single or Multiple Species

For the rhizospheric soil microcosms, a sandy soil was selected with a low *in situ* availability of carbon. Metabolic activity and growth of bacteria were restricted without the addition of ARE to this soil (Figure S1). In the presence of the supplied carbon source, the growth and CO$_2$ production of different rhizobacterial strains was assessed (Figure S2). Finally, five phylogenetic different bacterial strains (*Burkholderia sp*. AD024, *Dyella sp*. AD056, *Janthinobacterium sp*. AD080, *Pseudomonas sp*. AD021, and *Paenibacillus sp*. AD087) were selected. Those strains, except for *Paenibacillus sp*. AD087, showed a similar CO$_2$ production profile. A maximal CO$_2$ production was reached after 6 days of incubation (Figure S2) which suggested that the supplied ARE as sole carbon source were fully consumed. Furthermore, the selected Gram-negative strains multiplied to a maximum of about 10^8 CFU per g soil in the microcosms supplied with ARE (Figure S2). A quantification of 16S rRNA gene copy numbers revealed that the growth of *Burkholderia sp*. AD024, *Dyella sp*. AD056, and *Pseudomonas sp*. AD021

was significantly reduced in incubations with other bacteria in the ARE supplied soil (**Figure 1**). The Gram-positive strain *Paenibacillus sp.* AD087 grew poorly in the rhizospheric soil microcosms, both in monoculture and mixture (**Figure 1** and Figure S2). However, in presence of *Paenibacillus sp.* AD087 the growth of the other bacteria in the mixture was affected. For instance, the 16S rRNA gene copy number (no.) of *Dyella sp.* AD056 and *Pseudomonas sp.* AD021 was significantly higher and for *Janthinobacterium sp.* AD080 significantly lower as compared to incubations without *Paenibacillus sp.* AD087 (**Figure 1**).
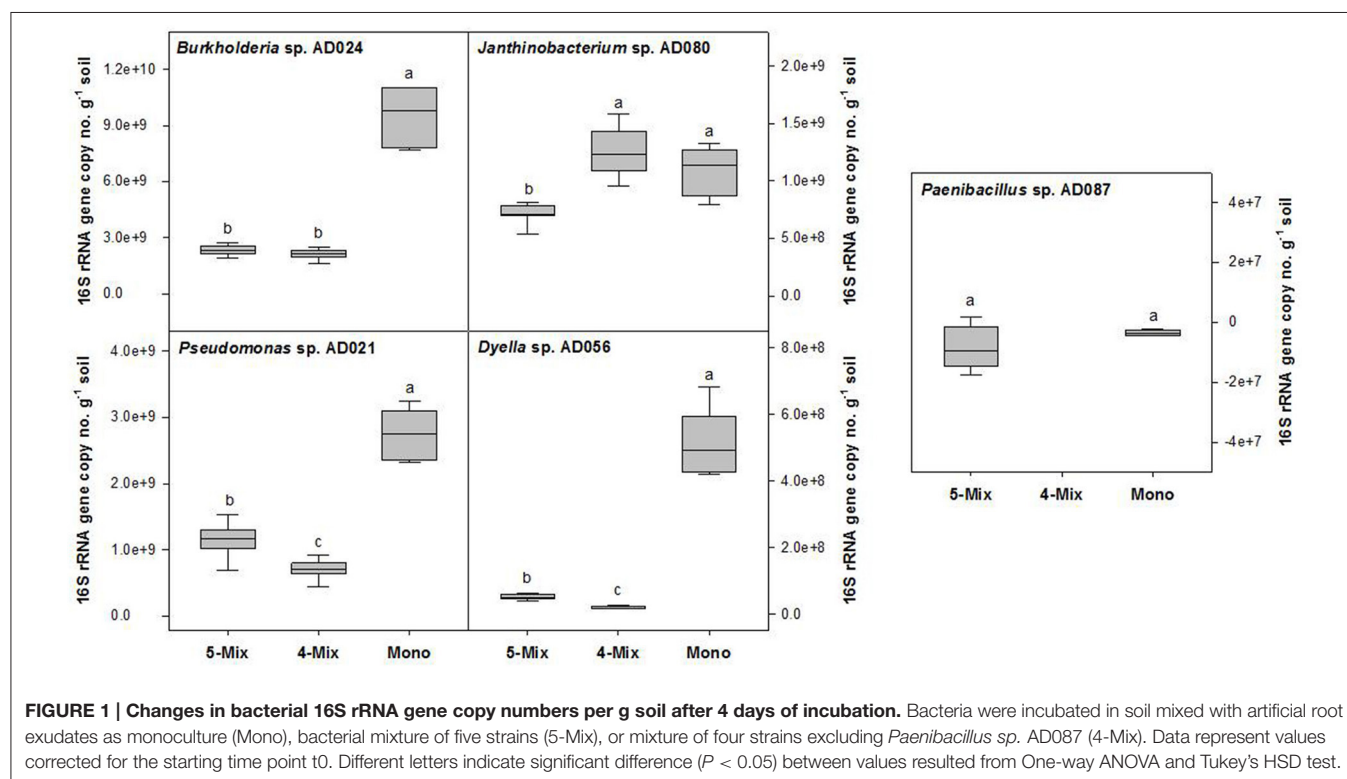
A different blend of VOCs was produced by the bacterial mixture in comparison to the monocultures (**Figure 2**). The VOCs profile was different when the bacterial mixture consisted of five or four strains without *Paenibacillus sp.* AD087 (**Figure 2**). Volatiles released by the bacterial mixture consisted of alcohols, ketones, and esters as well as aromatic and organosulfur compounds. Some of those were also produced by the monocultures (**Table 2**). Most VOCs only released by the mixture of four bacterial strains excluding *Paenibacillus sp.* AD087 were alcohols and organic acids whereas VOCs released by for the mixture of all five strains consisted mainly of ketones, ester, and aromatic compounds. However, also numerous unknown volatile compounds were detected, especially for the bacterial mixture consisting of five strains (**Table 2**).
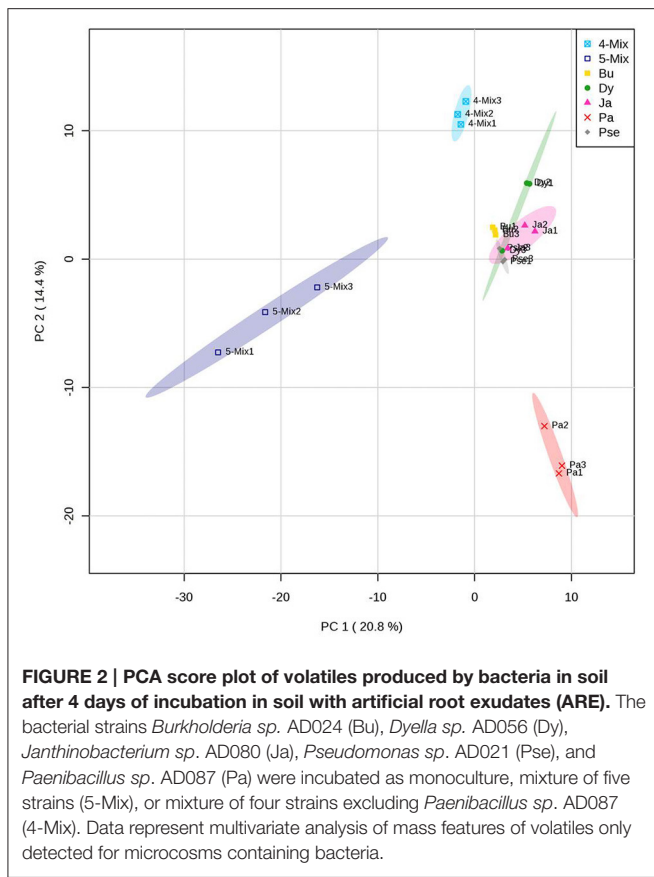
Effect of Microbial Volatiles on Starving Bacteria

To test if volatiles produced during microbial interactions in a rhizosphere environment can stimulate starving microbes in the nutrient-depleted surrounding soil, soil microcosm experiments in two-compartment Petri-dishes with a mixture of all five bacterial strains were performed. Bacteria without additional carbon-source (compartment C4 of Treatment 1 and compartment C6 of Treatment 2) were exposed to volatiles produced by bacteria supplied with ARE (compartment C3 and C5, respectively) (**Figure 3A** and Figure S3A).

The 16S rRNA copy no. per g soil increased over time for bacteria supplied with ARE as well as for bacteria in the nutrient-depleted soil that were exposed to microbial VOCs (**Figure 3B** and Figure S3B). For the control microcosms, in which the ARE supplied compartment did not contain bacteria (**Figure 3A** and Figure S3A), the bacterial 16S rRNA copy no. per g soil did not significantly change over time. After 6 days of incubation, the 16S rRNA copy no. per g soil was significantly higher for all five bacterial strains in the nutrient-depleted soil exposed to microbial VOCs as compared to the control without exposure to microbial VOCs (**Figure 3B**). In case of *Dyella sp.* AD056 and *Paenibacillus sp.* AD087, the increase in 16S rRNA copy no. per g soil after 6 days of incubation was 1.5 and 12 times higher, respectively, when starving bacteria were exposed to microbial VOCs (compartment C4) as compared to bacteria growing in the ARE containing compartment (compartment C3) (**Figure 3B**). A similar trend was observed when bacteria were pre-incubated for 2 days in the soil before exposure to microbial VOCs (Figure S3B). Hence, volatiles released by mixtures of ARE-consuming bacteria significantly affected the activity of starving bacteria without ARE, based on an increase in 16S rRNA.

FIGURE 1 | Changes in bacterial 16S rRNA gene copy numbers per g soil after 4 days of incubation. Bacteria were incubated in soil mixed with artificial root exudates as monoculture (Mono), bacterial mixture of five strains (5-Mix), or mixture of four strains excluding *Paenibacillus sp.* AD087 (4-Mix). Data represent values corrected for the starting time point t0. Different letters indicate significant difference ($P < 0.05$) between values resulted from One-way ANOVA and Tukey's HSD test.

FIGURE 2 | PCA score plot of volatiles produced by bacteria in soil after 4 days of incubation in soil with artificial root exudates (ARE). The bacterial strains *Burkholderia sp.* AD024 (Bu), *Dyella sp.* AD056 (Dy), *Janthinobacterium sp.* AD080 (Ja), *Pseudomonas sp.* AD021 (Pse), and *Paenibacillus sp.* AD087 (Pa) were incubated as monoculture, mixture of five strains (5-Mix), or mixture of four strains excluding *Paenibacillus sp.* AD087 (4-Mix). Data represent multivariate analysis of mass features of volatiles only detected for microcosms containing bacteria.

The 16S rRNA gene copy no. per g soil was measured for *Burkholderia sp.* AD024, *Dyella sp.* AD056, and *Paenibacillus sp.* AD087 to determine potential growth stimulation by exposure to bacterial VOCs under starving conditions. In case of *Burkholderia sp.* AD024 and *Dyella sp.* AD056, a significantly higher increase in 16S rRNA gene copy no. per g soil was observed for starving bacteria exposed to bacterial VOCs in comparison to the control (**Figure 3C**). In contrast, the 16S rRNA gene copy no. per g soil decreased over time for the strain *Paenibacillus sp.* AD087, even when nutrients were available or bacteria were exposed to VOCs (**Figure 3C**). Thus, the increase in activity by exposure to bacterial VOCs is coinciding with growth for the strains *Burkholderia sp.* AD024 and *Dyella sp.* AD056 but not for the Gram-positive *Paenibacillus sp.* AD087.

DISCUSSION

Most of the current knowledge about the possible functioning of microbial volatiles is based on *in vitro* studies under nutrient rich conditions (e.g., Kai et al., 2007; Vespermann et al., 2007; Zou et al., 2007). This is different from the nutrient-limiting conditions with which microbes have to deal in most soil environments. Here, we developed a soil model system mimicking more closely the natural situation occurring in and around the rhizosphere to reveal new insights into the ecological role of volatiles in microbial interactions in soil.

For bacteria incubated in rhizospheric soil microcosms, it was observed that the volatiles produced by the mixture differed from those produced by each strain. Some volatiles were only emitted by the bacterial mixture and not by the monocultures. The shift in the volatile blend can be due to competitive interactions (Garbeva et al., 2014a). This was indicated by a significant decrease in 16S rRNA gene copy number of *Burkholderia sp.*, *Dyella sp.*, and *Pseudomonas sp.* when they were growing in mixture with other bacterial strains. Besides inter-specific competitive interactions it was also observed that shifts in the bacterial community composition, i.e., bacterial mixture consistent of four or five different strains, influenced the volatile production. Therefore, as proposed by Mc Neal and Herbert (2009) changes in the VOCs profile can be a potential indicator of microbial community composition shifts. Indeed, recent studies pointed at a relationship between the composition of soil bacterial communities and that of VOCs (Hol et al., 2015; Van Agtmaal et al., 2015). Interestingly, the volatile emission by the bacterial mixture was strongly affected by the presence of the poorly-growing, non-abundant *Paenibacillus sp.* This is in line with recent studies revealing that low abundant microbes can play an important role in ecosystem functioning (Hol et al., 2010, 2015; Lynch and Neufeld, 2015). Furthermore, we observed that in the presence of the slow-growing *Paenibacillus sp.*, the 16S rRNA gene copy number of *Dyella sp.* and *Pseudomonas sp.* was significantly higher. Hence, non-abundant species can promote the growth of dominant species. On the other hand, they may also trigger the production of various growth-suppressing secondary metabolites such as antimicrobial volatiles (Jousset et al., 2014; Hol et al., 2015). In the presence of *Paenibacillus sp.*, several additional volatiles were released by the bacterial mixture which might be involved in the growth suppression of *Janthinobacterium sp.* besides non-vol atile antimicrobial compounds released by the interacting bacteria. Among those volatiles 2,5-bis(1-methylethy)-pyrazine was produced. It was recently reported that *Paenibacillus* can produce pyrazines with broad spectrum antimicrobial activity against bacteria, fungi and yeast (Rybakova et al., 2015). Here, the pyrazine-derivate was only detected for the bacterial mixture but not for *Paenibacillus sp.* in monoculture. However, it is possible that due to competitive interactions in the mixture the Gram-positive strain became active and started to produce the pyrazine compound.

Microorganisms are thought to use VOCs to influence other organisms living at a distance in the same soil environment (Stahl and Parkin, 1996). In the present study, we tested if volatiles produced by bacterial mixtures growing on root exudates can affect the activity of starving bacteria unable to access those root exudates. While microbes in the rhizosphere benefit from a constant flow of organic substrates (Effmert et al., 2012) volatile compounds released from the rhizosphere can represent an important carbon source to microorganisms in the nutrient-poor surrounding soil (Owen et al., 2007; Gramss and Bergmann, 2008; Ramirez et al., 2009). Besides, it has been suggested that volatiles produced by rhizosphere microorganisms could act as chemoattractants to the nutrient-rich environment around the roots (Garbeva et al., 2014a). Our results revealed that after 2 days of incubation, the activity of the starving
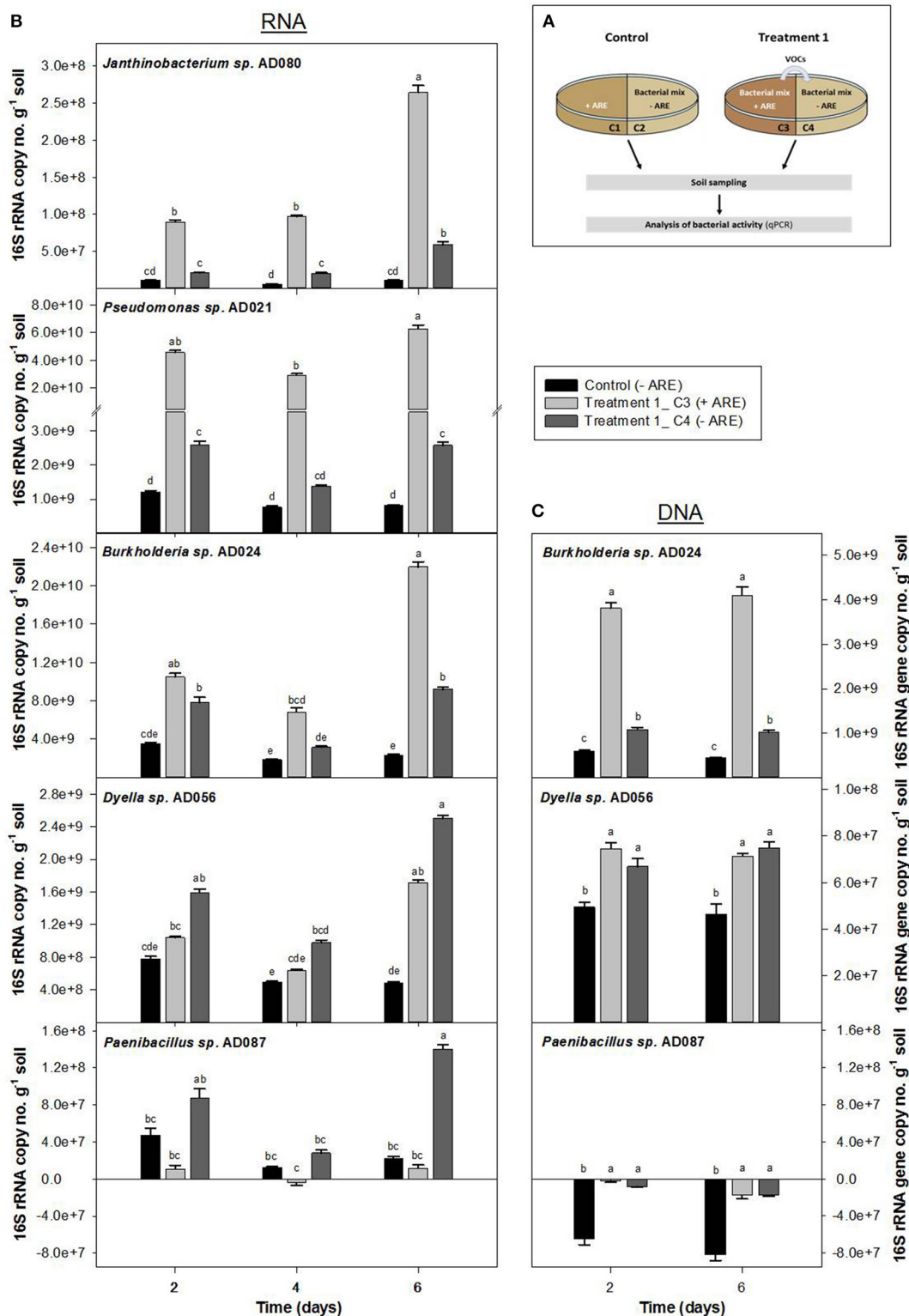
FIGURE 3 | Influence of bacterial volatiles on bacteria in nutrient-depleted soil. Increase in 16S rRNA copy number **(B)** and 16S rRNA gene copy number per g soil **(C)** were determined for a bacterial community consisting of *Burkholderia sp.* AD024, *Dyella sp.* AD056, *Janthinobacterium sp.* AD080, *Pseudomonas sp.* AD021, and *Paenibacillus sp.* AD087 which was incubated in nutrient-poor soil. **(A)** In Treatment 1, bacteria in nutrient-depleted soil in compartment C4 (dark gray) were exposed to volatiles produced by bacteria supplied with artificial root exudates (ARE) in compartment C3 (light gray). The control compartment C2 (black) represents bacteria in nutrient-depleted soil not exposed to bacterial volatiles. Data represent mean ($n = 6$) and standard error corrected for the starting time point t0. Different letters indicate significant differences ($P < 0.05$) between values resulted from One-way ANOVA and Tukey's HSD test.

bacteria *Burkholderia sp.*, *Dyella sp.*, and *Pseudomonas sp.* was already stimulated by exposure to microbial VOCs. After 6 days of incubation, the activity of all five bacterial strains in the nutrient-depleted soil was significant increased by the exposure to microbial VOCs. Thus, volatiles released by rhizosphere-inhabiting bacterial communities can stimulate the activity of the surrounding starving bacteria in nutrient-depleted soil. The mechanism behind the activation of bacteria by volatiles, however, remains unclear. It was reported that volatiles can function as growth-promoting agents (Wheatley, 2002; Horii and Ishii, 2006; Garbeva et al., 2014a) which might explain the increase in bacterial activity by exposure to microbial VOCs. Quantification of 16S rRNA gene copy number revealed that the growth of the starving *Burkholderia sp.* and *Dyella sp.* was induced by exposure to microbial VOCs. Hence, the growth of starving bacteria in soil can be promoted by volatiles released from the rhizosphere. Bacteria are able to detoxify VOCs and/or use them as carbon and energy source. For instance, it was shown that *Pseudomonas fluorescens* is able to degrade the volatile compound alpha-pinene and to use it as a sole carbon source (Best et al., 1987; Kleinheinz et al., 1999). Furthermore, *Burkholderia* was reported as a toluene-degrader (Chen et al., 2015) and *Dyella* was abundant on a biofilter exposed to terpenes (Moe et al., 2013). In the current study, various terpenes and butylated hydroxytoluene were released by the bacterial mixture supplied with ARE in soil. It can be assumed that some of these volatiles stimulated the growth of *Burkholderia sp.* and *Dyella sp.* in the nutrient-depleted soil. This, however, needs to be verified in future studies.

Interestingly, while the activity of *Paenibacillus sp.* was strongly induced by exposure to microbial VOCs the growth was suppressed. As previously reported some VOCs can act as growth-inhibiting, toxic agents (Peñuelas et al., 2014) and induce stress response (Kim et al., 2013; Garbeva et al., 2014a). This might explain the strong induction in the activity and at the same time growth-suppression of *Paenibacillus sp.* by exposure to microbial VOCs. Another possible scenario for the strong increase in activity of *Paenibacillus sp.* is the induction of motility to escape from the toxic environment. A stimulation of motility by exposure to microbial VOCs was already reported for several bacterial species (Kim et al., 2013; Hagai et al., 2014;

Garbeva et al., 2014a). For microbes living in the heterogeneous soil environment, an activation of motility by volatiles may be important to move toward nutrient-rich regions or to escape from hostile areas. Hence, volatiles in soil can provide important information on the quality of the nearby surroundings.

Besides the role as growth-promoting or growth-suppressing agents (Peñuelas et al., 2014), volatiles can also induce the production of non-volatile secondary metabolites. For instance, it was recently reported that microbial volatiles induce the production of inhibiting secondary metabolites in *Pseudomonas fluorescence* against Gram-positive bacteria (Garbeva et al., 2014a). Thus, the growth suppression of *Paenibacillus sp.* may be also due to non-volatile secondary metabolites which production was activated by microbial volatiles.

In conclusion, this study revealed that the blend of released volatiles from the rhizosphere is affected by inter-specific competitive interactions and shifts in the microbial community composition. Moreover, the presence of non-abundant, slow-growing species can strongly influence the volatile production by other dominant species. Based on our results and other recent studies it is evident that microbial volatiles in soil can serve multiple roles as C-source, defense metabolites, chemoattractant, repellants or other unknown so far. Hence, volatiles released by rhizosphere-inhabiting microbial communities can have a significant long distance effect on starving microorganisms in the surrounding, nutrient-depleted soil.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.01212

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A meta-analysis approach for assessing the diversity and specificity of belowground root and microbial volatiles

Denis Schenkel[1,2], Marie C. Lemfack[3], Birgit Piechulla[3] and Richard Splivallo[1,2]*

[1] Institute for Molecular Biosciences, Goethe University Frankfurt, Frankfurt, Germany, [2] Integrative Fungal Research Cluster, Frankfurt, Germany, [3] Institute for Biological Sciences, University of Rostock, Rostock, Germany

Volatile organic compounds are secondary metabolites emitted by all organisms, especially by plants and microbes. Their role as aboveground signals has been established for decades. Recent evidence suggests that they might have a non-negligible role belowground and might be involved in root–root and root–microbial/pest interactions. Our aim here was to make a comprehensive review of belowground volatile diversity using a meta-analysis approach. At first we synthesized current literature knowledge on plant root volatiles and classified them in terms of chemical diversity. In a second step, relying on the mVOC database of microbial volatiles, we classified volatiles based on their emitters (bacteria vs. fungi) and their specific ecological niche (i.e., rhizosphere, soil). Our results highlight similarities and differences among root and microbial volatiles and also suggest that some might be niche specific. We further explored the possibility that volatiles might be involved in intra- and inter-specific root–root communication and discuss the ecological implications of such scenario. Overall this work synthesizes current knowledge on the belowground volatilome and the potential signaling role of its constituents. It also highlights that the total diversity of belowground volatiles might be orders of magnitude larger that the few hundreds of compounds described to date.

Keywords: microbes, fungi, bacteria, volatiles, diversity, rhizosphere, mycorrhizas, roots

Introduction

Secondary metabolites are small molecules that are produced by all living organisms. Unlike primary metabolites which are directly involved in regular growth and development, secondary metabolites might be produced only at specific developmental stages or under certain circumstances; hence they might provide a functional readout of cellular state (Patti et al., 2012). Tens of 1000s of secondary metabolites derived from plants and microbes are known to humans as drugs, food additives or flavors, and fragrances; yet, their ecological functions remain poorly understood.

Secondary metabolites indeed play a central role in inter-organismic interactions. In numerous cases volatile and non-volatile secondary metabolites have been implicated in defense

and communication among organisms. Recently, volatiles have attracted sustained attention, especially in belowground communication, due to their ability to travel further distances than non-volatile metabolites (Rasmann et al., 2005; Wenke et al., 2010; Peñuelas et al., 2014). Because of their potent biological activities on plants, the use of volatiles in agriculture have been suggested as a possible alternative to pesticides (Bitas et al., 2013; Kanchiswamy et al., 2015). A search through literature and databases allows estimating the known structural diversity of volatiles derived from plant flowers – about 1700 volatiles from 991 species (Knudsen et al., 2006; Dunkel et al., 2009) – and from microbes, including fungi and bacteria – 1093 volatiles from 491 microbes at the time of this study (Lemfack et al., 2014). Yet considering that 10^7–10^9 bacterial species (Schloss and Handelsman, 2004), 1.5 million fungal species (Hawksworth, 2001) and 2,98,000 of plant species (Mora et al., 2011) might exist on earth, the number of volatiles will increase as new species are being characterized and discovered.

In the past 5 years, the ecological role of volatiles in above- and belowground interactions among plants, fungi, bacteria, and insects has been addressed in a series of comprehensive reviews (Wenke et al., 2010, 2012; Bailly and Weisskopf, 2012; Effmert et al., 2012; Davis et al., 2013; Farag et al., 2013; Audrain et al., 2015; Kanchiswamy et al., 2015; Schmidt et al., 2015). The latest of these reviews (Kanchiswamy et al., 2015) covered literature up to the beginning of 2015. Most recently a further example of belowground volatile based communication has been brought to light for plants and the ectomycorrhizal fungus *Laccaria bicolor* (Ditengou et al., 2015). Some volatile sesquiterpenoids emitted by the latter fungus were shown to induce root branching in poplar, a host plant which can enter into symbiotic interactions with the fungus, but also in *Arabidopsis*, a non-host plant unable of symbiosis with *Laccaria*. Remarkably not all fungal sesquiterpenoids induced root branching: the volatile (–)-thujopsene was implicated in the root morphological change but β-caryophyllene, another sesquiterpenoid also emitted by maize roots (Rasmann et al., 2005), had no effect on branching. These observations raise questions about the specificity of belowground signals as well as the ability of the target organisms to perceive and react to volatiles.

Soil is actually a highly colonized inhomogeneous substrate. Non-homogeneity is not only reflected in terms of structure and porosity but also in terms of nutritional differences (Schoenholtz et al., 2000). Besides, organisms present in the soil might also provide specific niches for defined microbes, thus exerting a community structuring effect. Belowground community structuring has indeed been observed in numerous cases. A textbook example includes root nodules in legumes which are exclusively colonized by nitrogen fixing rhizobacteria (Gage, 2004). More recent examples are provided by *Arabidopsis*' root endophytic microbial community made of *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria* (Bulgarelli et al., 2012) and by truffle's fruiting bodies which host bacterial communities clearly distinct from those of the surrounding soil (Antony-Babu et al., 2014). This belowground community structuring might explain why some volatiles could act as successful signaling cues within such communities, however evidence that specific volatiles are emitted in defined habitats/niches is currently limited.

The aim of this paper is to quantify the diversity and explore the specificity of belowground volatiles produced by microbes and plant roots. For this purpose we synthesized existing literature on plant root volatiles and relied on the "mVOC database" of microbial volatiles (Lemfack et al., 2014) to address questions such as: how structurally diverse are plant root and microbial volatiles? Which volatiles are common and specific to microbes and plant roots? Is their emission influenced by microbial phylogeny or habitat; and finally do root volatiles serve as signals for neighboring plants? Overall our aim was to shed more light on belowground volatiles diversity and functions by essentially using a quantitative approach to diversity and by integrating information on the phylogeny and the habitat of the emitters.

Materials and Methods

Diversity of Plant Root Volatiles

Volatile organic compounds (VOCs) released by plant roots have been investigated in a limited number of species. Here we gathered information relative to volatile diversity in barely – *Hordeum vulgare* – (29 compounds; Gfeller et al., 2013), the model plant *Arabidopsis thaliana* (eight compounds; Steeghs et al., 2004), maize – *Zea mays* (one compound; Rasmann et al., 2005) and the bean *Vicia faba* (one compound; Babikova et al., 2013). Overall these plant roots emitted 39 volatiles, which have been grouped in **Figure 1** based on their biosynthetic origins/chemical classes (i.e., terpenoids, alcohols).

Diversity of Microbial Volatiles

The diversity of microbial volatiles was investigated using the mVOC database (Lemfack et al., 2014). At the time of this study, the database comprised 1093 volatiles emitted by 135 fungi and 356 bacteria. As for plant roots, volatiles were classified according to chemical classes/biosynthetic origins (**Figure 2**).

Specificity of Microbial Volatiles Linked to Taxonomy and Ecological Niches

To understand how specific or common volatiles were in microbes, bacteria and fungi of the mVOC database were classified in taxonomical units either at the phylum or class level. Gaining insight into niche specificity was achieved by classifying the microbes of the mVOC database based on their habitat. Because of our focus on belowground interactions, classification was made in five categories: fungi or bacteria living in the rhizosphere, fungi, or bacteria living in the soil (excluding the rhizosphere), and microbes living in any other habitat (i.e. animals, marine habitats, and microorganisms associated to above-ground plant parts). Classification in specific niches/habitats was based on various data sources which will be shortly included in the mVOC database. Because we were interested in habitat/niche specificity, microbes which were ubiquitous to more than one habitat/niche were excluded from the analysis.
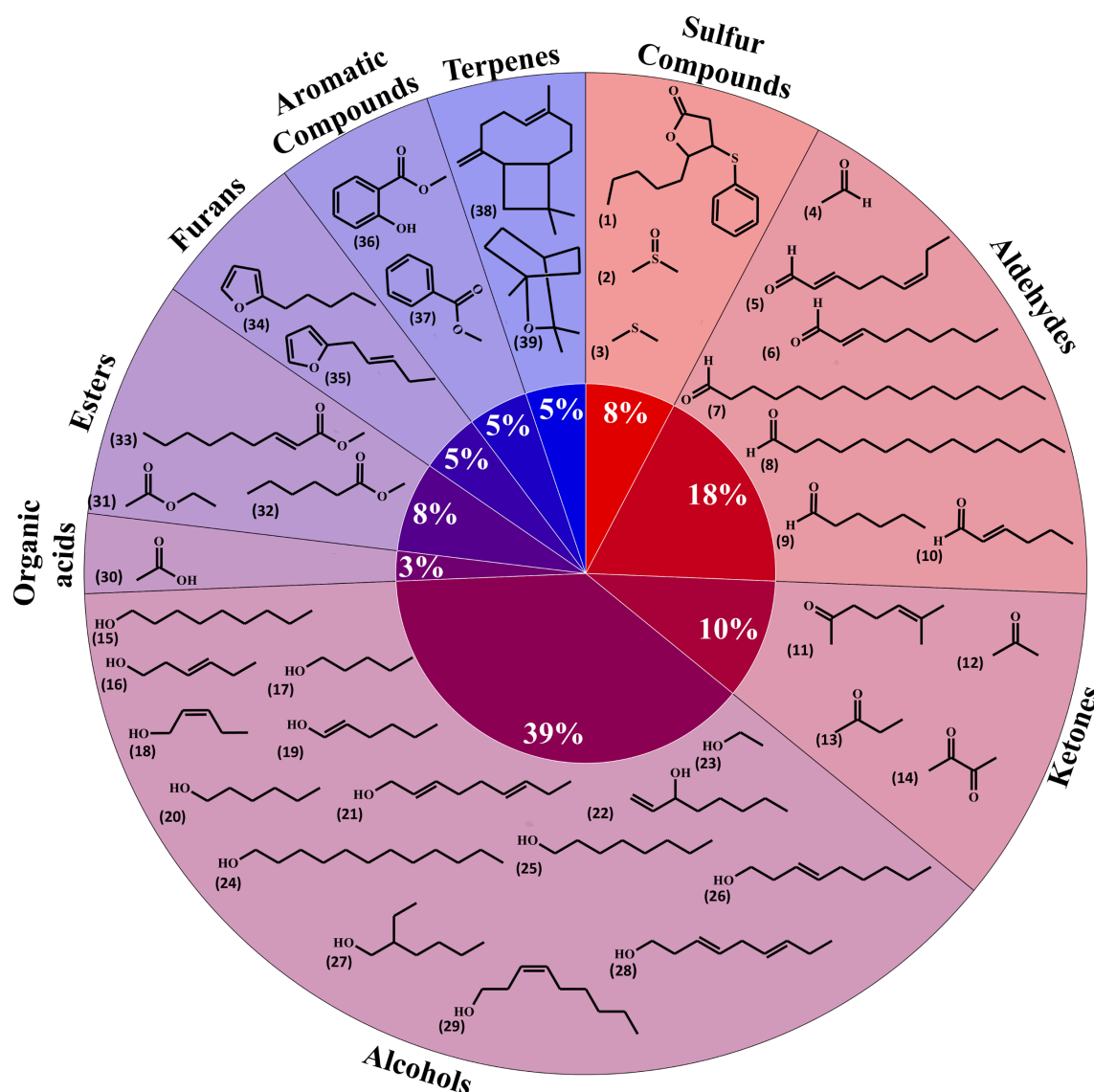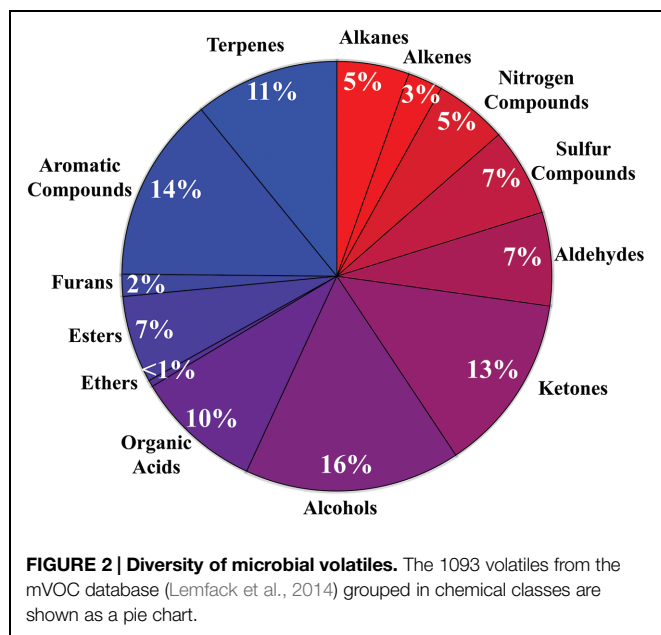
FIGURE 1 | Diversity of plant root volatiles. A total of 39 volatiles released from barley, maize, *Arabidopsis* or fava bean roots were classified based on their chemical classes and shown here in a pie chart. 5-Pentyl-4-phenylsulfanyloxolan-2-one (1); dimethyl sulfoxide (2); dimethyl sulfide (3); acetaldehyde (4); (2E,6Z)-nona-2,6-dienal (5); (E)-non-2-enal (6); hexadecanal (7); tetradecanal (8); hexanal (9); (2E)-hex-2-enal (10); 6-methylhept-5-en-2-one (11); acetone (12); butan-2-one (13); butane-2,3-dione (14); nonan-1-ol (15); (3E)-hex-3-en-1-ol (16); pentan-1-ol (17); (2Z)-pent-2-en-1-ol (18); (1E)-hex-1-en-1-ol (19); hexan-1-ol (20); nona-2,6-dien-1-ol (21); oct-1-en-3-ol (22); ethanol (23); dodecan-1-ol (24); octan-1-ol (25); (E)-non-3-en-1-ol (26); 2-ethylhexan-1-ol (27); nona-3,6-dien-1-ol (28); (Z)-non-3-en-1-ol (29); acetic acid (30); ethyl acetate (31); methyl hexanoate (32); methyl (E)-non-2-enoate (33); 2-pentylfuran (34); 2-pent-2-enylfuran (35); methyl salicylate (36); methyl-benzoate (37); β-caryophyllene (38); 1,8-cineole (39).

Effect of Neighboring Plant on Root Development

The influence of neighboring plants on root development was investigated by compiling data from 18 publications (Mahall and Callaway, 1992; Gersani et al., 2001; Maina et al., 2002; Day et al., 2003; Falik et al., 2003, 2006; Gruntman and Novoplansky, 2004; O'Brien et al., 2005; Dudley and File, 2007; Murphy and Dudley, 2007, 2009; Semchenko et al., 2007, 2014; Broz et al., 2008; Milla et al., 2009; Fang et al., 2013; Schmid et al., 2013a). In all those works root development

(biomass or root length depending on the parameter reported) of a plant subjected to neighboring plants was compared to root development of a plant without neighbors. The effects on roots were classified as "increase, decrease, no effect" based on the statistics reported in the papers. Subject plants were grouped either based on genetic relatedness with the interacting plants (as kin, conspecific but not kin, and foreign species) or as monocots and dicots. Cases in which the kinship of individuals of the same species was unspecified were categorized as conspecific.

FIGURE 2 | Diversity of microbial volatiles. The 1093 volatiles from the mVOC database (Lemfack et al., 2014) grouped in chemical classes are shown as a pie chart.

Results

Diversity of Plant Root and Microbial Volatiles

Whereas publications investigating volatiles emitted by aboveground plant organs abound, only a few papers have been published on root volatiles, most likely due to the technical difficulties in sampling volatiles in soil matrices. Compiling the information from root volatiles emitted by maize (Rasmann et al., 2005), barley (Gfeller et al., 2013), *Arabidopsis thaliana* (Steeghs et al., 2004), and the bean *Vicia faba* (Babikova et al., 2013) revealed an overall diversity of 39 volatiles belonging to nine chemical/biosynthetic groups (**Figure 1**). With 66% of all volatiles, alcohols, aldehydes, and ketones represented the major share of root volatiles. The remaining 44% was composed of minor groups (sulfur compounds, terpenoids, aromatic compounds, furans, esters, and organic acids) each represented by a single or two compounds. By contrast to the scarce information on root volatiles, microbial volatiles have been investigated more thoroughly. An effort to synthesize the large amount of information on microbial volatiles has recently been made through the mVOC database (Lemfack et al., 2014), which also served as the basis of the present study. Here a total of 1093 microbial volatiles from the mVOC database have been grouped according to chemical classes/biosynthetic pathways and the resulting data is presented as a pie chart in **Figure 2**. Even though some volatiles like ketones, esters, sulfur-containing compounds, and furans appeared with a comparable frequency as in plants roots and microorganisms, the microbial volatilome comprised a greater structural complexity of organic acids, aromatic compounds, and terpenes than plant roots, at least considering the currently available data (**Figures 1** and **2**). Five groups of microbial volatiles (terpenes, alcohols, ketones, aromatic compounds, and organic acids) represented each 10% or more of the volatiles, overall accounting for 64% of the total

diversity. Aldehydes, sulfur and nitrogen containing compounds, alkanes, alkenes, furans, ester, and ethers represent minor groups accounting together for almost 37% of the total diversity.

Which Microbes Produce Plant Root Volatiles?

A total of 28 plant root volatiles were also produced by microbes. These volatiles included 11 alcohols (dodecan-1-ol; ethanol; 2-ethyl-1-hexanol; hexan-1-ol; 2-hexen-1-ol; 3-hexen-1-ol; 1-nonanol; 1-octanol; 1-octen-3-ol; pentanol; 2-penten-1-ol), 4 aldehydes (acetaldehyde; hexanal; 2-hexenal; tetradecanal), two aromatic compounds (methyl benzoate; methyl salicylate), two esters (ethyl acetate; methyl hexanoate), one furan (2-pentylfuran), four ketones (acetone; butanone; butanedione; 6-methyl-5-hepten-2-one), one organic acid (acetic acid), two sulfur compounds (dimethyl sulfide; sulfinylbismethane), and one terpene (β-caryophyllene). Our aim was to understand if these volatiles were preferentially produced by specific bacterial or fungal phyla/classes. For this purpose, microbes emitting plant root volatiles were grouped in phyla and in some cases in classes. The heatmap in **Figure 3** represents the percentage of microbes, which are emitters of the plant root volatiles of **Figure 1**.

In terms of volatile groups, 14 fungal and 22 bacterial phyla emitted plant root volatiles at a rather low frequency (<10% as shown from the color scale on the heatmap of **Figure 3**). Alcohols were the most frequent and were emitted by four of the seven bacterial phyla and by all the fungal phyla. Volatiles belonging to remaining groups were similarly emitted at a low frequency by 50% of all phyla (fungal and bacterial). Interestingly all volatile groups occurred in at least one fungal and one bacterial phylum. Furans were produced by a fair percentage of fungi belonging to the three fungal phyla considered here whereas it was emitted at low frequency within a single bacterial phylum (*Firmicutes*, specifically the *Bacilli* class).

Considering the data in terms of phyla highlighted that members of the *Firmicutes* and *Proteobacteria* bacterial phyla and *Ascomycetes* fungi emitted volatiles belonging to most of the chemical groups. Zooming into bacterial classes revealed that among the *Firmicutes*, *Bacilli* were the most frequent emitters of plant root volatiles while among the *Proteobacteria*, β-, and γ-*Proteobacteria* were the most frequent emitters. Interestingly, acetic acid (the only molecule in the category "organic acid") was produced by about 50% of all *Bacilli* and an even higher percentage of *Clostridia* (the highest percentage with Negativicutes reflects the fact that this class has a single representative).

Overall these results highlight that numerous microbes are capable of emitting the same volatiles as plant roots. They also suggest that some phyla might be better than others at producing these volatiles. Bacteria belonging to the *Firmicutes* (*Bacilli*), to the *Proteobacteria* (β- and γ-*Proteobacteria*) and *Ascomycetes* fungi specifically stand out for their ability to produce a large variety of plant root volatiles.

Common and Specific Volatiles to Plant Roots, Bacteria, and Fungi

The microbial volatiles of the mVOC database and the plant root volatiles of **Figure 1** have been presented according to
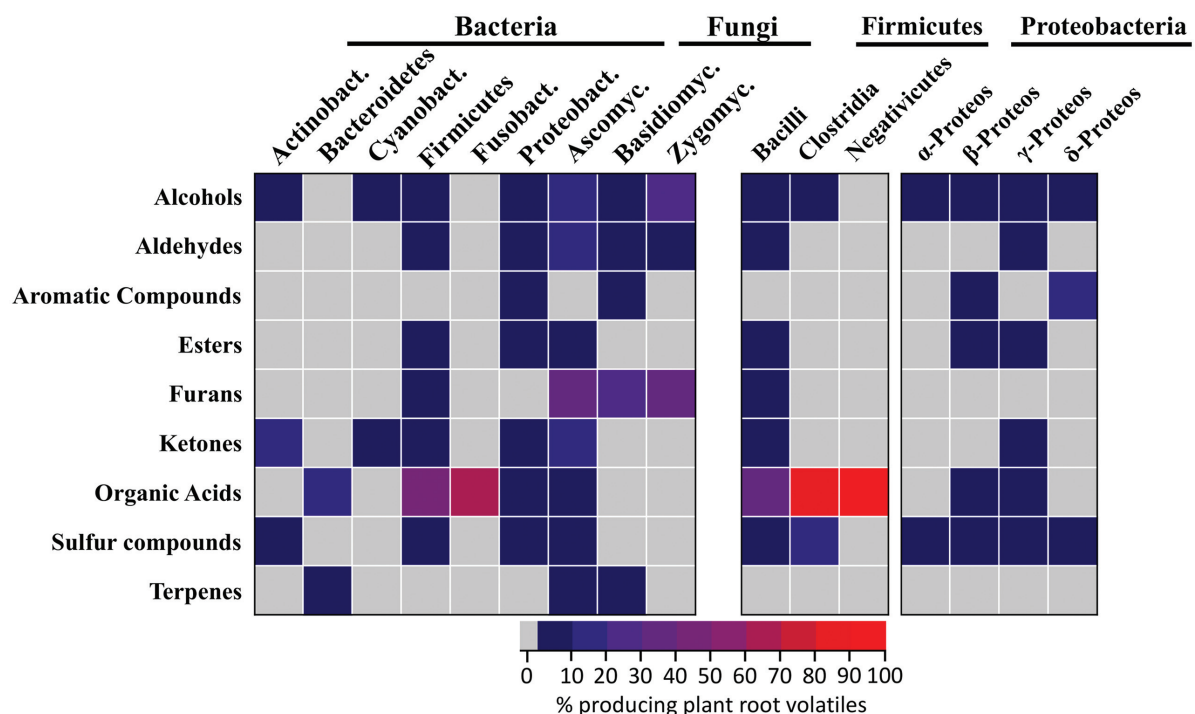
FIGURE 3 | Which microbes produce plant root volatiles? Bacteria and fungi producing the same volatiles as plant roots (**Figure 1**) were identified from the mVOC database. The results are displayed for the different phyla or classes of bacteria and fungi as a heatmap where each cells represents the percentage of microbes emitting volatiles of **Figure 1**. Actinobact.: *Actinobacteria* (n = 64), *Bacteroidetes* (n = 44), Cyanobact: *Cyanobacteria* (n = 19), *Bacilli* (n = 11), *Clostridia* (n = 10), *Negativicutes* (n = 1), Fusobact: *Fusobacteria* (n = 3), α-Proteos: α-*Proteobacteria* (n = 25), β-Proteos: β-*Proteobacteria* (n = 43), γ-Proteos: γ-*Proteobacteria* (n = 62), δ-Proteos: δ-*Proteobacteria* (n = 17), Ascomyc: *Ascomycota* (n = 104), Basidiomyc: *Basidiomycota* (n = 28), Zygomyc: *Zygomycota* (n = 3).

the potential origin/habitat of their emitters. These origins have been regrouped here in five categories as plant roots (39 volatiles), rhizosphere fungi (261 volatiles), rhizosphere bacteria (209 volatiles), soil fungi (187 volatiles), soil bacteria (483 volatiles). The data is presented as a Venn diagram highlighting the number of specific and common volatiles among groups (**Figure 3**).
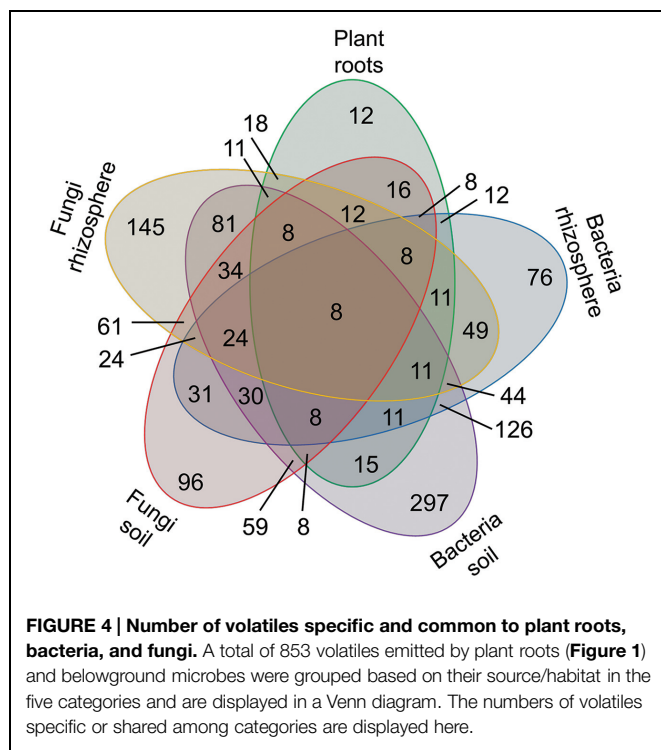
A total of 853 volatiles were emitted by plant roots and belowground microbes. Considering the five groups defined here, all groups shared eight volatiles; however, the majority of volatiles were unique to distinct origins/habitats. For example, of the 39 volatiles produced by plant roots (**Figure 1**), 12 (or 31%) were solely produced by roots and not by any other microbes. Depending on their habitat fungi produced 145 (rhizosphere) and 96 (soil) unique volatiles not shared by any other groups; by contrast soil and rhizosphere fungi had 61 volatiles in common. The same argument can be made for bacteria, which produced 76 (rhizosphere) and 297 (soil) unique volatiles, and shared 126 of them.

Overall this data exemplifies the specificity but also the extent of the overlap in volatile signals emitted by plant roots and microbes. It highlights the existence of a core volatilome for bacteria and plant roots but also the fact that a high proportion of volatiles are specific to organisms in defined habitats.

Are Microbial Volatiles Niche Specific?

Fungi and bacteria from the mVOC database were regrouped according to their lifestyle/habitat. Similarly to **Figure 4** three categories were considered in relation to possible interactions with plants: organisms typically found in the soil (S), microbes associated with the rhizosphere (R) and organisms which did not fall in those two categories (N) (i.e., either associated to above plant organs or with animals). Only volatiles occurring in at least 10 microbes are shown here. Values in the heatmap represent the percentage of microbes emitting a specific volatile in each category.

In terms of chemical classes/groups, numerous terpenes, aromatic compounds, nitrogen, and sulfur containing compounds, alkanes and alkenes were predominantly produced by bacteria compared to fungi. Some volatiles such as nitrogen containing compounds were actually almost exclusively produced by bacteria. By contrast no volatiles were exclusively produced by fungi. In most cases habitat specificity (i.e., soil, rhizosphere) seemed to have little influence on volatiles patterns. Volatiles belonging to a few groups were, however, predominantly produced by rhizosphere (R) organisms (in opposite to soil (S) and "other" (N) organisms). This was the case for example in fungi for alcohols, sulfur compounds, some aromatic compounds (i.e., 2-phenylethanol) and some ketones (i.e., octan-3-one). Similarly in bacteria nitrogen containing

FIGURE 4 | Number of volatiles specific and common to plant roots, bacteria, and fungi. A total of 853 volatiles emitted by plant roots (**Figure 1**) and belowground microbes were grouped based on their source/habitat in the five categories and are displayed in a Venn diagram. The numbers of volatiles specific or shared among categories are displayed here.

compounds production seemed slightly higher in rhizosphere organisms.

Plant root volatiles shown in bold were marked with an asterisk in **Figure 5**. With the exception of 1-octen-3-ol, most of these volatiles were emitted by microbes in most/all categories. Nevertheless it is noteworthy that six of the eight plant root volatiles shown here [2-pentylfuran; dimethyl sulfide (syn. (methylsulfanyl)methane]; ethyl acetate; acetone; ethanol; 1-octen-3-ol) were emitted by a comparatively higher percentage of rhizospheric fungi compared to fungi and bacteria colonizing different habitats.

These results demonstrate marked differences in terms of volatile production patterns among bacteria and fungi. This suggests that bacteria might be capable of synthesizing structurally more diverse volatiles than fungi. They also indicate that microbes belonging to specific niches/habitats, especially to the rhizosphere, might preferentially produce volatile signals, including many of the volatiles also emitted by plant roots.

Could Root Volatiles be Perceived by Neighboring Plants?

There is a mounting body of evidence that neighboring plants can communicate with each other through their roots (Dudley and File, 2007; Bhatt et al., 2011; Fang et al., 2013; Schmid et al., 2013b). Obvious signals for such communication might be volatile molecules. Additionally, volatile emission patterns of aboveground plant organs were shown to be dependent on genetic relatedness. For the sake of clarity, kin plants by definition share the same parents/ancestors, as opposed to conspecific plants which, besides belonging to one species, do not have common parents/ancestors. Recently volatile profiles of kins

were shown to be more similar to each other than those of plants without kinship (conspecific plants) (Karban et al., 2014). This led us to question whether plant roots react differently to neighboring plants based on their genetic relatedness (i.e., kins, conspecific but not kins, or foreign (different species) – see cartoon of **Figure 6**). Furthermore we also questioned if differences existed among monocots and dicots.

To answer these questions we gathered publications, which compared root development of one plant with a neighbor to a single plant without neighbor. A total of 30 observations from 18 publications were taken into account and their outcomes have been synthesized in **Figure 6**.

Comparing kins to conspecifics revealed that the roots of more than 50% of kins were unaffected by their neighbors compared to only 21% for conspecifics. When an effect was observed, this predominantly corresponded to an increased root biomass for both categories. The opposite was true for plants subjected to a foreign neighbor. These predominantly (43% of all observations) reacted to the neighbor by decreasing or shortening their roots. Patterns were less obvious with monocots and dicots. Indeed the number of cases in which roots were either affected (increase or decrease in biomass/root length) or unaffected were comparable.

Overall these results highlight that among kin, foreign and conspecific plants, roots of kins are the less likely to be influenced by a neighbor. They also exemplify that plants sharing the same genotype (kins and conspecifics) might predominantly react to each other by increasing their root biomass/root length, while plants with a foreign neighbor might commonly decrease their root biomass.

Discussion

During the past decade VOCs have gained recognition as essential signals in inter-organismic interactions. Especially belowground volatiles might convey information among plant roots, microbes, and insects. The diversity of volatiles and effects on their target organisms have been recently synthesized in a series of comprehensive reviews (Wenke et al., 2010, 2012; Bailly and Weisskopf, 2012; Effmert et al., 2012; Davis et al., 2013; Kanchiswamy et al., 2015). Our aim here was to bring this synthesis one step further by using a quantitative meta-analysis approach and integrating data about phylogeny and potential habitat of the emitters.

Diversity of Belowground Volatiles

Adding up volatiles emitted by plant roots to volatiles emitted by soil/root microbes brings the total diversity of belowground volatiles to 853. Considering the scarce information on plant root volatiles (only a few existing publications), and the huge unexplored diversity of soil microbes, the overall diversity of belowground volatiles might be orders of magnitudes higher than the few 100s of compounds described to date.

A note of caution should nevertheless be used when estimating diversity from literature data, since most studies describing volatiles from microbes or plant roots have been conducted under laboratory (and sometimes axenic) conditions. Indeed it
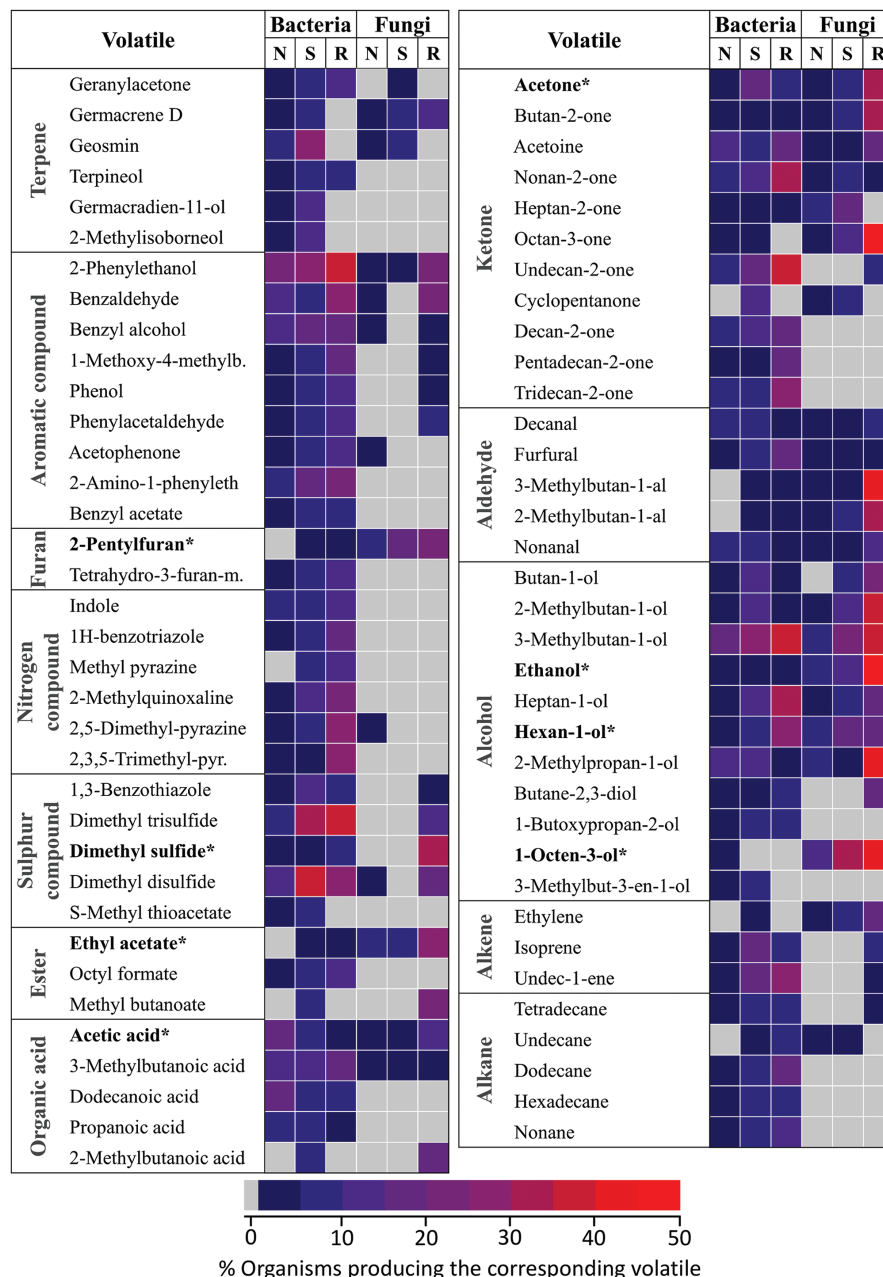
FIGURE 5 | Niche specificity of bacterial and fungal volatiles. Volatiles emitted by at least 10 different organisms from the mVOC database are shown. Microbes were classified based on their lifestyle/habitat as "soil" (120 bacteria, 36 fungi), "rhizosphere" (rhizo: 43 bacteria, 26 fungi) and "other" (157 bacteria, 64 fungi). The color code shows the frequency of each volatile in each group. *These volatiles are also emitted by plant roots.

is well known that media composition, culture conditions or interacting organisms might influence secondary metabolism (Blom et al., 2011; Brakhage and Schroeckh, 2011). Hence if the presence of one volatile in the mVOC database reflects the ability of specific organisms to produce that volatile, its absence does not exclude that it might be produced under natural conditions. The reverse is certainly also true. Overall estimating the total diversity of belowground volatiles will require isolating and characterizing more microbes/plant roots

but also analyzing full soil communities under both laboratory and natural conditions. It should be highlighted that profiling volatiles from soil is much more complicated than from any other system. Indeed soil is a highly complex matrix which requires the most advanced instrumentation in terms of resolution and sensitivity (i.e., high resolution MS or proton transfer MS) as well as powerful data processing for harnessing the complexity of its volatilome (Peñuelas et al., 2014; Mancuso et al., 2015).

FIGURE 6 | Influence of a neighboring plant on root development. Data presented in a bar chart summarizes the observations of several studies which compared root development of a plant with and without neighbor. The cartoon on the right illustrates the interactions considered here. "Increase, decrease, no effect" refer to the root biomass of a plant with neighbor compared to the one without. Kin: 20 observations in which interacting plants are kins. Conspecies: 27 observations where tested plants are from the same species (but not kins). Foreign: 45 observations where interacting plants belong to different species. Monocots: 28 observations involving monocotyledonous plants. Dicot: 64 observations involving dicotyledonous plants.

Differences and Similarities in Volatile Profiles of Roots, Bacteria, and Fungi

Our analysis highlighted similarities and differences among plant root and belowground microbial volatiles. In terms of similarities, numerous microbes were capable of emitting the same volatiles as plant roots; however, bacteria belonging to the *Firmicutes* (*Bacilli*), to the *Proteobacteria* (β- and γ-*Proteobacteria*) and *Ascomycete* fungi especially distinguished themselves in this regard. Interestingly *Firmicutes* and β- and γ-*Proteobacteria* tend to be dominant root endophytes in rice and sugarcane (Fischer et al., 2011; Sessitsch et al., 2012). Ascomycete fungi also include numerous members which live in close association with plant roots (i.e., truffles forming ectomycorrhizas; Martin et al., 2010). It is therefore tempting to speculate that resemblance in terms of volatile profiles might translate into closer associations between microbes and plant roots. Testing this hypothesis will require characterizing the volatile profiles of numerous plant roots and microbial strains under natural conditions.

How Specific are Belowground Volatile Signals?

Determining how specific volatile signals might be in terms of interactions requires understanding the nature of the interaction and also the habitat in which it takes place. In terms of molecules, terpenoids are not only important volatiles for floral scent (Knudsen et al., 1993, 2006; Pichersky and Gershenzon, 2002), but as illustrated by β-caryophyllene in maize, they might serve as an alarm signal upon attack by root pests (Rasmann et al., 2005). This volatile is also emitted by a bacterium belonging to the *Bacteroidetes* phylum and by some fungi (**Figure 3**; Lemfack et al., 2014). It has additionally been reported from the fungus *Fusarium oxysporum* colonized by ectosymbiotic bacteria, and it is responsible of the growth promoting effect observed in lettuce colonized by the latter fungus (Minerdi et al., 2011). This example illustrates that one volatile might be produced by numerous

organisms to a different end. Another terpenoid, geosmin, which is produced by numerous microbes (Lemfack et al., 2014) was also recently reported from beet roots (*Beta vulgaris* sp. *vulgaris*) (Freidig and Goldman, 2014). We had originally not included this compound in our list of plant root volatiles because they were suspicions that it might not have been produced by beet root itself but by microbes colonizing beet roots tissues; however, the data presented by Freidig and Goldman (2014) suggests that this might be otherwise. This highlights that characterizing the volatile profiles of existing plant roots might greatly increase the diversity of plant root volatiles.

Sulfur containing volatiles are also important signals in plant–microbe interactions. Indeed it has recently been demonstrated that dimethyl disulfide produced by Bacillus bacteria naturally colonizing tobacco roots promoted plant growth by enhancing sulfur assimilation (Meldau et al., 2013). Our data highlights that this volatile is predominantly produced by bacteria (essentially soil bacteria) and to a lesser extent by fungi (**Figure 5**). A tempting interpretation might be that numerous soil bacteria might use this volatile for plant growth promotion. Other bacterial volatiles might also serve this purpose, indeed 2,3-butanediol promotes plant growth in Arabidopsis (Ryu et al., 2003). Nevertheless the overall effect of microbial volatiles on plant growth depends on the total volatile blend and cultural conditions of the microbes (Blom et al., 2011; Peñuelas et al., 2014). Therefore understanding the specificity of a signal also requires characterizing the context in which it is emitted as well as the bioactivity of the total volatile blend.

Eight carbon containing volatiles are characteristic of fungi, and its major representative, 1-octen-3-ol is indeed responsible of the typical fungal smell perceived by humans (Wnuk et al., 1983; Mosandl et al., 1986). Our data indicates that 1-octen-3-ol and octan-3-one are predominantly produced by rhizospheric fungi (**Figure 5**). Since numerous of these fungi live in

symbiotic association with plant roots (i.e., truffles), eight carbon-containing volatiles might serve as symbiotic signals to a potential host plant. In terms of biological activity high concentrations of these volatiles have been shown to inhibit seed germination and seedling development in *Arabidopsis* and *Cistus incanus*, a host plant to truffles (Splivallo et al., 2007; Hung et al., 2014). Nevertheless lower concentrations of 1-octen-3-ol was shown to induce plant defense genes in *Arabidopsis* (Kishimoto et al., 2007). These volatile signals might therefore modulate the host–plant fitness, however, how effective this modulation might be in nature remains to be investigated.

Another group of potential signaling molecules are nitrogen-containing volatiles. Interestingly, these volatiles seem essentially produced by bacteria but not by fungi. In relation to their habitat, rhizosphere bacteria were the best producers of these volatiles (**Figure 5**). Since these bacteria include the Rhizobium genus, members of which are able to fix atmospheric nitrogen and hence literally serve as natural fertilizers for legumes when colonizing their roots (Gage, 2004), it can be speculated that nitrogen-containing volatiles are involved in signaling between Rhizobium and legumes. As in the case of dimethyl disulfide (Meldau et al., 2013), nitrogen-containing volatiles might be directly assimilated by legumes for nutritional purposes, however, they might serve other purposes as well. Demonstrating their exact role as signaling agents will first require deciphering their biosynthesis.

The examples above illustrate how specific or unspecific belowground volatile signals might be. The various ecological roles highlighted here and, in some cases, the ability of different organisms to emit the same signals, suggest the existence of complex volatile-based interaction networks. Demonstrating their specificity will require characterizing full networks of interacting organisms but also concentrations-activity ratios as well as the persistence of volatile signals in soil.

Could Root Volatiles be Perceived by Neighboring Plants?

Plants are able to communicate belowground with their neighbors through some unknown signals (Dudley and File, 2007; Bhatt et al., 2011; Fang et al., 2013; Schmid et al., 2013b). Genetic relatedness has recently emerged as an important factor governing belowground root–root interactions. For example roots of rice plants belonging to the same genotype were show to grow toward each other whereas those of different genotypes seem to avoid each other (Fang et al., 2013). Another study involving *Cakile edentula* plants illustrated that plant root allocation is influenced by kinship; indeed the authors observed lower root allocation in kin pairs than stranger pairs (Bhatt et al., 2011). The nature of the signals involved in root–root communication has not yet been fully identified, however, root exudates have been recently suggested as possible candidates (Semchenko et al., 2014). Because volatiles can essentially diffuse further in the soil than root exudates, they might also act as signaling agents in root–root communication. We explored this possibility relying here on indirect evidence. Indeed volatile emission patterns of aboveground plant organs were recently shown to be dependent on kinship, with volatile profiles of kins being more similar to each other than those of plants without kinship (but of the same species) (Karban et al., 2014). Our data demonstrates that how plants respond in terms of root biomass/structure to the presence of a neighboring plant is actually influenced to a certain extent by kinship and genetic relatedness (**Figure 6**). Taken as a whole this suggests that volatile signals might indeed be involved in belowground root–root communications. Demonstrating their exact involvement will require profiling root volatiles as a function of genetic relatedness, identifying the signaling agents, and demonstrating their activity.

Conclusion

The past decade has seen an increasing interest in belowground volatile-based communication among organisms (Kai et al., 2009; Wenke et al., 2010, 2012; Bailly and Weisskopf, 2012; Piechulla and Degenhardt, 2014). Because of the high heterogeneity and large organismic diversity present in the soil, and the potentially humongous diversity of belowground volatiles, it is essential to apply a holistic approach to understand diversity. Such an attempt has been made here essentially relying on the recently published mVOC database of microbial volatiles (Lemfack et al., 2014) and on a limited number of papers describing plant root volatiles. Although our analysis highlights interesting patterns in belowground volatile diversity and distribution, it also cries out for more data. Essentially we might be looking at the tip of the iceberg and estimating total belowground volatile diversity will require characterizing both the emitters and their full volatile spectra. This will be a major challenge considering the huge number of undescribed soil microbes.

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Chemical diversity of microbial volatiles and their potential for plant growth and productivity

Chidananda Nagamangala Kanchiswamy[1]*, Mickael Malnoy[1] and Massimo E. Maffei[2]

[1] *Research and Innovation Center, Biology and Genomic of Fruit Plants, Fondazione Edmund Mach, Trento, Italy,* [2] *Plant Physiology Unit, Department of Life Sciences and Systems Biology, University of Turin, Turin, Italy*

Microbial volatile organic compounds (MVOCs) are produced by a wide array of microorganisms ranging from bacteria to fungi. A growing body of evidence indicates that MVOCs are ecofriendly and can be exploited as a cost-effective sustainable strategy for use in agricultural practice as agents that enhance plant growth, productivity, and disease resistance. As naturally occurring chemicals, MVOCs have potential as possible alternatives to harmful pesticides, fungicides, and bactericides as well as genetic modification. Recent studies performed under open field conditions demonstrate that efficiently adopting MVOCs may contribute to sustainable crop protection and production. We review here the chemical diversity of MVOCs by describing microbial–plants and microbial–microbial interactions. Furthermore, we discuss MVOCs role in inducing phenotypic plant responses and their potential physiological effects on crops. Finally, we analyze potential and actual limitations for MVOC use and deployment in field conditions as a sustainable strategy for improving productivity and reducing pesticide use.

Keywords: microbial volatile compounds, bacteria, fungi, plant productivity, sustainable agriculture

Introduction

Volatile Organic Compounds (VOCs) typically occur as a complex mixture of low-molecular weight lipophilic compounds derived from different biosynthetic pathways. To describe their complexity the term "volatilome" has been recently proposed (Maffei et al., 2011). In nature, VOCs are responsible for inter- and intra-organismic communication, partaking in innumerable interactions between plants, antagonists, and mutualistic symbionts both below and above ground (Maffei, 2010; Maffei et al., 2011; Garbeva et al., 2014b; Lemfack et al., 2014; Kanchiswamy et al., 2015).

Volatile Organic Compounds can travel far from the point of production through the atmosphere, porous soils, and liquid, making them ideal info-chemicals for mediating both short- and long-distance intercellular and organismal interactions (Maffei et al., 2011). In the past there was less focus on volatiles of microorganisms with respect to VOCs from species of the plant and animal kingdom (Stahl and Parkin, 1996; Schulz and Dickschat, 2007; Korpi et al., 2009; Effmert et al., 2012; Junker and Tholl, 2013; Weisskopf, 2013; Penuelas et al., 2014).

The cosmopolitan distribution of microorganisms creates a context for frequent and frequently overlooked, biotic responses to microbial emissions. In some ecosystems, bacterial or fungal emissions can also incite biotic aggregations, and often a single microorganism or emission can have different effects on biota behaviors, especially across

species, ontogenies, and habitats (Davis et al., 2013). These interactions prompt coevolution, whose process among biota – including viruses, fungi, bacteria, plants, nematodes, insects, and mammals, is considered by many biologists to have generated much of the earth's biological diversity (Occhipinti, 2013).

Microbial volatile organic compounds (MVOCs) are a type of VOCs produced by all microorganisms as part of their normal metabolism. They serve as chemical windows through which the fundamental information about the molecular basis of microbial activities is released (Liang et al., 2008; Korpi et al., 2009; Thorn and Greenman, 2012). There appears to be a multipartite basis for organisms responses to MVOCs, and complex trophic interactions can result from the production of MVOCs. Moreover, species-specific MVOCs may also serve as marker compounds for the selective detection of fungal and bacterial species in the environment (Fiedler et al., 2001). Other transformations may, however, occur in detoxification processes (Marmulla and Harder, 2014). Many of the formed MVOCs are produced by soil microorganisms, and it would be a challenge to investigate soil microbial communities by studying their MVOC profile (Insam and Seewald, 2010). To date, 100s of bacteria and fungi were described as soil MVOC producers (Effmert et al., 2012).

The comparative analysis of experimental data has shown that volatile metabolites make a much greater contribution to the microbial interactions than non-volatile ones. It has been found that interaction of microorganisms via the volatiles they release occurs frequently and is typical of a number of microorganisms (Tirranen and Gitelson, 2006). Understanding of the MVOCs, as well as the insights into the molecular basis associated with the MVOCs, can provide a real-world capabilities for better control and utilization of microorganisms (Liang et al., 2008). Systematic exploration of MVOCs and the characterization of their biological functions and ecological roles will likely uncover novel mechanisms for controlling diverse biological processes critical to plant health and will also offer tangible practical benefits in addressing agricultural and environmental problems (Bitas et al., 2013).

Volatile metabolites released by microorganisms produce an inhibitory, sometimes bactericidal, effect on the vital functions of bacteria, while the stimulating action occurs 6–8 times less frequently (Tirranen and Gitelson, 2006). To quote a few examples, furfural, butanoic acid, propanoic acid, 5-hydroxymethyl-furfural, β-caryophyllene, geosmin, 2-methyl isoborneol, 1-octen-3-ol, α-pinene, camphene, camphor, methanol, and acetaldehyde (**Figure 1**) are among the most frequently emitted compounds (Stahl and Parkin, 1996; Li et al., 2004; Muller et al., 2004a,b; Leff and Fierer, 2008; Gray et al., 2010; Insam and Seewald, 2010; Ramirez et al., 2010; Wenke et al., 2010; Perl et al., 2011; Juenger et al., 2012; Sundberg et al., 2013).

Recent developments in analytical instrumentation and bioinformatics software have established metabolomics as an important research tool for studying ecological interactions between organisms (Kuske et al., 2005; Scotter et al., 2005; Bunge et al., 2008; Liang et al., 2008; Wihlborg et al., 2008; Mcneal and Herbert, 2009; O'Hara and Mayhew, 2009; LeBouf et al., 2010; Ramirez et al., 2010; Rasanen et al., 2010;

Dolch et al., 2012; Juenger et al., 2012; Thorn and Greenman, 2012; Aponte et al., 2013; Kunze et al., 2013), while recent reviews described MVOCs biosynthesis (Davis et al., 2012; Penuelas et al., 2014).

In this review, we will give an overview of the chemical diversity of microbial volatiles and we will address the important issue of the exploitation of these bioactive molecules to improve plant growth, development, and health in a sustainable agricultural context.

Overview of Bioactive Bacterial Volatiles

Bacteria emit a wealth of volatiles. During the past few years, an increasing awareness concerning the emission of an unexpected high number of bacterial volatiles has been registered and recent investigations have clearly demonstrated that bacteria employ their volatiles during interactions with other organisms in order to influence populations and communities (Kai et al., 2009; Romoli et al., 2014). A wide array of compounds has been identified in bacterial emission of a large number of chemicals. Bacteria are known to either positively or negatively affect other organisms' fitness and recent studies have suggested that bacterial volatiles play an important role in bacterial–plant, bacterial–bacterial, and bacterial–fungal interactions.

MVOC in Bacterial–Plant Interactions

Hundreds of different bacterial MVOCs have been identified, comprising alkanes, alkenes, alcohols, esters, ketones, sulfur compounds, and terpenoids. The appearance of a characteristic volatile profile or compound is attributable to the specific metabolism or metabolic pathway(s) active in the bacteria (Kai et al., 2009). Such volatiles are ideal infochemicals because they occur in the biosphere over a range of concentrations and can act over long distances (Wheatley, 2002).

Some bacteria preferentially live in the soil closely associated with the plant roots, exploiting the rich nutrient exudates that plants deliver into the soil. These bacteria are collectively defined rhizobacteria and many of them promote plant growth (Bhattacharyya and Jha, 2012), whereas the root environment they colonize is called the rhizosphere (Mendes et al., 2013). Among rhizobacteria, Pseudomonads have been considered to be important rhizosphere organisms (Goswami et al., 2013). VOCs produced by rhizobacteria are involved in their interaction with plant-pathogenic microorganisms and host plants and show antimicrobial and plant-growth modulating activities (Vespermann et al., 2007). Fluorescent *Pseudomonas* strains help in maintenance of soil health and protect the crops from pathogens (Hol et al., 2013). Rhizospheric bacterial strains can modulate both plant growth promotion and root-system architecture by differential VOC emission (Gutierrez-Luna et al., 2010). Recent analytical developments have provided a most comprehensive profile of rhizobacterial volatiles. These MVOCs exhibit molecular masses below 300 Da and are rather lipophilic with relatively low boiling points.

FIGURE 1 | Most frequently emitted Microbial volatile organic compounds (MVOCs).
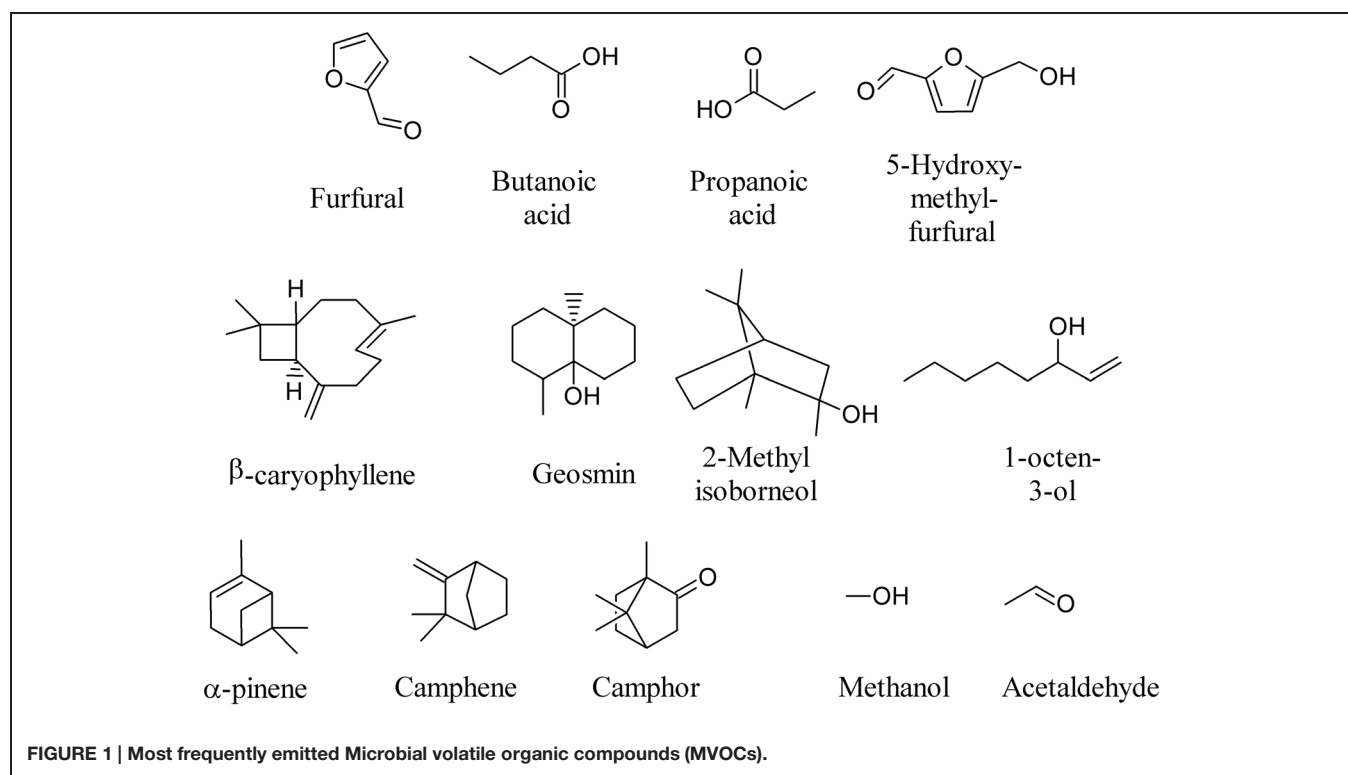
As well as commonly known bacterial VOCs such as 2-pentanone, 4-heptanone, 2-heptanol, 2-undecanone, 2-tridecanone, and 2-pentadecanone (Schulz and Dickschat, 2007; Weise et al., 2014), well known compounds like sodorifen, a bicyclic oligomethyl octadiene produced by *Serratia odorifera* (Kai et al., 2010), are able to interfere with plants (**Figure 2**). Forty-two soil-borne bacterial strains were screened for their volatile-mediated effect on 6-day-old seedlings of *Arabidopsis thaliana*. Thirty-six compounds of bacterial origin were selected for further analysis and among these 1-hexanol, indole,

and pentadecane stimulated plant growth (Blom et al., 2011). Co-cultivation of *A. thaliana* with *S. odorifera* in bi-partite Petri dishes, which only allowed volatiles to diffuse from one side to the other, resulted in dramatic growth inhibition of plants (Vespermann et al., 2007). Dimethyl disulfide (DMDS) and ammonia are among the most bioactive compounds (Kai et al., 2010).

Application of DMDS produced by a *Bacillus cereus* strain significantly protected tobacco (*Nicotiana tabacum*) and corn (*Zea mays*) plants against *Botrytis cinerea* and *Cochliobolus*

FIGURE 2 | Common bacterial volatile organic compounds (VOCs) and the bicyclic oligomethyl octadiene sodorifen.
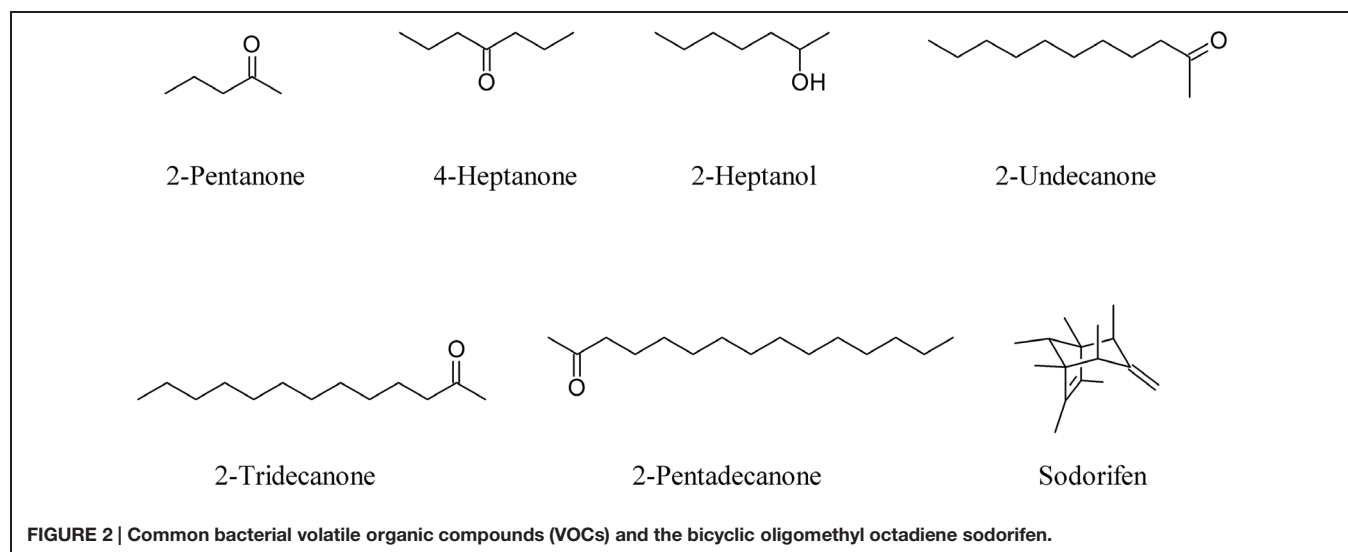
heterostrophus, respectively (Huang et al., 2012). Furthermore, DMDS supplementation significantly reduced the expression of *Nicotiana attenuata* sulfur-assimilation genes, as well as methionine biosynthesis and recycling (Meldau et al., 2013). Two compounds, 3-hydroxy-2-butanone also known as acetoin and 2,3-butanediol (2,3-BD), were released consistently from strains of *Bacillus subtilis* and *B. amyloliquefaciens* and were found to significantly enhance total leaf surface area and induced systemic resistance (ISR) of *A. thaliana* (Ryu et al., 2003; Rudrappa et al., 2010). 2,3-BD was also one of the major MVOCs produced by *Enterobacter aerogenes*, an endophytic bacterium that colonizes corn plants. The production of 2,3-BD by *E. aerogenes* rendered corn plants more resistant against the Northern corn leaf blight fungus *Setosphaeria turcica* (D'Alessandro et al., 2014).

The differential emission of acetophenone, tridecanal, tetradecanal, 6,10,14-trimethyl 2-pentadecanone and benzaldehyde produced by different lemon rhizobacteria showed that the effect observed in *Arabidopsis* roots is proportional to the type and amount of compounds produced by the bacteria (Gutierrez-Luna et al., 2010). 3-Hexanone produced by strains of *Burkholderia ambifaria* significantly increased *Arabidopsis* biomass, as did acetophenone and DMDS (Groenhagen et al., 2013). **Figure 3** depicts some bacterial VOCs able to induce plant responses.

A broad phylogenetic spectrum of bacteria, including α-, β-, and γ-Proteobacteria, high-G+C-content Gram-positive bacteria, and microbes belonging to the Fibrobacteres/Acidobacteria group live inside special cells surrounding the root vascular cylinder of vetiver (*Vetiveria zizanioides*; Maffei, 2002). Most of them are able to grow by using oil sesquiterpenes as a carbon source and to metabolize them releasing into the environment a large number of compounds typically found in commercial Vetiver oils

(Del Giudice et al., 2008; Alifano et al., 2010; Maffei et al., 2011). When the sesquiterpene cuparene, for instance, was fed to Vetiver root-associated bacteria an amazing number of other unrelated sesquiterpenes were produced, including β-bourbonene, β-copaene, β-humulene, ledene, α-muurolene, δ-cadinene, spathulenol, viridiflorol (**Figure 4**), and β-caryophyllene (**Figure 1**; Del Giudice et al., 2008). These results underline the ability of bacteria to biotransform *in vivo* complex MVOCs.

MVOC in Bacterial–Bacterial Interactions

Volatiles of bacteria can influence the metabolism of other bacteria but the role of volatiles in interactions between bacterial species has been studied very little. Given the physically separated distribution of bacterial populations (micro-colonies) in the porous soil matrix it has recently been suggested that MVOCs play key roles in interspecific bacterial interactions (Garbeva et al., 2014a). It is expected that rhizosphere-inhabiting bacteria might invest a substantial part of the energy obtained from metabolizing root-exudates to produce bioactive MVOCs.

Volatiles produced by *Collimonas pratensis* and *Serratia plymuthica* stimulated the growth of *Pseudomonas fluorescens*, whereas volatiles emitted by *Paenibacillus* sp., *Pedobacter* sp. and the mix of all four bacteria did not affect *P. fluorescens* growth (Garbeva et al., 2014a). The highest numbers of unique volatile compounds were emitted by *C. pratensis* and *S. plymuthica*, including *S*-methyl thioacetate, methyl thiocyanate, benzonitrile (**Figure 5**) and DMDS (**Figure 3**). Specific MVOCs produced by *C. pratensis* included among others: 3-hexanone (**Figure 3**), 2-methyl propanal, ethenyl acetate, 3-methyl 2-pentanoene, methyl 2-methylbutanoate, methyl 3-methylbutanoate, 4-methyl 3-penten-2-one, 3-methyl 2-heptanone, myrcene, terpinene, and methyl salicylate (**Figure 5**). Specific MVOCs produced by *S.*
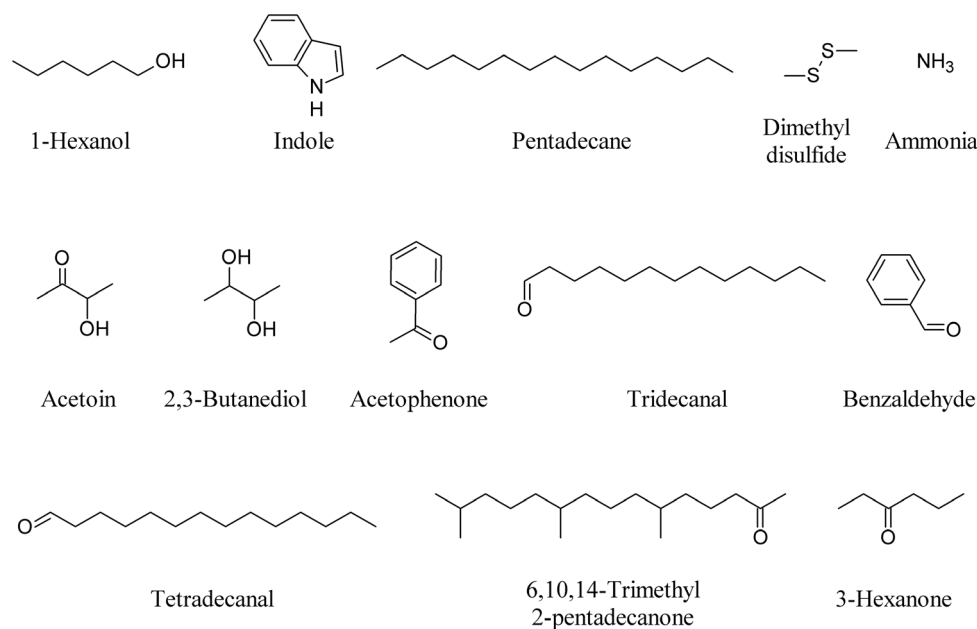
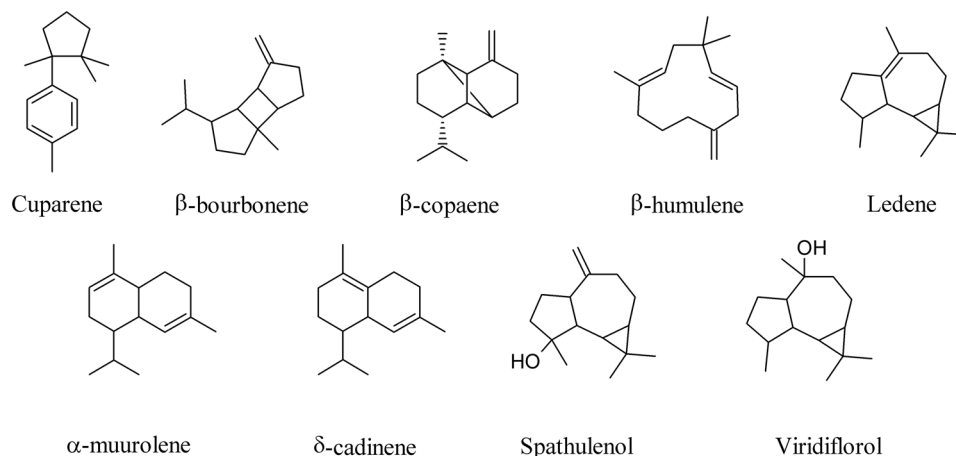

FIGURE 3 | Bacterial VOCs able to induce plant responses.

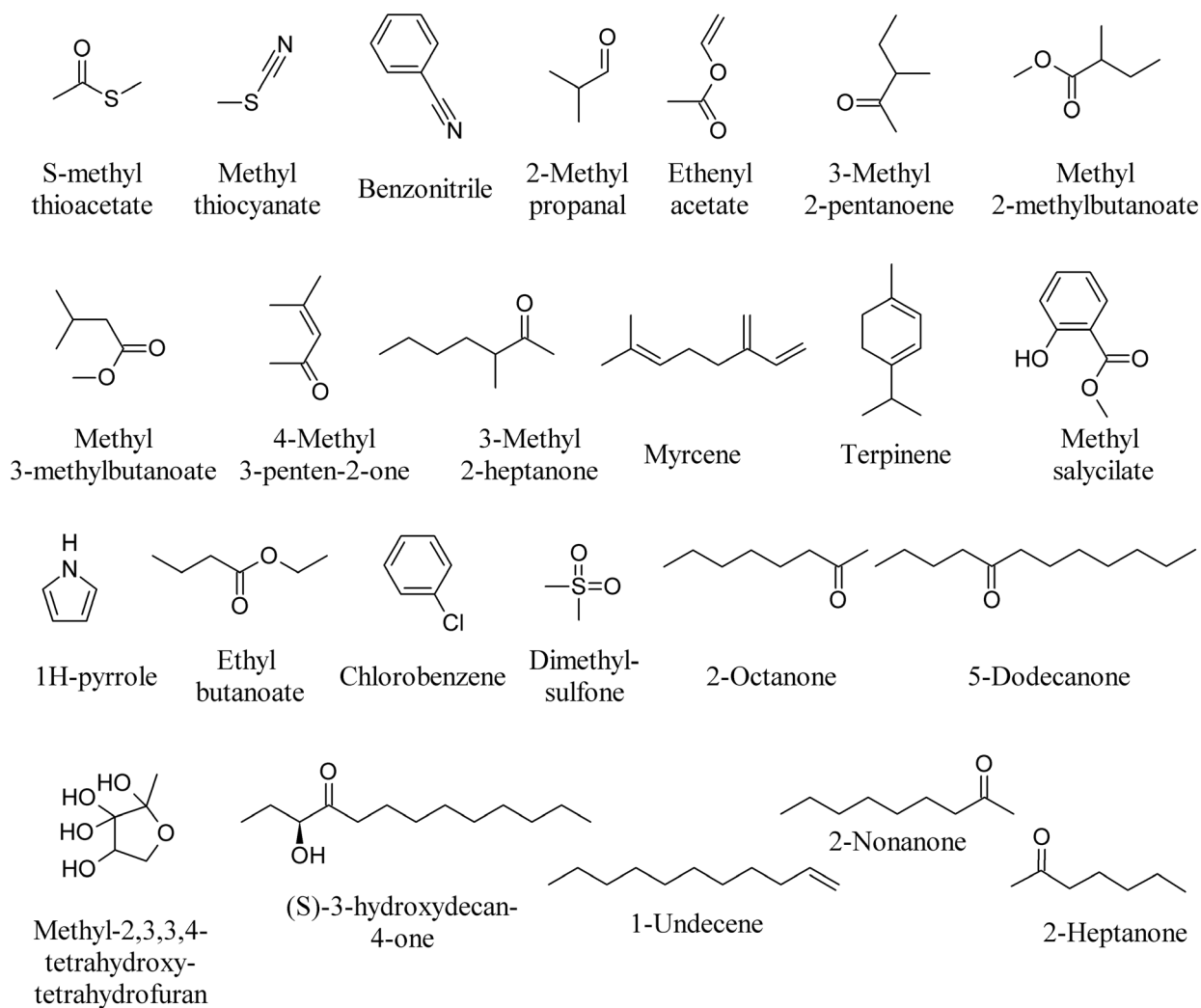**FIGURE 4 | Biotransformation products of cuparene by Vetiver endobacteria.**

FIGURE 5 | Bacterial volatiles able to induce bacterial responses.

plymuthica included among others: 2-pentadecanone (**Figure 2**), 1H-pyrrole, ethyl butanoate, chlorobenzene, dimethylsulfone, 2-octanone, and 5-dodecanone (**Figure 5**; Garbeva et al., 2014a).

It has recently been demonstrated that bacteria are able to inhibit the growth of *Burkholderia cepacia* complex (Bcc) strains through the synthesis of MVOCs (Papaleo et al., 2012, 2013; Orlandini et al., 2014). Reported data show that two *Pseudoalteromonas* strains were able to completely inhibit the growth of most Bcc strains (Romoli et al., 2011). Methyl-2,3,3,4-tetrahydroxytetrahydrofuran (**Figure 5**), indole (**Figure 3**) and its derivatives, quinolones and (*S*)-3-hydroxytridecan-4-one (**Figure 5**) as signals have also been described (Diggle et al., 2006; Ryan and Dow, 2008).

Expressions of phenotypic characteristics in Gram-negative bacteria such as bioluminescence, biofilm formation and production of virulence factors, exoenzymes, antibiotics and pigments are often regulated by a cell density-dependent cell-to-cell communication quorum-sensing (QS) network mediated by *N*-acyl homoserine lactone (AHL) signal molecules (Bassler, 2002; Waters and Bassler, 2005; Ng and Bassler, 2009). AHLs are used by Gram-negative bacteria to monitor population density, a term commonly referred to as QS (Ortiz-Castro et al., 2008). AHLs belong to a class of bacteria-produced amino compound-containing lipids. AHL signals have been described in many plant-associated bacteria, including plant growth-promoting rhizobacteria (PGPR; see below). The ability to disrupt QS networks is termed quorum quenching (QQ), and is an important mechanism of competition between bacteria (Chernin et al., 2011). An elucidation of the mechanisms governing the QQ phenomenon might help in developing new approaches to controlling plant pathogens (Rasmussen and Givskov, 2006). AHL-mediated communication between individual bacterial cells has been detected in the rhizosphere, and rhizospheric bacteria have been shown able to persist and produce MVOCs inside the plant.

Microbial volatile organic compounds produced by some *P. fluorescens* and *S. plymuthica* strains inhibited the growth of *Agrobacterium tumefaciens* and *A. vitis* strains *in vitro*. DMDS was the principal headspace volatile produced by *S. plymuthica*; it strongly suppressed *Agrobacterium* growth *in vitro* and was emitted by tomato plants treated with *S. plymuthica*. 1-Undecene (**Figure 5**) was the main volatile emitted by the *P. fluorescens* strain. It was concluded that MVOCs, and specifically DMDS, might be involved in the suppression of oncogenicity in plants (Dandurishvili et al., 2011). The main VOCs emitted by the *P. chlororaphis* strain 449 were 1-undecene, 2-nonanone, and 2-heptanone (**Figure 5**) along with and 2-undecanone (**Figure 2**) and lower amounts of DMDS. The composition of MVOCs produced by the *S. proteamaculans* 94 strain differed significantly from that emitted by *P. chlororaphis* strain 449, with DMDS being the main headspace MVOC emitted by the former (Popova et al., 2014). When these MVOCs were tested on the *A. tumefaciens* strain C58 and the cyanobacterium *Synechococcus* sp. strain PCC 7942, a strong *A. tumefaciens* bacteriostatic effect of DMDS (**25**) was confirmed and completely suppressed the growth of the cyanobacterium strain *Synechococcus* sp. 2-Nonanone and 2-heptanone (**Figure 5**) were effective on both microorganisms, whereas 2-undecanone completely inhibited the

growth of *Synechococcus*, but did not appreciably affect *A. tumefaciens*. 1-Undecene (**Figure 5**) did not significantly affect the growth of any of the two microorganisms tested (Popova et al., 2014).

MVOCs in Bacterial–Fungal Interactions

With respect to the functioning of soil microbial volatiles, most attention has been given to the suppressive effects of bacterial volatiles on soil eukaryotes that are harmful to agricultural crops; e.g., plant-pathogenic fungi (Zou et al., 2007; Verginer et al., 2010; Garbeva et al., 2014a,b). Rhizobacterial isolates comprising *S. plymuthica*, *S. odorifera*, *Stenotrophomonas maltophilia*, *Stenotrophomonas rhizophila*, *P. fluorescens*, and *P. trivialis* synthesize and emit complex blends of MVOCs that inhibit growth of many phytopathogenic and non-phytopathogenic fungi (Vespermann et al., 2007; Kai et al., 2010). The role of MVOCs in positive and antagonistic interactions between rhizobacteria and mycorrhizal fungi and their ecological significance has been described recently in an excellent review by Effmert et al. (2012).

One key antibiotic in soil is pyrrolnitrin (PRN, **Figure 6**). PRN is a chlorinated phenylpyrrol antibiotic that was first isolated from *Burkholderia pyrrocina* and was later found in other genera, such as *Pseudomonas*, *Enterobacter*, *Myxococcus*, and *Serratia* (Garbeva et al., 2004). PRN has shown broad-spectrum activity against a range of fungi belonging to the Basidiomycota, Deuteromycota, and Ascomycota, including several economically important phytopathogens such as *Rhizoctonia solani*, *Botrytis cinerea*, *Verticillium dahliae*, and *Sclerotinia sclerotiorum*. For example, PRN production by *Burkholderia cepacia* strain 5.5B was related to the suppression of stem rot of poinsettia (*Euphorbia pulcherrima*) caused by *R. solani* (Hwang et al., 2002). PRN has been used as a lead structure in the development of a new phenylpyrrol agricultural fungicide (Ligon et al., 2000).

The mycelium of *Tuber borchii*, a commercial truffle species, is used as a model system for *in vitro* ectomycorrhizal synthesis, infected seedling production and biotechnological applications (see below for truffle volatiles). Bacteria with unusual biological activities could be a major problem during large-scale production of inoculum for truffle-infected seedling. For instance, a *Staphylococcus pasteuri* strain shows notable antifungal activity against *T. borchii* mycelium due to the production of MVOCs. Interesting molecules emitted by the fungal–bacterial interaction were γ-patchoulene, known for its antifungal activity, 3-methyl butanal (**Figure 6**) and 1-octen 3-ol (**Figure 1**). Typical metabolites of the *Staphylococcus* sp. were 2-undecanone (**Figure 2**), 2-nonanone (**Figure 5**), 3-methylbutanoate, 2-methylbutan-1-ol, 4-methyl-2-heptanone, ethanethioic acid, and dimethyl trisulfide (**Figure 6**; Barbieri et al., 2005).

A strong negative influence on the mycelial growth of the soil-borne phytopathogenic fungus *R. solani* (99–80%) was observed under the test conditions by MVOCs emitted by *Stenotrophomonas maltophilia* R3089, *Serratia plymuthica* HRO-C48, *Stenotrophomonas rhizophila* P69, *Serratia odorifera* 4Rx13, *Pseudomonas trivialis* 3Re2-7, *S. plymuthica* 3Re4-18, and *Bacillus subtilis* B2g. Although many of the bacterial VOCs
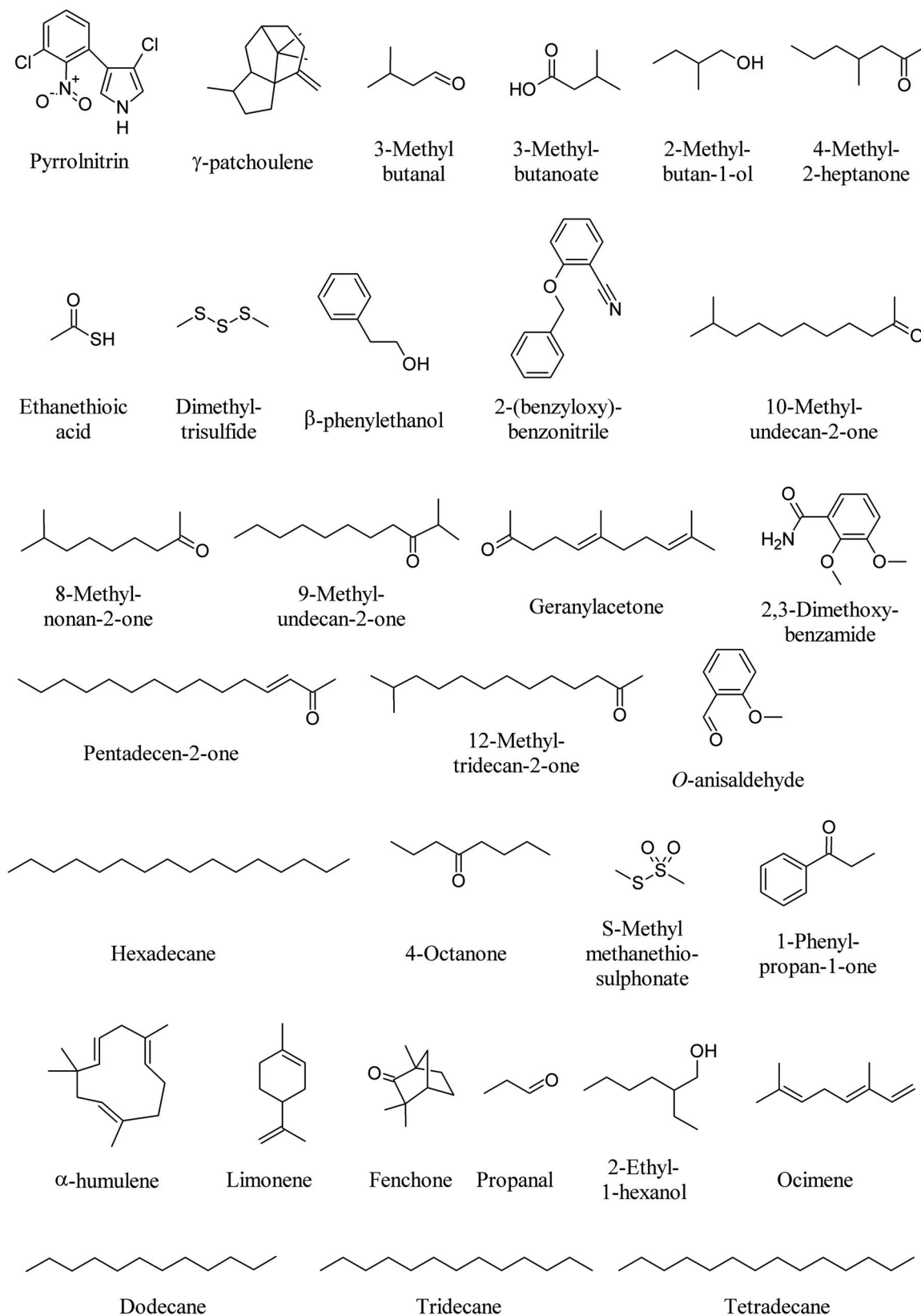
FIGURE 6 | Bacterial volatiles that interfere with fungi.

could not be identified due to no matches being found with mass-spectra of volatiles in the databases, most of them were species-specific, and overlapping MVOCs patterns were found for *Serratia* sp. and *Pseudomonas* sp. (Kai et al., 2007). For example, β-phenylethanol could be detected in *S. rhizophila*, *S. epidermidis*, *S. plymuthica*, and *S. odorifera*, while other unidentified compounds were only emitted from pseudomonads, such as 2-(benzyloxy)benzonitrile (**Figure 6**; Kai et al., 2007).

The MVOC emission profile of *Xanthomonas campestris* pv. *vesicatoria* 85-10 consists of more than 50 compounds, the majority consisting of ketones and methylketones while the dominant compound is 10-methylundecan-2-one followed by 8-methylnonan-2-one, 9-methylundecan-2-one, geranylacetone, pentadecen-2-one, and 12-methyltridecan-2-one (**Figure 6**). However, when some of these compounds were tested on the fungus *R. solani* contradictory results were found with either promoting and inhibiting effects (Weise et al., 2012).

The volatile compounds produced by *Bacillus atrophaeus* CAB-1 include a range of alcohols, phenols, amines, and alkane amides. Hexadecane, 2,3-dimethoxybenzamide and *O*-anisaldehyde (**Figure 6**), were among the most abundant MVOCs. *O*-anisaldehyde was found to exert the highest inhibition on the mycelial growth of the fungal pathogen *Botrytis cinerea* (Zhang et al., 2013).

Significant growth inhibition of two phytopathogenic fungi, *R. solani* and *Alternaria alternata*, was observed with high concentrations of DMDS (**Figure 3**), 2-undecanone (**Figure 2**), dimethyl trisulfide, 4-octanone, *S*-methyl methanethiosulphonate, and 1-phenylpropan-1-one (**Figure 6**) emitted by *Burkholderia ambifaria* (Groenhagen et al., 2013). Fifteen *Burkholderia tropica* strains significantly inhibited the mycelial growth of four plant pathogenic fungi, *Colletotrichm gloesporioides*, *Fusarium culmorum*, *F. oxysporum*, and *Sclerotum rolffsi*. The volatile profile of *B. tropica* strain MTo431 showed the presence of several MVOCs know to play an important role in the antagonistic antifungal mechanism, including α-pinene (**Figure 1**), DMDS (**Figure 3**), ocimene, limonene, and fenchone (**Figure 6**; Tenorio-Salgado et al., 2013). *Bulkholderia gladioli* pv. *agaricola* strains produced MVOCs which inhibited fungal growth and reduced the growth rate of *F. oxysporum* and *R. solani*. Limonene was the most effective compound (Elshafie et al., 2012).

Several ectosymbiotic bacterial species live in association with fungi. The fungus *F. oxysporum* MSA 35 [wild-type (WT) strain] is a nonpathogenic *Fusarium* strain, which exhibits antagonistic activity to plant pathogenic *F. oxysporum* isolates (Minerdi et al., 2009). The fungus lives in association with a consortium of ectosymbiotic bacteria including *Serratia* sp. strain DM1 and *Achromobacter* sp. strain MM1. The WT strain, when cured of the bacterial symbionts, is pathogenic to lactuce, causing wilt symptoms similar to those of pathogenic *F. oxysporum* f. sp. *lactucae* (Minerdi et al., 2011). The major MVOC of *Achromobacter* sp. strain MM1 was DMDS, whereas *Serratia* sp. strain DM1 MVOC profile was made of DMDS, propanal, 2-ethyl-1-hexanol, dodecane, tridecane, and tetradecane (**Figure 6**). These volatiles had no effect on lettuce seedling growth. However, the WT strain of *F. oxysporum* MSA 35 produced a higher amount of α-humulene

(**Figure 6**) and β-caryophyllene (**Figure 1**) with respect to cured *F. oxysporum*. β-Caryophyllene was found to be responsible for the lactuce growth promotion (Minerdi et al., 2011).

Overview of Bioactive Fungal Volatiles

Several fungi show the ability to synthesize and emit MVOCs. Grass endophytic *Epichloë* species (Clavicipitaceae, Ascomycota; Schiestl et al., 2006), rust fungi including *Puccinia monoica* and *Uromyces pisi* (Pucciniaceae, Uredinales, Basidiomycota; Kaiser, 2006), truffles (*Tuber* sp., Pezizales, Ascomycota; Splivallo et al., 2011), some soil saprophytes (*Trichoderma* sp.; Yuan et al., 2012), mushrooms (sporocarps; Fraatz and Zom, 2010), fungi isolated from humid building materials (Schleibinger et al., 2008; Araki et al., 2009), wood and diseased plants (*Ceratocystis fimbriata*, Ascomycota) have the potential to generate MVOCs. Most of fungal MVOCs also exert either potent inhibitory or stimulatory effects on plants (Hung et al., 2013).

MVOC of Fungal Endophytes

Endophytes constitute an important group of plant-associated fungal symbionts, which occur in both below-ground and above-ground tissues (Yuan et al., 2010; Waqas et al., 2014; Zhou et al., 2014). Volatile-producing endophytes (VPEs) may be of primary interest because of their production of antibiotic or pleasant MVOCs (Khan et al., 2014). From a biotechnological viewpoint, VPEs produce a broad spectrum of odorous compounds with different physicochemical and biological properties that make them useful in both industry and agriculture (Yuan et al., 2012).

Endophytic fungi of several Ascomycota lineages are capable of producing MVOCs, but members of the Xylariaceae family may be an especially rich source. *Aspergillus niger* produces 2-phenylethanol on the host plant *Rosa damascena* (Wani et al., 2010); *Botrytis* sp. BTF21 produces 2-methyl butane, β-butyrolactone, and 2-butene dinitrile on the host plant *Musa* sp. (Ting et al., 2010); *Phomopsis* sp. produces sabinene, 3-methylbutan-1-ol, 2-methylpropan-1-ol and acetone on the host plant *Odontoglossum* sp. (Singh et al., 2011); whereas *Nodulisporium* sp. produces β-elemene, α-selinene, β-selinene, and 2,5-dihydrotoluene (**Figure 7**) on the host plant *Cinnamomum loureirii* (Park et al., 2010).

Several species of *Muscodor* growing on different plant species produce different MVOCs including among others: naphthalene, tetrahydofuran, 2-methylfuran, azulene, α-phellandrene, β-phellandrene, 2-pentylfuran, aromadendrene (**Figure 7**), and β-caryophyllene (**Figure 1**) on different host plants such as *Actinidia chinensis*, *Ananas ananassoides*, *Ginkgo biloba*, and *Myristica fragrans* (Macias-Rubalcava et al., 2010; Yuan et al., 2012). The use of *Muscodor* MVOCs has been suggested as a promising option to replace methyl bromide fumigation as a means of controlling soil-borne plant diseases (Strobel, 2006). β-caryophyllene (**Figure 1**) is also emitted by the endophyte *Phialocephala fortinii* (Kramer and Abraham, 2012).

Among the least studied taxa of plant-associated fungal endophytes are the unspecialized, widespread soil-borne fungal endophytes belonging to the genus *Acremonium*. It was recently found

FIGURE 7 | Volatiles of fungal endophytes.
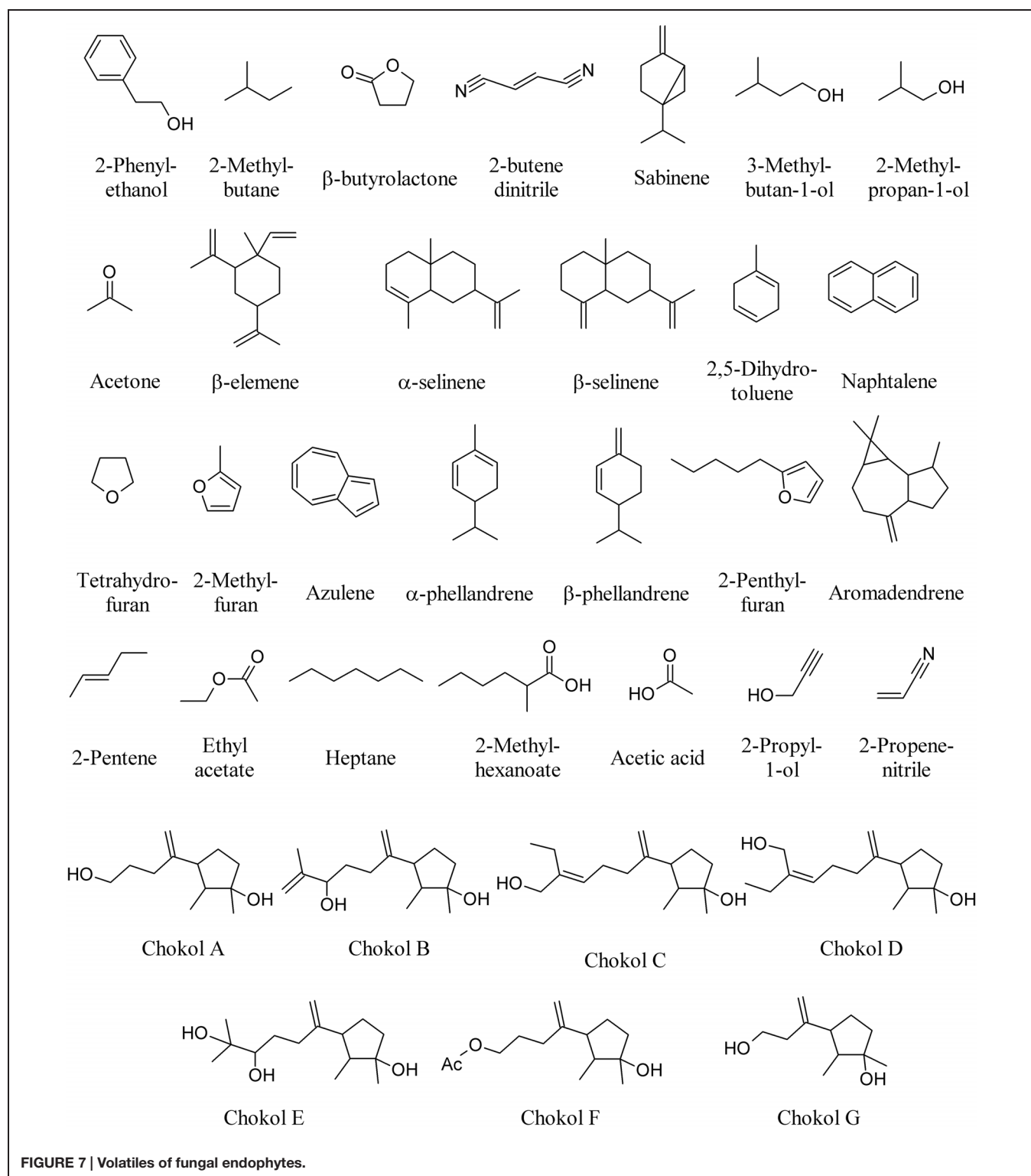
that endophyte inoculated tomato plants emitted diverse terpenes and sesquiterpenes at significantly lower amounts as compared to endophyte free-plants, demonstrating that *Acremonium strictum* is able to induce changes in volatile emissions of the host plants (Jallow et al., 2008).

The fungal endophyte NRRL 50072, isolated from *Eucryphia cordifolia* in northern Patagonia, produces a variety of medium-chain and highly branched, with a third of the short- and medium-chain compounds also produced when cultures grow on a cellulose substrate. Among these are

2-pentene, 3-methylbutan-1-ol, ethyl acetate, heptane, and 2-methylhexanoate (**Figure 7**); collectively, these and other MVOCs have been highlighted for their potential as fuel alternatives as myco-diesel (Griffin et al., 2010).

Sesquiterpenes, chokols A–G (**Figure 7**) have been isolated from *Epichloe typhina* an endophytic fungus of *Phleum pratense*, and have been found to be fungitoxic to the leaf spot disease pathogen *Cladosporium phlei* (Kumar and Kaushik, 2012). Other enophytic fungi isolated from plum (*Prunus domestica*) leaves show antagonistic activity against *Monilinia fructicola*. Here, the most frequently isolated species is *Phaeosphaeria nodorum* and four isolates produced inhibitory volatiles to *M. fructicola*. The volatiles produced by these fungi were identified as ethyl acetate, 3-methylbutan-1-ol, acetic acid, 2-propyl-1-ol, and 2-propenenitrile (**Figure 7**). The fungal volatiles inhibited growth and reduced the width of the hyphae, and caused the disintegration of the hyphal content (Pimenta et al., 2012).

Current perspectives on the volatile-producing fungal endophytes have been recently reviewed (Yuan et al., 2012).

MVOCs of Truffles

Truffles use volatile signals throughout their life cycle to regulate their interactions with other organisms. Despite this fascinating aspect, the functional role of truffle volatiles in nature has rarely been investigated. In truffles, more than 200 VOCs have been described from various truffle species in the presymbiotic mycelial stage, during the mycorrhizal stage when the fungus enters a symbiosis with plant roots, and during the reproductive stage (Splivallo et al., 2011). The volatile profiles have been studied in the three most representative species: *Tuber melanosporum*, *T. magnatum*, and *T. borchii*. 2-Octenal (**Figure 8**) seems to be specific to symbiotic fungi as it has been reported in *T. borchii*, *T. melanosporum*, and *T. indicum* as well as in other mycorrhizal fungi (Splivallo et al., 2007). DMDS, 3-Methylbutanal (**Figure 6**), 2-methylbutanal, and dimethyl sulfide have also been found in most truffles investigated to date, while 2-methyl 4,5-dihydrothiophene has only been described from fruiting bodies of *T. borchii* (**Figure 8**; Splivallo et al., 2011). Bis(methylthio)methane, is the major contributor to the aroma of the white truffle *T. magnatum*, while other volatiles include dimethyl sulfide, methyl(methylthio)methyl disulfide, benzothiazole, methanethiol, and some terpenoids including, carveol, guaiene, *p*-cymene, cumene hydroperoxide (**Figure 8**) and limonene (**Figure 6**; Gioacchini et al., 2005, 2008). 1-Octen-3-ol (**Figure 1**) is produced by both truffle mycelium and fruiting bodies (Menotta et al., 2004; Splivallo et al., 2007). Mycelial cultures of *T. borchii* were found to emit camphor (**Figure 1**), *n*-undecane, 2-ethyl-1-butanol, 2-tert-butoxyethanol, 4,5-dimethyl resorcinol, 5-hexen-3-ol, and 3-(methylthio)propanal (**Figure 8**). Submerged fermentation colures of *T. melanosporum* were found

FIGURE 8 | Major volatiles emitted by truffles.
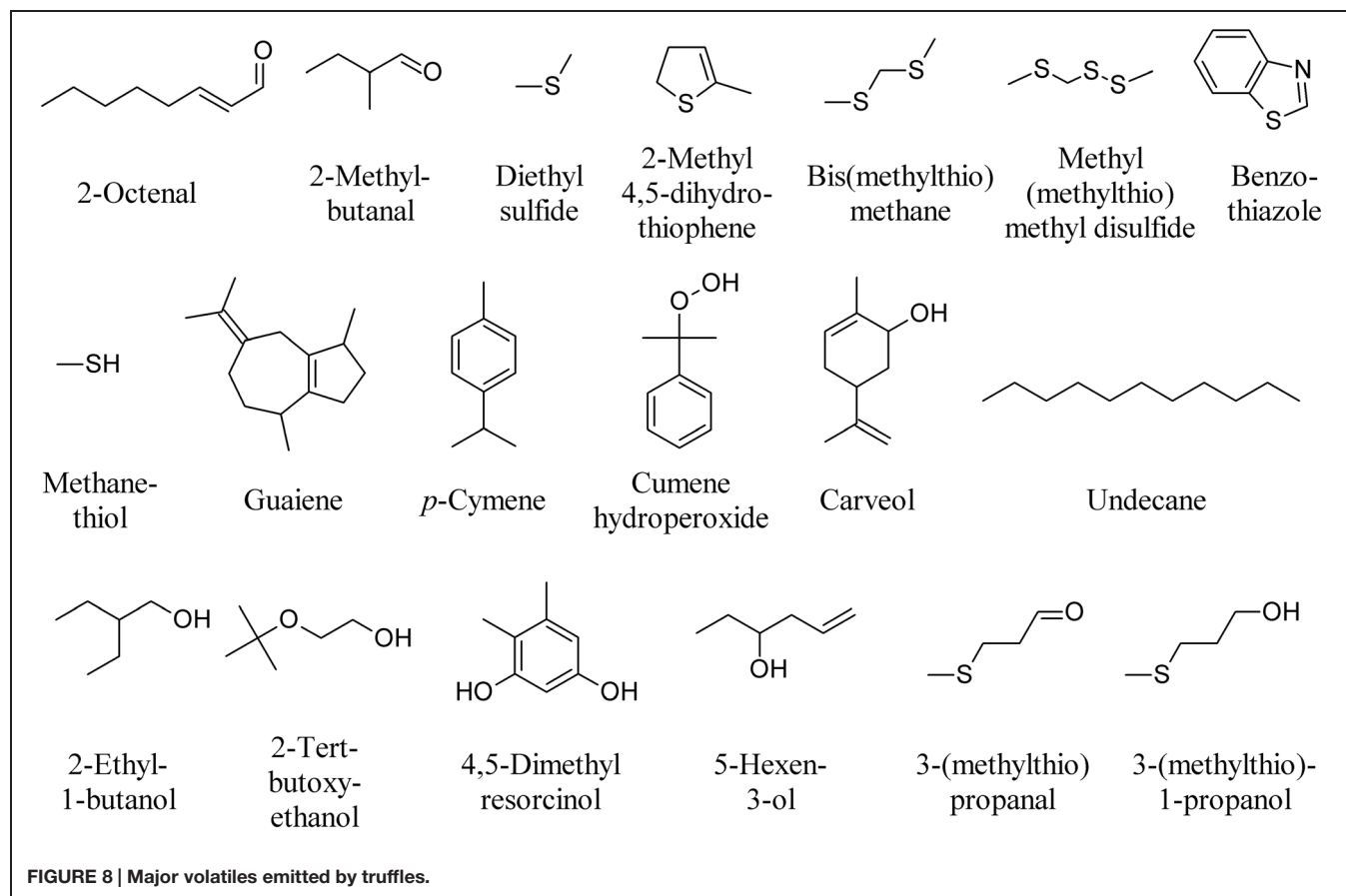
to emit dimethyl sulfide, dimethyl trisulfide (**Figure 6**), DMDS, methanethiol, 3-(methylthio)propanal, and 3-(methylthio)-1-propanol (**Figure 8**; Liu et al., 2013).

Bioactive MVOCs Emitted by Other Fungi

Volatile organic compounds produced by fungi provide an alternative diagnostic approach for the identification of fungal strains (Zhang et al., 2014). Many other MVOCs have been identified from several fungal species, most of which exerting a significant effect on plants and plant-associated organisms. *m*-Cresol and methyl benzoate (**Figure 9**) were identified as major active volatile compounds from *Ampelomyces* sp. and *Cladosporium* sp., respectively, and found to elicit *Arabidopsis* ISR against the pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (Naznin et al., 2014). 1-Octen-3-ol (**Figure 1**), 3-octanone, and 3-octanol (**Figure 9**) are emitted by the fungus *Fomes fomentarius* and found to induce contrasting behavior of the fungivorous beetle *Bolitophagus reticulatus* in olfactometer bioassays (Holighaus et al., 2014).

The pine weevil *Hylobius abietis* is a severe pest of conifer seedlings. The isolation of a fungus (*Penicillium expansum*) from feces and frass of *H. abietis* and the biological activity of its volatile metabolites styrene and 3-methylanisole showed that styrene significantly reduced male and female pine weevils' attraction to cut pieces of Scots pine twigs, whereas 3-methyl anisole (**Figure 9**) only reduced male weevil attraction to pine twigs (Azeem et al., 2013).

Microbial volatile organic compounds emitted by *F. culmorum* and *Cochliobolus sativus* significantly decreased barley (*Hordeum vulgare*) leaf surface and mean root length. Among bioactive compounds emitted [including germacrene A, longifolene, α-bisabolene, and β-acoradiene], sativene was found the most emitted compound (**Figure 9**; Fiers et al., 2013).

Microbial volatile organic compounds of *Trichoderma viride* cultured on Petri plates in a shared atmosphere with *Arabidopsis* without direct physical contact, prompted plants with taller, bigger, and earlier flowered plants with more lateral roots. The most abundant MVOCs were isobutyl alcohol (**163**), isopentyl alcohol (**164**), and farnesene (**Figure 9**), along with the presence of 3-methylbutanal and geranylacetone (**Figure 6**; Hung et al., 2013).

Microbial volatile organic compounds were collected from the headspace of four ectomycorrhizal, three pathogenic, and two saprophytic fungi. Principal component and cluster analyses revealed that fungal species differ in their odor profiles, particularly in the pattern of sesquiterpenes. Among ectomycorrhizal fungi, 3,5-dimethyl anisole was uniquely released by *Laccaria bicolor*, whereas β-caryophyllene (**Figure 1**) and 3-cyclohepten-1-one were unique to *Paxillus involutus*. Among pathogenic fungi, longipinene was the unique sesquiterpene released by *Armillaria mellea*, which along with the other pathogens (*Pholiota squarrosa* and *Verticillium longisporum*) emitted geosmin (**Figure 1**), γ-muurolene, α-bisabolene, and epizonarene (**Figure 9**).

With regards to the terpenoid emission, the saprophytic species *Stropharia rugosoannulata* had unique emissions of α-muurolene (**Figure 4**), γ-selinene, and 3-phenylpropan-1-ol

(**Figure 9**). *Trichoderma viride* was the only one releasing α-ylangene, α-bergamotene, bicyclogermacrene, zingiberene, β-sesquiphellandrene, and valencene (**Figure 9**; Müller et al., 2013). β-Caryophyllene (**Figure 1**) has been also detected in MVOCs emissions of saprophyte *F. oxysporum* (Campos et al., 2010), non-toxigenic strain of the saprophyte *Penicillium roqueforti* and the coprophyte *Coprinus cinereus* (Wihlborg et al., 2008), the pathogen *Phialophora fastigiata*, the mold *Penicillium caseifulvum*, and the saprophyte *Trichoderma pseudokoningii* (Müller et al., 2013). The production of many other fungal sesquiterpenes has been recently reviewed (Kramer and Abraham, 2012).

The potential roles of α-pinene, β-caryophyllene (**Figure 1**), tetrahydro-2,2,5,5-tetramethylfuran, dehydroaromadendrene, and sativene (**Figure 9**) produced by *Cladosporium cladosporioides* was evaluated on growth of tobacco seedlings *in vitro* when co-cultivated without physical contact (Paul and Park, 2013).

Microbial volatile organic compounds generated by *Streptomyces alboflavus* inhibit storage fungi *F. moniliforme*, *Aspergillus flavus*, *Aspergillus ochraceus*, *Aspergillus niger*, and *Penicillum citrinum in vitro*. The main MVOCs emitted by *S. alboflavus* fermentation broth were 2-methyl isoborneol (**Figure 1**), 1,4-dimethyladamantane and 1,2,3-trimethyl benzene (**Figure 9**; Wang et al., 2013).

The MVOCs produced by the yeast *Saccharomyces cerevisiae* strain CR-1 are able to inhibit the vegetative development of the fungus *Guignardia citricarpa*, causal agent of the disease citrus black spot. 3-Methylbutan-1-ol, 2-methylbutan-1-ol (**Figure 6**), 2-phenylethanol, ethyl acetate (**Figure 7**), and ethyl octanoate (**Figure 9**), which were naturally found in the atmosphere produced by the yeast, were found to considerably inhibit the mycelial development and interfered negatively with the production of the morphogenesis-related enzymes (Fialho et al., 2011).

Tea bushes entangled by rhizomorphs of *Marasmius crinisequi* are mostly devoid of leaves. This is due to the emission of a defoliation-inducing MVOC by the rhizomorphs. The MVOCs emitted by *M. crinisequi* were identified as 3-oxo-β-ionol and 2-phenyl-3,4,5,6-tetramethylpyridine (**Figure 9**; Su et al., 2011).

Microbial volatile organic compounds emitted by the yeast *Saccharomyces cerevisiae* were studied as a chemical control effectiveness of citrus black spot, caused by the fungus *G. citricarpa* at postharvest. Ethyl acetate, 2-methylbutan-1-ol (**Figure 6**), 3-methylbutan-1-ol, 2-phenylethanol (**Figure 7**), and ethyl octanoate (**Figure 9**) were the main MVOCs emitted. An artificial MVOCs mixture prepared on the basis of the composition of the above volatiles mimicked the inhibitory effects on *G. citricarpa* of the natural MVOCs released by *S. cerevisiae* (Fialho et al., 2010).

MVOCs Role in Inducing Phenotypic Plant Responses

Considerable progress is also being made in understanding the important role of MVOCs. Bacterial and/or fungal MVOCs modulate plant growth and defense, interspecies interaction between plant, bacteria, fungi, and nematodes, play a role as attractants
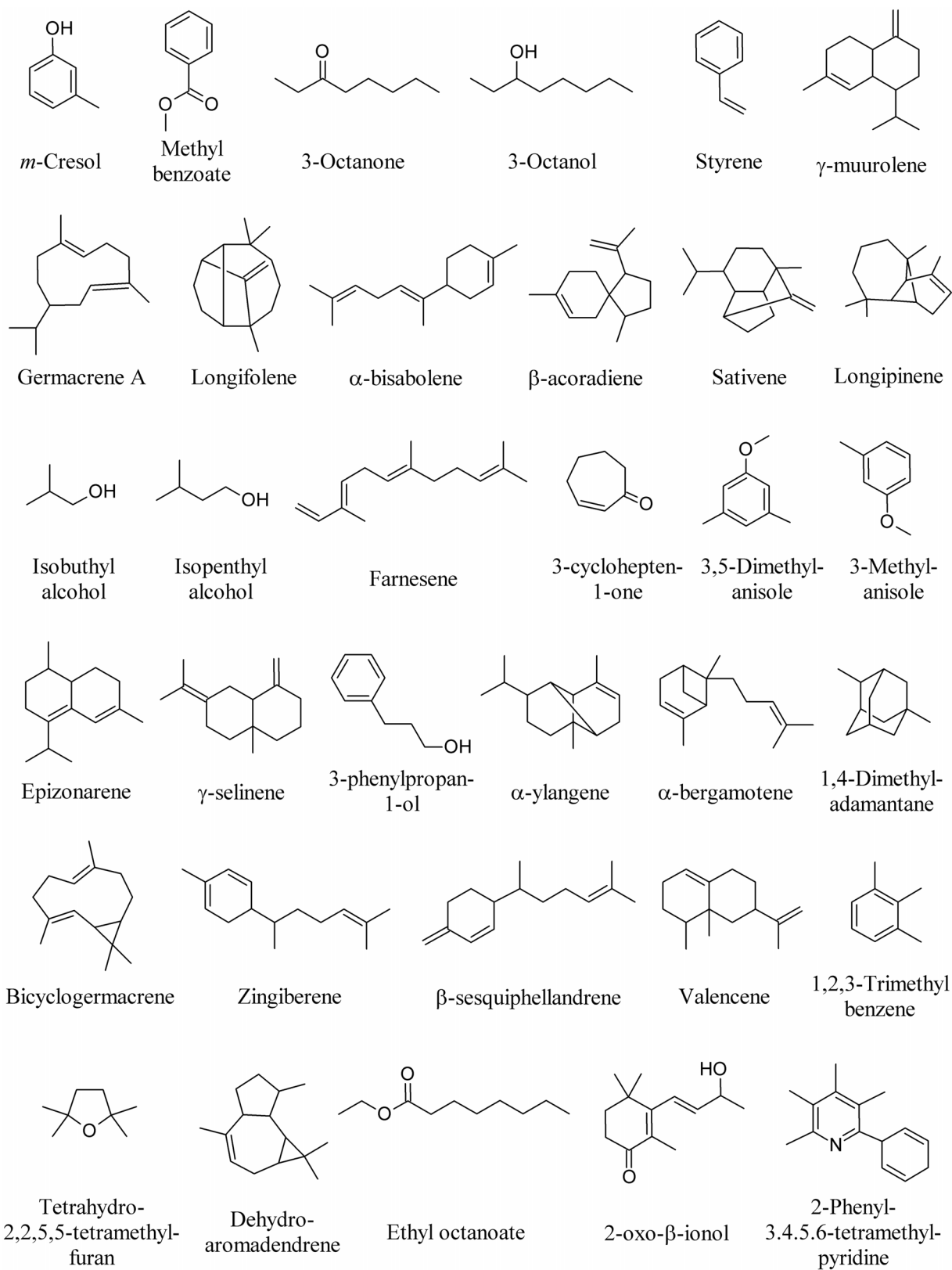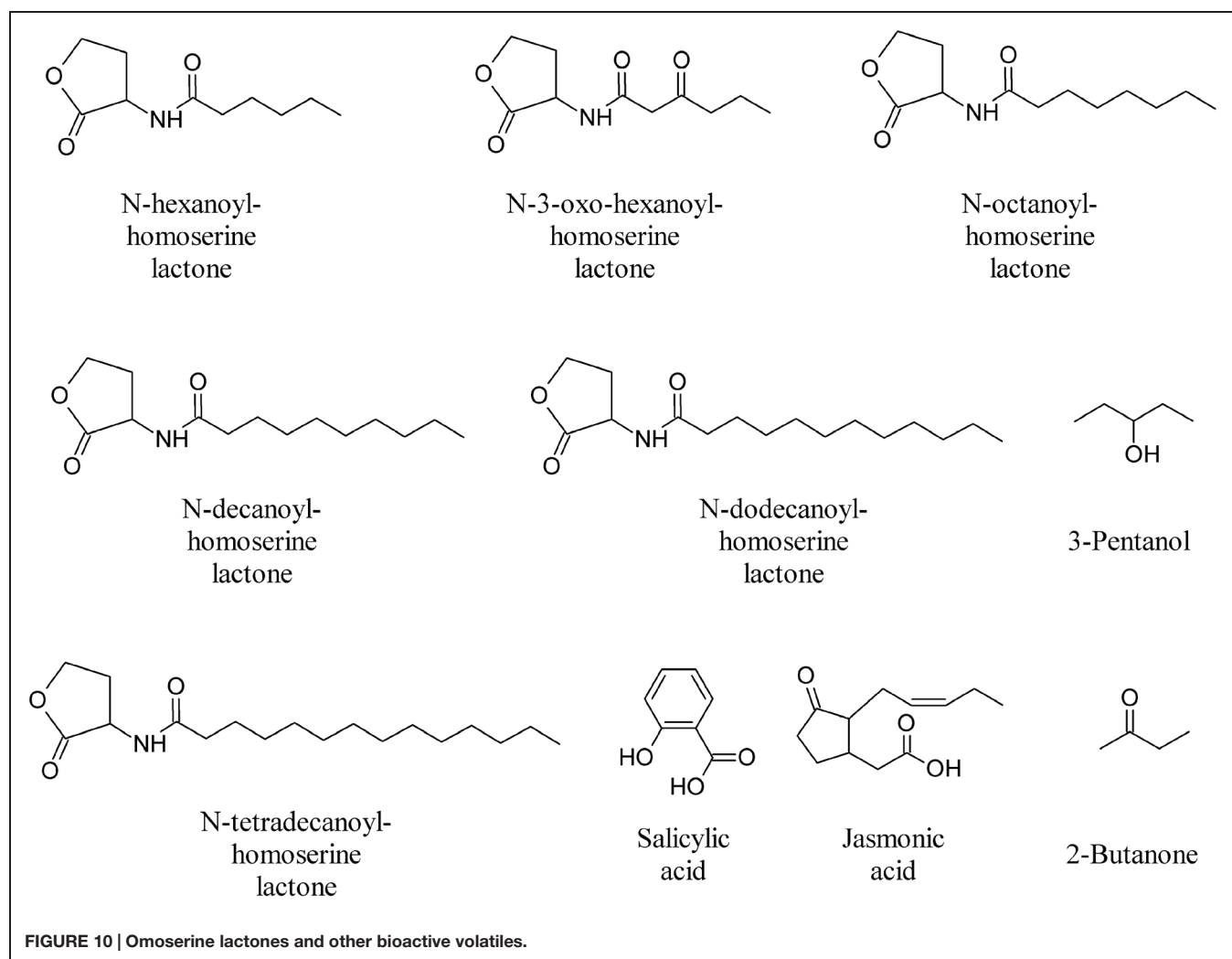
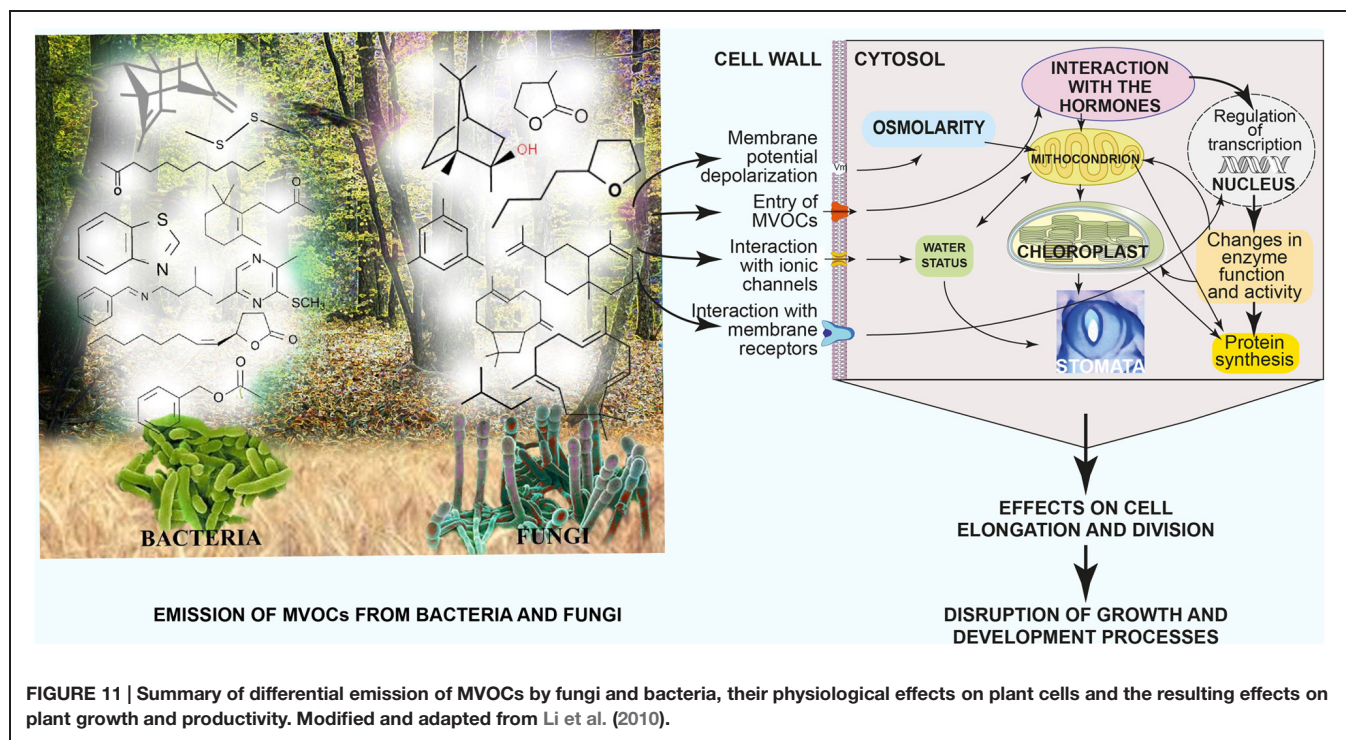**FIGURE 9 | Bioactive MVOCs produced by other fungi.**

of natural enemies, as bio-control agents and find suitable applications as pest/insect/herbivore management (Leroy et al., 2011; Davis et al., 2013; Weise et al., 2013; D'Alessandro et al., 2014). These progressive studies on MVOCs illustrate their critical roles in multitrophic interactions and their importance in both the ecosystem and sustainable agriculture systems.

During the different stages of plant development extensive communication occurs between soil microorganisms and plants in which signal molecules from the two partners play important roles. MVOCs involved in multifaceted inter and intraspecific interactions, above and below ground, result in genetic, phenotypic and morphologic alteration of the interacting organisms (Effmert et al., 2012; Piechulla and Degenhardt, 2014; Penuelas et al., 2014). Fungal and bacterial species are able to detect the plant host and initiate their colonization strategies in the rhizosphere by producing canonical plant growth regulating substance such as auxins and/or cytokinins (Ortiz-Castro et al., 2009). Additional signals from microbes play a role in plant morphogenetic processes, as discussed in relation to AHLs. These compounds enable bacterial cells to regulate gene expression depending on population density

(Ortiz-Castro et al., 2009). Very recently it was found that AHLs can be recognized by plants, alter gene expression in roots and shoots and modulate defense and cell growth responses (Ortiz-Castro et al., 2008; von Rad et al., 2008). In particular, medium (C_8–C_{14})-chained AHL compounds (N-hexanoyl-homoserine lactone, N-3-oxo-hexanoyl-homoserine lactone, N-octanoyl-homoserine lactone, N-decanoyl-homoserine lactone, N-dodecanoyl-homoserine lactone, and N-tetradecanoyl-homoserine lactone, **Figure 10**) showed a dose-dependent effect on root architecture, altering primary root growth, lateral root formation, and root hair development of *Arabidopsis* (Ortiz-Castro et al., 2008; von Rad et al., 2008).

The majority of bacteria that activate ISR appear to do so via a salicylic acid (SA)-independent pathway involving jasmonate (JA) and ethylene signals (**Figure 10**). VOCs from *Bacillus amyloliquefaciens* strain IN937a triggered ISR through an ethylene-independent signaling pathway, whereas MVOCs from *Bacillus subtilis* strain GB03 appear, however, to operate through an ethylene-dependent pathway, albeit independent of the SA or JA signaling pathways (Ryu et al., 2004). This finding provides new insight into the role of MVOCs as

FIGURE 10 | Omoserine lactones and other bioactive volatiles.

FIGURE 11 | Summary of differential emission of MVOCs by fungi and bacteria, their physiological effects on plant cells and the resulting effects on plant growth and productivity. Modified and adapted from Li et al. (2010).

initiators of defense responses in plants. Initially, in the process of developing an assay system to assess the growth promotion capacity of rhizobacteria *in vitro*, Ryu et al. (2003, 2004) found that bacterial volatiles are involved in plant growth promotion. An assessment of growth promotion induced by bacterial volatiles in *Arabidopsis* revealed that inoculation with the above mentioned GB03 or IN937a strains significantly promoted growth of *Arabidopsis*, as compared to water control or treatment with the *Escherichia coli* strain DH5α (Ryu et al., 2004). The two most abundant compounds released from cultures of strains GB03 and IN937a, albeit not from cultures of the other strains, were identified as 2,3-BD and its precursor acetoin (**Figure 3**; Ryu et al., 2003, 2004). The qualitative and quantitative compositions of volatile blends emitted by the growth-promoting strains differ significantly from those of the null growth-promoting *E. coli* strain DH5α (Ryu et al., 2003). Exogenous application of commercial acetoin and 2,3-BD result in the dose-dependent stimulation of plant growth, which simulates the effects of the volatile blend produced by the two *Bacillus* sp.

Microbial volatile organic compounds of plant growth promoting fungi (PGPF) Phoma sp. GS8-3 significantly enhanced the growth of tobacco seedlings (Naznin et al., 2014) and *Talaromyces wortmannii* FS2 MVOCs results in komatsuna (*Brassica campestris* L. var. *perviridis*) growth promotion and induced resistance against *Colletotrichum higginsianum* (Yamagiwa et al., 2011).

Priming the defense pathways with external signals enables the potentiated induction of defense response without immediately activating the defense signaling cascades that would be accompanied by energy expenditure for defense mobilization

(Pare et al., 2005). In the case of PGPR priming of plant defenses, induction of the primed state is thought to result in an increase in the amount or activity of cellular components that play important roles in defense signaling; while this process is not associated with direct changes in gene expression in leaves (Lee et al., 2012). The priming activity of 2,3-B, thus reducing plant susceptibility to disease, has been confirmed in several studies (Ryu et al., 2004; Cortes-Barco et al., 2010a,b; Park et al., 2013; D'Alessandro et al., 2014). For example, in controlled environment tests, application 2,3-BD to the soil reduced the diseased leaf area of *Agrostis stolonifera* by 20–40% for the fungal pathogens *Microdochium nivale*, *R. solani*, or *Sclerotinia homoeocarpa* compared to the water control (Cortes-Barco et al., 2010b).

In a separate study, the application of 2,3-BD failed to elicit ISR against *Pseudomonas syringae* pv. *tabaci* but did induce the ISR response against *P. carotovora* subsp. *carotovora*, suggesting that different defensive cascades are elicited in response to different pathogens. The precursor acetoin was on the other hand shown to trigger ISR against *P. syringae* in *Arabidopsis* (Rudrappa et al., 2010). Application of 3-pentanol and 2-butanone (**Figure 10**) on cucumber seedlings consistently triggered plant systemic defense responses against *P. syringae* pv. *lachrymans*. These compounds induce gene expression of plant green leaf volatile signaling pathway to attract natural enemies of pests, an indirect defense strategy that protects plants from herbivores (Scala et al., 2013). Also these compounds do not affect plant growth but increase fruit yields and resulted, unexpectedly, in a significant increase in the number of ladybird beetles, *Coccinella septempunctata*, a natural enemy of aphids (Song and Ryu, 2013).

Microbial volatile organic compoundsof *Serratia plymuthica* and *Stenotrophomonas maltophilia* significantly inhibited growth and induced H_2O_2 production in *Arabidopsis* in dual culture. Expression studies performed with different timing revealed altered transcript levels for 889 genes and 655 genes in response to *S. plymuthica* or *S. maltophilia* volatiles, respectively. Furthermore, specifically volatile-responsive genes were significantly overlapped with those affected by abiotic stress and genes responsive to both treatments were enriched for W-box motifs in their promoters and transcription factors (ERF2, ZAT10, MYB73, and WRKY18). Interestingly, the susceptibility of *wrky18* mutant lines to volatiles was significantly delayed, suggesting an indispensable role for WRKY18 in bacterial volatile responses (Wenke et al., 2012).

Volatiles released from different microbial species ranging from Gram-negative and Gram-positive bacteria to fungi exert an effect on leaf starch metabolism. Surprisingly, all microbial species tested emitted MVOCs that strongly promoted starch accumulation in leaves of both mono-and dicotyledonous plants. Starch content in leaves of plants treated for two days with MVOCs was comparable with or even higher than that of reserve organs such as potato tubers. Transcriptome and enzyme activity analyses of potato leaves exposed to volatiles emitted by *Alternaria alternata* revealed that starch over-accumulation was accompanied by up-regulation of sucrose synthase, invertase inhibitors, starch synthase (SS) class III and IV, starch branching enzyme and glucose-6-phosphate transporter. This phenomenon, which was designated as MVOC-ISAP (MVOC-induced starch accumulation process), was also accompanied by down-regulation of acid invertase, plastidial thioredoxins, starch breakdown enzymes, proteins involved in internal amino acid provision and less well defined mechanisms involving a bacterial-type stringent response (Ezquer et al., 2010). Time-course analyses of starch accumulation in *Arabidopsis* leaves exposed to fungal MVOCs emitted by *A. alternata* also revealed stimulation of starch biosynthesis during illumination. The increase of starch content in illuminated leaves of MVOCs-treated *hy1/cry1*, *hy1/cry2*, and *hy1/cry1/cry2 Arabidopsis* mutants was many-fold lower than that of WT leaves, indicating that MVOCISAP is subjected to photoreceptor-mediated control. This phenomenon was inhibited by cordycepin and accompanied by drastic changes in the *Arabidopsis* transcriptome. The use of different *Arabidopsis* knockout mutants revealed that the magnitude of the MVOCs-induced starch accumulation was low in mutants impaired in SS classes III and IV and plastidial NADP-thioredoxin reductase C (NTRC). The overall data thus showed that *Arabidopsis* MVOC-ISAP involves a photo-controlled, transcriptionally and post-translationally regulated network wherein photoreceptor-, SSIII-, SSIV-, and NTRC-mediated changes in redox status of plastidial enzymes play important roles (Li et al., 2011). The discovery that microbial volatiles trigger starch accumulation enhancement in leaves constitutes an unreported mechanism for the elucidation of plant carbohydrate metabolism by microbes (Ezquer et al., 2010; Li et al., 2011).

Table 1 summarizes the effect of microbial volatiles on plants, bacteria, and fungi.

Exploiting MVOCs for Sustainable Crop Protection and Production

Diverse and rapidly evolving pathogens and global climate changes threaten the world crop yield and food security. The increased use of synthetic pesticides and fertilizers provides immediate solutions for the plant disease and crop yield problems, respectively, but in the end, they drastically affect human and environment health. Although bio-pesticides, bio-fertilizers, and bio-control agents derived from living microbes are becoming suitable replacements for the hazardous synthetic pesticides and fertilizers, their reduced efficiency, still high costs and inconstent field performance generally relegate them to niche products (Glare et al., 2012).

Over the past decade, research on MVOCs–plant interactions has led to an increasingly conceptual understanding of the intriguingly complex and dynamic nature of MVOCs, by stressing their potential role in enhancing crop protection and productivity in a sustainable way. As discussed above, exposing plants to MVOCs results in a significant modulation of plant metabolomics, physiology, and transcriptional status, which leads to the assumption that plants have the ability to perceive and respond to MVOCs. Most of the studies have, however, been conducted under lab conditions. Only recently have a few studies been performed in open field conditions to demonstrate efficient adoption of MVOCs for a sustainable crop protection and production (Cortes-Barco et al., 2010a,b; Song and Ryu, 2013). These studies clearly demonstrate the need for implementation of MVOCs application in open field conditions and stress their multiple roles to increase pathogen resistance, protection against herbivores and in general as bio-control agents. We now have the means to begin a new era of MVOCs applications for a sustainable crop protection and production strategies as a possible substitute for synthetic and hazardous chemical pesticides and fertilizers. Effective deployment of MVOCs still, however, remains a major challenge.

Challenges for the Deployment of MVOCs under field conditions

Microbial volatile organic compounds as plant defense and growth modulators is still in its infancy. Up to now, only 10,000 microbial species described of the millions of species on Earth and only a 1000 MVOCs released by 400 bacteria and fungi have been described in the literature (Lemfack et al., 2014).

The effect of volatile compounds varies from lab to field conditions, as discussed in relation to 2,3-BD (**Figure 3**; Han et al., 2006; Cortes-Barco et al., 2010a,b; Song and Ryu, 2013). The contrasting results reported in the literature suggest that some of the MVOCs may modulate growth/defense in a species-dependent manner. For instance, 2,3-BD used at field conditions has been show to exert its effect only as a modulator of defense and none of the studies demonstrated its effect as growth modulator. Thus, before generalizing MVOCs as growth or defense modulators, it is necessary to evaluate either single MVOCs or MVOCs mixtures on different crop species both at lab and field

TABLE 1 | Summary of effects of microbial volatiles on plants, bacteria, and fungi.

Phylum	Species	Effective on	Effect	Molecule(s)	References
Bacteria	*Serratia marcescens* MG-1	Fungi and plants	Growth inhibition	N.d.	Vespermann et al. (2007)
Bacteria	*Stenotrophomonas maltophilia* R3089	Fungi and plants	Growth inhibition	N.d.	Vespermann et al. (2007)
Bacteria	*Stenotrophomonas rhizospehila* P69	Fungi and plants	Growth inhibition	N.d.	Vespermann et al. (2007)
Bacteria	*Pseudomonas aeruginosa* PAO1, PAO14, Tb, TBCF10839, and PUPa3	Plants	Growth inhibition	HCN	Blom et al. (2011)
Bacteria	*Pseudomonas trivialis* 3Re2-7	Plants	Growth inhibition	N.d.	Vespermann et al. (2007)
Bacteria	*Serratia odorifera*	Plants	Growth inhibition	Sodorifen	Vespermann et al. (2007)
Bacteria	*Serratia plymuthica* 3Re4-18, HRO-C48, IC14	Plants	Growth inhibition	N.d.	Vespermann et al. (2007), Blom et al. (2011)
Bacteria	*Pseudomonas fluorescens* A112	Plants	Growth inhibition (shoot and root)	N.d.	Åström and Gerhardson (1989)
Bacteria	Rhizosphere strains (more than 42 strains predominantly from *Burkholderia* genus)	Plants	Growth inhibition or promotion (dose dependent)	N.d.	Blom et al. (2011)
Bacteria	Rhizosphere strains (isolated from rhizosphere of lemon plants) L263, L266, L272a, L254, L265a, and L265b	Plants	Growth promoting and modulation of root architecture	Volatile mixture	Gutierrez-Luna et al. (2010)
Bacteria	*Arthobacter agilis* UMCV2	Plants	Growth promotion	*N,N*-dimethyl-hexadecanamine	Velazquez-Becerra et al. (2011)
Bacteria	*Bacillus megaterium* XTBG34	Plants	Growth promotion	2-Penthylfuran	Zou et al. (2010)
Bacteria	*Bacillus amyloliquefaciens* IN937a	Plants	Growth promotion and induced systemic resistance (ISR)	2,3-Butanediol; acetoin	Ryu et al. (2003, 2004)
Bacteria	*Bacillus subtilis* GBO3	Plants	Growth promotion and ISR	2,3-Butanediol; acetoin	Ryu et al. (2003, 2004)
Bacteria	*Pseudomonas chlororaphis* O6	Plants	Growth promotion, ISR, and drought stress tolerant	2,3-Butanediol	Han et al. (2006), Cho et al. (2008)
Fungi	*Muscodor yucatanensis*	Fungi and plants	Allelochemical effects against other endophytic fungi, phytopathogenic fungi, and plants	Mixture of volatile organic compounds (VOCs)	Macias-Rubalcava et al. (2010)
Fungi	*Muscodor albus*	Fungi and bacteria	Collectively they acted synergistically to kill a broad range of plant- and human-pathogenic fungi and bacteria	Isoamyl acetate	Strobel et al. (2011)

(Continued)

TABLE 1 | Continued

Phylum	Species	Effective on	Effect	Molecule(s)	References
Fungi	*Muscodor crispans*	Fungi and bacteria	Effective against a wide range of plant pathogens, including the fungi *Pythium ultimum*, *Phytophthora cinnamomi*, *Sclerotinia sclerotiorum*, and *Mycosphaerella fijiensis* (the black sigatoka pathogen of bananas), and the serious bacterial pathogen of citrus, *Xanthomonas axonopodis* pv. *citri*. In addition, the VOCs of *M. crispans* killed several human pathogens, including *Yersinia pestis*, *Mycobacterium tuberculosis*, and *Staphylococcus aureus*.	Mixture of volatile compounds	Mitchell et al. (2010)
Fungi	*Tuber melanosporum*, *Tuber indicum*, and *Tuber borchii* (truffles)	Plants	Growth inhibition	2-Octenal	Splivallo et al. (2007)
Fungi	*Trichoderma virens*	Fungi and plants	Growth promotion and induction of defense responses of *Arabidopsis thaliana* against *Botrytis cinerea*	β-Caryophyllene; β-elemene; germacrene D; δ-cadinene	Angel Contreras-Cornejo et al. (2014)
Fungi	Mold fungi	Plants	Induced defense and protection against *Botrytis cinerea*	1-Octen-3-ol	Kishimoto et al. (2007)
Fungi	*Fusarium oxysporum* MSA 35	Plants	Induced shoot length, root length, and fresh weight of lettuce seedlings	β-Caryophyllene	Minerdi et al. (2011)
Fungi	*Phomopsis* sp.	Fungi	Possess antifungal properties and an artificial mixture of the VOCs mimicked the antibiotic effects of this organism with the greatest bioactivity against a wide range of plant pathogenic test fungi including: *Pythium*, *Phytophthora*, *Sclerotinia*, *Rhizoctonia*, *Fusarium*, *Botrytis*, *Verticillium*, and *Colletotrichum*.	Sabinene; isoamyla alcohol; 2-methyl propanol; 2-propanone	Singh et al. (2011)
Fungi	*Trametes gibbosa*	Fungi	Serves as attractant for fungus eating beetles	1-Octen-3-ol	Thakeow et al. (2008)
Fungi	*Trametes versicolor*	Fungi	Serves as attractant for fungus eating beetles	γ-Patchoulene; δ-cadinene; isoledene; β-guaiene	Drilling and Dettner (2009)
Fungi	*Phoma* sp.	Fungi	The volatiles of *Phoma* sp. possess antifungal and fuel properties	Unique mixture of VOCs, including a series of sesquiterpenoids, some alcohols, and several reduced naphthalene derivatives.	Strobel et al. (2011)
Fungi	*Muscodor albus*	Fungi and bacteria	Volatile mixture were effectively used to control postharvest plant diseases	2-Methyl butanol; isobutyric acid	Mercier and Jimenez (2004)

N.d., not determined.

conditions. Microbes produce a plethora of MVOCs and their effect on plant physiology is immense. This implies not only the need for application of single molecules, but also the experimentation of blends of different MVOCs able to modulate growth and defense of crop plants. Therefore, if a MVOCs mixture has an effect on plant resistance to diseases, it may at the same time exert a positive effect on plant growth and development. This means we will have an abundance of options available for fine-tuning blends of MVOCs.

Another challenging aspect is the manner of application of MVOCs and their exploitation in open field conditions. Most of MOVOCs have rapid evaporation rates, which makes them difficult to use at open field conditions. As discussed above, drench application of 2,3-BD, 3-pentanol, and 2-butanone (**Figure 10**) results in consistent reproducible field trails on a few crop plants (Cortes-Barco et al., 2010a,b; Song and Ryu, 2013), but we are still lacking an appropriate, durable method of MVOCs delivery in the field. Future studies are needed to understand better cost effective and efficient delivery of MVOCs.

Another concern on MVOCs use at field condition is the 'allocation of fitness costs' or 'trade-off'; i.e., side effects of competing metabolic demands and requirement of energy and resources for the synthesis (Heil, 2001; Heil and Baldwin, 2002). MVOCs use for crop protection and productivity depend on the characterization of bioactive molecules, their proper bioactive dosage, and their role on plant growth and defense (Piechulla and Degenhardt, 2014). It should be considered that many MVOCs exert inhibitory effects; some of them are also toxic. However, after due assessing of the dose-response effect on specific crops their use can be safely managed.

Microbial volatile organic compounds can be applied at low concentration, are fully biodegradable and have no hazardous effects of the kind found in synthetic pesticides or fertilizers. In our opinion, the exploitation of MVOCs in the field as biocontrol agents in the field is only just evolving and its broad potential is only now beginning to be demonstrated (Song and Ryu, 2013). More open field studies and further physiological and molecular studies are required to show their full potential as substitutes and as potential candidates for a sustainable crop protection

and production. When the most bioactive MVOCs are identified, caged molecules or pro-bioactive compounds that are readily degradable to bioactive MVOCs can be developed in chemical forms that allow handling, storing and safe delivering to crop fields.

Concluding Remarks

Microbial volatile organic compounds form a bioactive interface between plants and a myriad of microorganisms above and below ground where most of the interactions take place. MVOCs are intriguingly complex and dynamic and understanding their ecology and evolution is the key to bioprospecting suitable tools for crop protection and production for sustainable agriculture perspective. New understanding of the importance of MVOCs for crop plants both at the lab and open field conditions will make possible to adopt and implement sustainable crop protection and to develop production strategies.

Many of the current insights on MVOCs have been carried out under lab conditions and on a limited number of microorganisms and molecules, but they have shown profound effects on growth, development, and defense system of plants. Numerous MVOCs contribute to these dynamic processes, leading to countless interactions between plants, antagonists, and mutualistic symbionts. For a better understanding of the role of MVOCs at field level, more studies should be conducted to provide further scientific evidence that can be used to assess the cost effective, ecofriendly, and sustainable use of naturally produced MVOCs for crop welfare.

Figure 11 summarizes the differential emission of MVOCs by fungi and bacteria and the physiological effects of MVOCs on plant cells and their resulting outcomes on plant growth and productivity.

Among the next forthcoming research areas we foresee: expanding the knowledge on the MVOC biodiversity (by implementing the existing data bases), exploring the holistic action of MVOC mixtures with respect to single compound effects, establishing high-throughput analyses of plant responses to MVOCs, and producing non-volatile biodegradable precursors of bioactive MVOCs for an efficient delivery to crop fields.

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