Volatile-mediated interactions in the rhizosphere

Viviane Cordovez da Cunha

Thesis committee

Promotors

Prof. Dr F.P.M. Govers
Personal Chair at the Laboratory of Phytopathology
Wageningen University

Prof. Dr J. M. Raaijmakers
Professor of Microbial Ecology
Leiden University
Head of the Department of Microbial Ecology
Netherlands Institute of Ecology, Wageningen

Co-promotor

Dr V. J. Carrion
Researcher at the Department of Microbial Ecology
Netherlands Institute of Ecology, Wageningen

Other members

Prof. Dr J.J.A. van Loon, Wageningen University
Dr K. Vrieling, Leiden University
Prof. Dr J.S. Dickschat, University of Bonn, Germany
Prof. Dr L. Eberl, University of Zürich, Switzerland

This research was conducted under the auspices of the Graduate School of Experimental Plant Sciences.

Volatile-mediated interactions in the rhizosphere

Viviane Cordovez da Cunha

Thesis
submitted in fulfillment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. Dr A.P.J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Friday 4th November 2016
at 4 p.m. in the Aula.

Viviane Cordovez da Cunha
Volatile-mediated interactions in the rhizosphere, 220 pages.

PhD thesis, Wageningen University, Wageningen, NL (2016)
With references, with summaries in English, Dutch and Portuguese

ISBN 978-94-6257-901-9
DOI: http://dx.doi.org/10.18174/388360

Table of Contents

Chapter 1

General introduction and thesis outline

Volatile organic compounds (VOCs) are carbon containing molecules of low molecular weight and high vapor pressure that can easily evaporate and diffuse through soil and atmosphere. Due to these physical-chemical properties, they can facilitate interactions between spatially separated organisms. Plants produce a wide variety of VOCs that mediate multiple interactions with their environment, ranging from the attraction of pollinators and seed dispersers (Dudareva *et al.*, 2004; Knudsen *et al.*, 2006) to plant defense and plant-plant communication (Paré & Tumlinson, 1999; Mumm & Dicke, 2010). Although VOCs produced by microorganisms have received attention only in the past decade, these compounds have been long described in literature. Zoller and Clark (1921) reported for the first time the production of VOCs by bacteria of the dysentery group. Five years later, Russian researchers reported that the 'soil air' should be recognized as an organic constituent, equally important as the soil aqueous and solid phases (Curl & Truelove, 1986). Several studies from the late 1960s and early 1970s described the presence of 'volatile inhibitors' in soil. For example, microbial volatile organic and inorganic compounds were reported as major factors in soil fungistasis, the widespread soil characteristic that prevents *via*ble fungal spores from germinating (Dobbs & Hinson, 1953). Fungistastic effects of the soil were relieved by partial sterilization and addition of antibiotics (Epstein & Lockwood, 1984), indicating that these VOC-mediated effects were of microbial origin. Hora and Baker (1972) demonstrated the production of a 'volatile inhibitor' of fungal spore germination in sterilized soils inoculated with soil actinomycetes and individual isolates of *Trichoderma*. Since then, several soil VOCs have been shown to reduce or inhibit spore germination and hyphal growth of fungi (Chuankun *et al.*, 2004; van Agtmaal *et al.*, 2015) and to influence plant growth and development (Vespermann *et al.*, 2007; Bailly & Weisskopf, 2012).

Diversity and biosynthesis of microbial volatiles

Currently, more than 1000 bacterial and fungal VOCs have been described in literature (Effmert *et al.*, 2012). Microbial VOCs typically include alkenes, alcohols, ketones, terpenes, benzenoids, pyrazines, acids, esters, and aldehydes (Piechulla & Degenhardt, 2014). Many of these compounds are produced by different soil and plant-associated bacteria and fungi (Fig. 1). Some of the microbial VOCs are common to several phylogenetic groups, while others seem to be unique for some species (Larsen & Frisvad, 1995; Schnürer *et al.*, 1999; Muller *et al.*, 2013). One of the most well-known microbial VOCs is geosmin, produced by most *Streptomyces* species, cyanobacteria and some fungi. Geosmin is a member of the terpene class of compounds and is responsible for the characteristic musty or earthy smell

of moist soils (Gerber, 1968; Jiang *et al.*, 2007). Although this compound has been identified already in 1891 and isolated in 1965, its ecological roles remain unknown to date (Berthelot & Andre, 1891; Gerber, 1968; Jiang *et al.*, 2007).

Microbial VOCs are produced *via* both primary and secondary metabolism (Korpi *et al.*, 2009). The biosynthetic pathways involved in VOC production by microorganisms are carbon metabolism, fatty acid degradation, fermentation, amino acid catabolism, terpenoid biosynthesis, and sulfur metabolism (Peñuelas *et al.*, 2014). Microbial production of aliphatic hydrocarbons is typically derived from fatty acids (Schulz & Dickschat, 2007). Decarboxylation of intermediates results in alkanes, alkenes or methyl ketones, whereas the reduction of the carboxyl group leads to the generation of aldehydes and alkanols (Peñuelas *et al.*, 2014). Eight-carbon VOCs, such as 1-octen-3-ol and 3-octanone, are products of the oxidation and cleavage of the fatty acid linoleic acid, and classified as oxylipins (Combet *et al.*, 2006). Acetoin (3-hydroxy-2-butanone) and its oxidized form 2,3-butanedione are derived from pyruvate fermentation under anaerobic conditions (Ryu *et al.*, 2003; Audrain *et al.*, 2015).

Short-chain-branched alcohols, such as 3-methyl-1-butanol and 2-methyl-1-butanol, are produced by enzymatic conversion of branched chain amino acids *via* the Ehrlich pathway (Marilley & Casey, 2004). Aromatic hydrocarbons, such as 2-phenylethanol, are generated in bacteria and fungi *via* the shikimic acid pathway, with L-phenylalanine as a precursor (Hazelwood et al., 2008; Kim et al., 2014). The bacterial production of indole, another aromatic hydrocarbon, is regulated by several environmental factors such as the availability of extracellular tryptophan, cell population density, catabolite repression, temperature and pH (Lee & Lee, 2010). The indole biosynthetic pathway is well characterized in Escherichia coli, where tryptophanase encoded by tnaA converts tryptophan into indole, pyruvate and ammonia (Newton & Snell, 1965; Pittard, 1996). Aliphatic amino acids, such as glutamate, aspartate, alanine, valine, leucine, isoleucine and methionine, serve as precursors for bacterial VOCs. Decarboxylation of these amino acids followed by deamination results in their respective 2-keto acids. After decarboxylation and in the presence of 2-keto acid decarboxylase, they are then transformed into alcohols and/or into ethyl or methyl esters (Roze et al., 2010).

Terpenes are the largest class of natural VOCs with over 50.000 known members (Conolly & Hill, 1991). They can mediate numerous antagonistic and beneficial interactions among (micro)organisms (Gershenzon & Dudareva, 2007; Dickschat *et al.*, 2014). Fungi and bacteria produce a variety of terpenoids which are transformed and rearranged to a multitude of compounds (Kramer & Abraham, 2011; Cane & Ikeda, 2012; Song *et al.*, 2015). Terpene synthases are the primary enzymes responsible for catalyzing the formation of

hemiterpenes (C5), monoterpenes (C10), sesquiterpenes (C15) or diterpenes (C20) from the substrates dimethylallyl diphosphate (DMAPP), geranyl diphosphate (GPP) farnesyl diphosphate (FPP) and geranylgeranly diphosphate (GGPP), respectively (Tholl, 2006; Dickschat *et al.*, 2014). Several terpene cyclases and synthases have been identified by mining of bacterial genomes, including those involved in the biosynthesis of 2-methylisoborneol (2-MIB) and 2-methylenebornane (Komatsu *et al.*, 2008; Chou *et al.*, 2010; Moody *et al.*, 2012; Yamada *et al.*, 2015).

Sulfur-containing VOCs range from relatively small compounds such as dimethyl sulfide, dimethyl disulphide and dimethyl trisulphide to more complex VOCs, such as 2-methyl-4,5-dihydrothiophene (Splivallo *et al.*, 2011; Effmert *et al.*, 2012). Two main biosynthetic pathways have been described for the production of sulfur-containing VOCs. The first pathway involves a one-step conversion of L-methionine to metanethiol by methionine lyases. The second one is initiated by L-methionine transamination to 4-methylthio- 2-oxobutyric acid, which is then converted to 3-(methylthio)propanal *via* decarboxylation or reduced to 4-methylthio-2-hydroxybutyric acid, with the ultimate formation of metanethiol (Bustos *et al.*, 2011; Splivallo *et al.*, 2011). Dimethyl sulfide is formed by the cleavage of dimethylsulfoniopropionate, which involves the enzymes encoded by the *dddL*, *dddP*, *dddD*, *dddQ*, *dddY* and *dddW* genes (Todd *et al.*, 2007; Curson *et al.*, 2008; Todd *et al.*, 2009; Curson *et al.*, 2011; Todd *et al.*, 2011; Peng *et al.*, 2012; Todd *et al.*, 2012)

Soil microorganisms also produce phytohormones, including the volatile ethylene. Three different pathways have been proposed for the production of ethylene by microorganisms. The first pathway uses methionine as a precursor, similarly to the ethylene production by plants, but it is rare in microorganisms. The second pathway occurs in several microorganisms and has been described for different soil fungi (Ladygina *et al.*, 2006). In this pathway, methionine is converted to 2-keto-4-methylthiobutyric acid, which is then oxidized to ethylene in the presence of hydroxyl radicals. The third known pathway described for fungi and bacteria uses 2-oxoglutaric acid as a precursor and the 'ethylene-forming enzyme' (Eckert *et al.*, 2014).

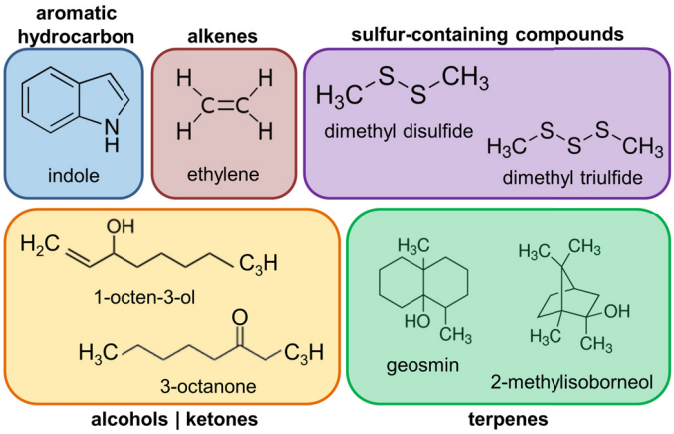


Fig. 1 | Chemical structure of common volatile organic compounds (VOCs) produced by several soil and plant-associated bacteria and fungi.

Volatile-mediated microbe-microbe interactions

Several bacterial VOCs stimulate or inhibit spore germination and mycelial growth of several fungi, including plant pathogenic fungi (Herrington *et al.*, 1987; Vespermann *et al.*, 2007; Kai *et al.*, 2009; Garbeva *et al.*, 2011; Garbeva *et al.*, 2014). Bacterial VOCs can also alter fungal morphology, enzyme activity and gene expression (Mackie & Wheatley, 1999). More recently, bacterial VOCs have been reported to attract swarming bacteria and to induce changes in bacterial motility and antibiotic resistance (Kim *et al.*, 2013; Hagai *et al.*, 2014). Among the bacterial species, *Pseudomonas* and *Bacillus* are the most frequently investigated species to date, but also VOCs from *Bulkholderia*, *Streptomyces* and *Serratia* species have been recently reported for their antimicrobial effects. For example, dimethyl disulfide, dimethyl trisulfide, benzothiazole, benzaldehyde, 2-undecanone and 2-nonannone have been described to adversely affect fungal growth (Weisskopf, 2013).

Compared to bacterial VOCs, fungal VOCs have been studied to a lesser extent. The compound 1-octen-3-ol, the most well-known fungal VOC and also referred as 'the mushroom smell', is produced by a wide range of filamentous fungi (Miyamoto *et al.*, 2014). This compound has been suggested to function as a developmental signal among fungi. In *Penicillium paneum*, 1-octen-3-ol was described as a self-inhibitor signal in spore germination at high densities (Chitarra *et al.*, 2004). In the biocontrol fungus *Trichoderma atroviride*, this compound acted as inducer of conidiation at concentrations from 0.1 µM to 100 µM, but as an inhibitor at 500 µM (Nemcovic *et al.*, 2008). Fungal VOCs have also been described to inhibit the growth of several plant and human pathogenic fungi (Strobel *et al.*,

2001) as well as to change swimming and swarming motility of bacteria (Schmidt *et al.*, 2015). For example, the fungal compounds heptanal, octanal and 2-methyl-1-butanol inhibited fungal growth, whereas the fungal terpenoids 3-carene and alpha-terpinene inhibited bacterial swarming motility (Wheatley *et al.*, 1996; Schmidt *et al.*, 2015). Fungal VOCs not only affect growth rate and motility of other microorganisms, but also change enzyme production in fungi. For example, VOCs emitted by *T. aureoviride* and *T. viride* affected synthesis of two proteins by the wood decay fungus *Serpula lacrymans* (Humphris *et al.*, 2002); interestingly, the protein synthesis was resumed after removal of the antagonistic microorganisms. Studies by Hynes *et al.* (2007) further showed that during the interaction of two wood decay fungi, *Hypholoma fasciculare* and *Resinicium bicolor*, the VOCs profile changed substantially, resulting in the production of VOCs that were not detected when these fungi were grown individually. These results suggest that fungi, and most likely also other microorganisms, may exhibit completely different activities in an ecological, multispecies context.

Volatile-mediated microbe-plant interactions

Although the production of VOCs by soil microorganisms has been revealed for many decades, the effects of microbial VOCs on plants have only been described in the past decade. Ryu *et al*. (2003, 2004) reported for the first time that bacterial VOCs can promote plant growth and elicit induced systemic resistance (ISR). The compound 2,3-butanediol, produced by *Bacillus subtilis* GB03, increased leaf area and disease resistance of *Arabidopsis* seedlings. Two widespread bacterial VOCs, indole and dimethyl disulfide, also showed plant growth-promoting effects (Bailly *et al.*, 2014). Tobacco exposure to dimethyl disulfide stimulated lateral root and root hair formation when seedlings were grown in a sulfate-deficient medium (Meldau *et al.*, 2013). Besides these positive effects, negative effects on plant growth caused by bacterial VOCs were also observed. For example, VOCs emitted by different *Serratia* species showed lethal effects on *Arabidopsis* seedlings (Vespermann *et al.*, 2007). Later studies showed that the deleterious effects on plant growth correlated with high concentrations of the VOC hydrogen cyanide (HCN) produced by some *Pseudomonas* and *Chromobacterium* species (Blom *et al.*, 2011).

Also fungal VOCs affect plant growth and development. VOCs emitted by the beneficial fungus *T. viride* and *Cladosporium cladosporioides* enhanced the growth of *Arabidopsis* and tobacco plants, respectively (Hung et al. 2013; Paul and Park 2013). Plants exposed to the fungal compound 1-octen-3-ol were less susceptible to leaf infection by the fungal pathogen *Botrytis cinerea* (Kishimoto et al. 2007). The same study further showed

that plant genes involved in ethylene and jasmonic acid pathways were up-regulated upon exposure to 1-octen-3-ol. At low concentrations, the fungal compound 1-hexanol exhibited plant growth-promoting effects, but at a higher concentration adverse effects on plant growth were observed (Spivallo et al. 2007; Blom et al. 2011). Another fungal VOC that displayed inhibitory effects on plant growth is 2-ethyl hexanal, which affected seed germination and growth of *Arabidopsis* (Hung et al. 2014).

While the effects of microbial VOCs on plant growth and development have become clear in the past decade, the VOC or VOC-blends responsible for these effects have only been identified in a few cases. Furthermore, the underlying mechanisms of VOC perception and signal transduction are largely unknown. Modulation of plant hormone pathways has been suggested as a possible mechanism of plant growth promotion mediated by microbial VOCs. Production of the volatile phytohormones indole-3-acetic and ethylene, which have a direct effect on plant growth and development, has been described for several soil and plant-associated microorganisms, including plant pathogens (Ilag & Curtis, 1968; Considine et al., 1977; Xu, 2013). More recently, indole has been described as an inter-kingdom signal that impacts plant development. Audrain *et al.* (2015) elegantly demonstrated that indole emitted by several soil bacteria promoted root development by modulating auxin-signaling in *Arabidopsis*.

Other studies suggested a central role for auxin-signaling in plant growth promotion mediated by microbial VOCs. For example, Zhang *et al.* (2007) found that VOCs emitted by *Bacillus* GB03 promoted growth of *Arabidopsis* by regulating auxin homeostasis. VOC–exposed plants showed up-regulation of genes involved in auxin biosynthesis in aerial tissues as well as of genes associated with cell wall remodeling and cell expansion. More recently, several strains of *Fusarium* were shown to promote the growth of *Arabidopsis* and tobacco plants by affecting auxin transport and signaling (Bitas *et al.*, 2015). Other hormone pathways have also been described to be affected by microbial VOCs. *Arabidopsis* genes involved in the abscisic acid (ABA) synthesis and ABA-responsive genes were down-regulated upon exposure to VOCs from *Bacillus* (Zhang *et al.*, 2007). In a recent study, VOCs from the fungal pathogen *Alternaria alternata* promoted growth and flowering by enhancing photosynthesis and accumulation of cytokinins and sugars of *Arabidopsis* plants (Sanchez-Lopez *et al.*, 2016). *Arabidopsis* genes involved in the biosynthesis of ethylene and in ethylene response have also been reported to respond to *Bacillus* VOCs at the transcriptional level; however ethylene emission by the bacteria could not be detected (Ryu *et al.*, 2003; Kwon *et al.*, 2010).

Currently, very few studies have described the mechanisms underlying the perception of microbial VOCs by plants. Evidence for the involvement of cytokinins was

provided by Ryu *et al.* (2003) who showed that *Arabidopsis* mutants deficient in the cytokinin receptor gene, *cre1*, were insensitive to VOCs from *Bacillus subtilis* GB03. Also, studies with the ethylene-insensitive *Arabidopsis* mutant *ein2* showed that plant growth is still promoted when plants were exposed to the VOCs from *B. subtilis* GB03 and the fungus *Piriformospora indica*, but not when exposed to VOCs from *Bacillus amyloliquefaciens* IN937a (Ryu *et al.*, 2003; Camehl *et al.*, 2010). These findings suggest that the perception of microbial VOCs by plants is dependent on the bacterial species.

Microbial VOCs can also function as a direct source of nutrition for plants. *Nicotiana attenuata* plants grown under sulfate deficiency displayed increased growth when exposed to the VOCs from *Bacillus* sp. B55. Among the VOCs, the sulfur-containing compounds, dimethyl disulfide was reported to be produced by *Bacillus* and incorporated into seedlings proteins (Meldau *et al.*, 2013). Sulfur-containing VOCs emitted by bacteria likely promote plant growth by increasing availability of reduced sulfur, which can be incorporated into proteins for plants growing in sulfur-deficient soils. Furthermore, the uptake of reduced sulfur allows plants to reduce the assimilation of sulfate, an energetic costly process, and invest in other physiological processes such as growth and reproduction. In another study, VOCs from *B. amyloliquefaciens* GB03 induced sulfur assimilation and accumulation in *Arabidopsis* plants, which resulted in increased levels of sulfur-rich aliphatic and indolic glucosinolates. Exposed plants further showed increased levels of glucosinolates and resistance against insect herbivory (Aziz *et al.*, 2016).

Collectively these studies highlight the diverse effects of microbial VOCs on plant growth, development and resistance by interfering with the plant's hormone signaling pathways and the plant's sulfur metabolism. Nonetheless, the underlying mechanisms of perception and signal transduction have yet to be elucidated.

Volatiles in soil ecosystems

Soils are complex and dynamic environments and host diverse microbial communities (Schloss & Handelsman, 2006). The rhizosphere, the area surrounding and influenced by plant roots, is inhabited by a dynamic population of microorganisms and is the 'playground' for numerous interactions between soil microorganisms and between microorganisms and plants (Raaijmakers *et al.*, 2009). These interactions and, in particular long-distance interactions, can be facilitated by VOCs as their physical-chemical properties allow the dispersion through the soil matrix (Moldrup *et al.*, 2000; Wheatley, 2008).

Microbial production of VOCs in soil is influenced by the community composition of soil microorganisms, but also by temperature and nutrient, water and oxygen

availability as well as the physiological state of the microorganisms (Asensio *et al.*, 2007; Insam & Seewald, 2010). Under micro-aerobic and anaerobic conditions, the diversity and amount of VOCs emitted increase due to fermentation processes (Moore-Landecker & Stotzky, 1973; Insam & Seewald, 2010). The quality of the substrate also impacts the composition of VOCs. For example, the soil pH influences the nutrient availability for microorganisms, which in turn affects their physiological state and the production of VOCs (Stotzky & Schenck, 1976).

Microbial VOCs have been proposed to play a role in disease suppressiveness of soils. Disease-suppressive soils are characterized by a low level of disease incidence despite the presence of a virulent pathogen and a susceptible host plant (Mazzola, 2002). These soils have been recognized for over 100 years, but the molecular mechanisms underlying specific disease suppression are still largely unknown. Different mechanisms have been proposed to contribute to soil suppressiveness, such as competition, antibiosis, allelopathy, hyperparasitism, and induction of plant disease resistance (Mazzola, 2002; Raaijmakers *et al.*, 2009). Several of these mechanisms have been reported to be mediated by microbial VOCs. In fact, a connection between soil fungistasis and suppressiveness of soil-borne plant diseases has been long proposed (Lockwood, 1977). The VOCs trimethylamine, 3-methyl-2-pentanone, dimethyl disulfide, methyl pyrazine, 2,5-dimethyl-pyrazine, benzaldehyde, N,N-dimethyloctylamine and nonadecane were detected in various fungistatic soils and some of these compounds inhibited the growth of different fungi (Chuankun *et al.*, 2004). Garbeva *et al.* (2011) highlighted the importance of activities of specific members of the microbial community in soil fungistasis. Also a more recent study showed that VOCs naturally emitted by soils suppress hyphal growth of the plant pathogenic oomycete *Pythium*. Remarkably, upon exposure to drastic stress, such as anaerobic disinfestation, these soils temporarily lost suppressiveness to the pathogens. Loss of suppressiveness was accompanied by dramatic shifts in soil microbial diversity and community composition (van Agtmaal *et al.*, 2015).

Conclusions

Current advances in chemical ecology have emphasized the importance of microbial VOCs in natural ecosystems as long distance signaling molecules in microbe-microbe and microbe-plant interactions. Despite the increasing body of evidence for the role of VOCs in microbe-microbe and microbe-plant interactions, the ecological roles of these compounds in soil and plant-associated environments, such as the rhizosphere, remain largely unknown. New methods for detection of VOCs in soil and on plant surfaces as well as

characterization of VOC blends involved in plant growth promotion and biological control of plant pests and diseases will contribute to better understand the behavior and functions of VOCs in complex ecosystems. Furthermore, transcriptome and proteome analyses will shed light on the underlying molecular mechanisms of VOC perception and signal transduction.

OUTSTANDING QUESTIONS

What are the ecological roles of microbial VOCs?
How do plants and microorganisms perceive VOCs?
Is plant perception of microbial VOCs tissue- or organ specific?
Can pathogens suppress plant immune system *via* the production of VOCs?
Can plants distinguish between VOCs from pathogenic and beneficial soil microorganisms?
What is the distribution of VOCs in soil?
What VOCs are responsible for the plant growth-promoting and antimicrobial effects?
How can microbial VOCs be used for sustainable agriculture?

Thesis outline

The research presented in this thesis aimed at elucidating how beneficial and pathogenic rhizosphere microorganisms modulate plant growth, development and resistance *via* the production of VOCs. To investigate the mechanisms underlying VOC-mediated interactions between plants and soil microorganisms, *in vitro* and *in vivo* bioassays were integrated with different 'omic' approaches including volatomics, transcriptomics and genomics (Fig. 2).

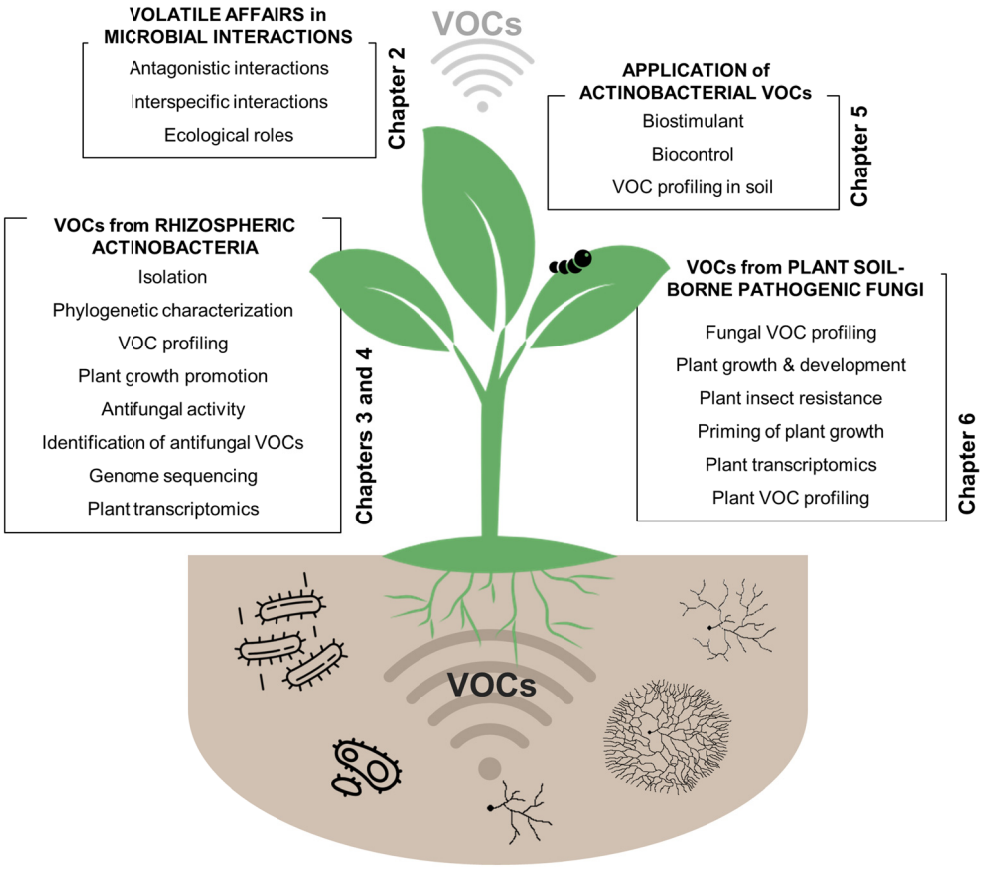


Fig. 2 | Schematic overview of the chapters presented in this thesis.

An increasing number of studies have described the different effects of microbial VOCs. To date, however, the ecological roles remain largely unknown. Chapter 2 reviews the current knowledge of the ecological roles of VOCs in microbial communities. This chapter describes how microorganisms can use VOCs for communication and competitiveness in long-distance relationships. Furthermore, it highlights the current challenges in studying microbial VOCs as well as their future applications.

Previous metagenomic studies revealed that Actinobacteria was the most abundant phylum in a soil suppressive to the soil-borne root pathogen *Rhizoctonia solani* (Mendes et al., 2011). Actinobacteria consist of Gram-positive and filamentous bacteria and can be found in diverse environments such as soils, rhizosphere, and marine ecosystems. Several plant-associated Actinobacteria promote plant growth and protect plant roots against infection by root pathogens. To investigate the effects of VOCs emitted by Actinobacteria on plant growth and plant protection against fungal infections, Actinobacteria were isolated from the rhizosphere of sugar beet plants grown in soil that is naturally suppressive to *R. solani*. Chapters 3 & 4 address the roles of VOCs of two genera of Actinobacteria, *Streptomyces* and *Microbacterium*. Chapter 3 also highlights the use of *Streptomyces* VOC profiling as a complementary tool for phylogenetic delineation of closely related strains. Chapter 4 focuses on VOC-mediated effects of *Microbacterium* species on the growth and resistance of plants. Specific attention is given to transcriptional changes in the plants upon exposure to the bacterial VOCs in order to better understand how plants perceive and respond to microbial VOCs. This chapter further combines genome and VOC profiling analysis of *Microbacterium* species to identify VOCs responsible for the plant growth-promoting effects.

Chapter 5 addresses the potential of VOCs emitted by *Microbacterium* as biostimulants and biocontrol of crop plants. Different experiments were designed to test how the plant growth-promoting effects by *Microbacterium* VOCs observed in *in vitro* experiments with *Arabidopsis* could be translated to crop species of economic importance such as lettuce and tomato. To get insight into the perception of microbial VOCs by plants, shoot and root tissues were exposed to VOCs from *Microbacterium*. Furthermore, Gas Chromatography-Quadrupole Time of Flight-Mass Spectrometry (GC-QTOF-MS) was used to investigate the production of VOCs in soils.

The plant growth-promoting effects seem to be a wide-spread phenomenon upon plant exposure to VOCs emitted by different soil bacteria and fungi. It is not clear, however, if plants can distinguish between VOCs from beneficial or plant pathogenic microorganisms. Chapter 6 investigates how a soil-borne pathogenic fungus modulates the growth, development and resistance of plants *via* the production of VOCs. *Arabidopsis*

thaliana seeds and seedlings were exposed to VOCs from the fungal pathogen *R. solani* to determine the effects on seed germination, plant growth, plant VOC emission and plant resistance to insects. To further understand the underlying mechanisms of VOC-mediated interactions between plants and the soil-borne fungal pathogen, VOCs produced by *R. solani* were profiled and a genome-wide transcriptome analysis of *A. thaliana* seedlings was performed. By combining these different approaches, this chapter further aimed at investigating if and how VOCs from a pathogenic fungus modulate the trade-off between plant growth and resistance.

Chapter 7 integrates the findings of this thesis and further discusses the role of microbial VOCs in growth and development of plants. Here, I discuss both the plants' and the microorganisms' perspectives and present a future outlook on the potential applications of microbial VOCs.

Chapter 2

Volatile affairs in microbial interactions

Ruth Schmidt[*], Viviane Cordovez[*], Wietse de Boer,
Jos Raaijmakers, Paolina Garbeva

The ISME Journal (2015) 9: 2329-2335 | doi:10.1038/ismej.2015.42

[*]*These authors contributed equally*

Abstract

Microorganisms are important factors in shaping our environment. One key characteristic that has been neglected for a long time is the ability of microorganisms to release chemically diverse volatile organic compounds (VOCs). At present, it is clear that the blend of VOCs released by microorganisms can be very complex and often includes many unknown compounds for which the chemical structures remain to be elucidated. The biggest challenge now is to unravel the biological and ecological functions of these microbial VOCs. There is increasing evidence that microbial VOCs can act as infochemicals in interactions among microbes and between microbes and their eukaryotic hosts. Here, we review and discuss recent advances in understanding the natural roles of VOCs in microbe-microbe interactions. Specific emphasis will be given to the antimicrobial activities of microbial VOCs and their effects on bacterial quorum sensing, motility, gene expression and antibiotic resistance.

Introduction

Microorganisms from diverse ecosystems produce a wide range of volatile organic compounds (VOCs). Compared with other secondary metabolites (for example, enzymes, antibiotics and toxins), VOCs are typically small compounds (up to C20) with low molecular mass (100-500 Daltons), high vapor pressure, low boiling point and a lipophilic moiety. These properties facilitate evaporation and diffusion through both water- and gas-filled pores in soil and rhizosphere environments. Hence, microbial VOCs have important roles in marine and terrestrial environments (Schulz *et al.*, 2010; Romoli *et al.*, 2014). To date, the chemical structure of ~ 1000 VOCs have been described originating from a wide range of bacterial and fungal genera and species (Effmert *et al.*, 2012; Lemfack *et al.*, 2014). Bacterial VOCs are typically dominated by alkenes, alcohols, ketones, terpenes, benzenoids, pyrazines, acids and esters, whereas fungal VOCs are dominated by alcohols, benzenoids, aldehydes, alkenes, acids, esters and ketones (Piechulla & Degenhardt, 2014). Most microbial VOCs are considered as side-products of primary and secondary metabolism. They are formed mainly by oxidation of glucose from various intermediates (Korpi *et al.*, 2009). The underlying biosynthetic pathways are aerobic, heterotrophic carbon metabolism, fermentation, amino acid catabolism, terpenoid biosynthesis, fatty acid degradation and sulfur reduction (Peñuelas *et al.*, 2014). The main metabolic pathways for microbial VOCs are summarized in Fig. 1.

Although there are common VOCs produced by different, often unrelated, microorganisms, other VOCs are unique for certain strains (Schulz & Dickschat, 2007; Garbeva *et al.*, 2014b; Garbeva *et al.*, 2014a). The amount and composition of VOCs produced by microorganisms can vary according to culturing conditions (Claeson *et al.*, 2007; Blom *et al.*, 2011; Garbeva *et al.*, 2014b; Garbeva *et al.*, 2014a). Other important factors influencing the production of VOCs are the physiological state of the producing microorganism, oxygen availability, moisture, temperature and pH (Insam & Seewald, 2010; Romoli *et al.*, 2014).

The importance of microbial VOCs for the ecology of microorganisms has been overlooked for a long time, probably due the lack of appropriate detection techniques. However, in the last 10 years the number of studies on microbial VOCs has increased substantially in different research areas such as food, medical, agricultural and environmental sciences. In this review, we focus on the ecological role of VOCs in microbe-microbe interactions. For more information on techniques used for VOC analyses and their role in microbe interactions with their eukaryotic hosts, we refer to several recent reviews (Effmert *et al.*, 2012; Farag *et al.*, 2013; Junker & Tholl, 2013; Peñuelas *et al.*, 2014).

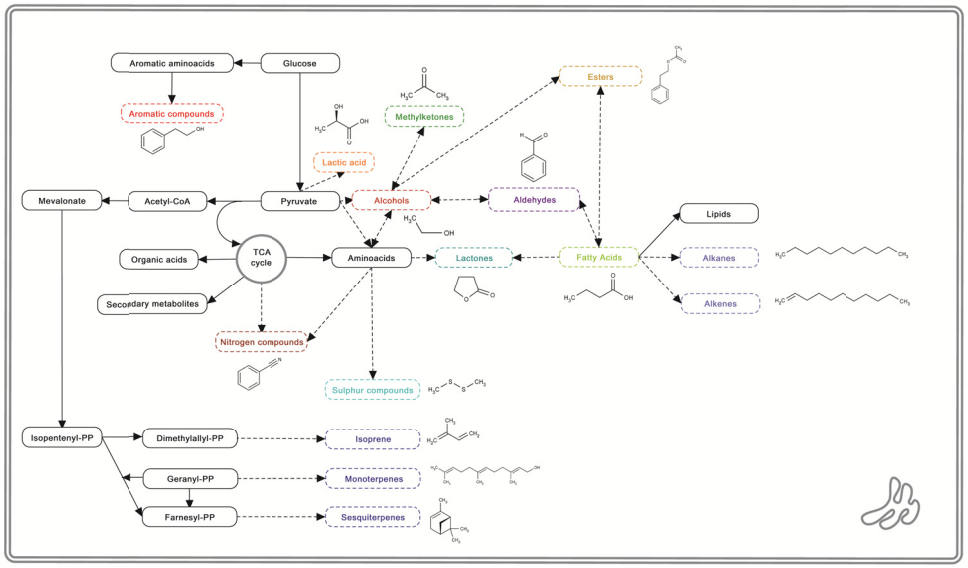


Fig. 1 | Main metabolic pathways for the production of microbial volatile organic compounds (VOCs). VOCs are depicted in colored dashed rectangles indicating different chemical classes. Representative examples are given per class: alcohols (for example, ethanol), aldehydes (for example, benzaldehyde), alkanes (for example, undecane), alkenes (1-undecene), aromatic compounds (for example, 2-phenylethanol), esters (for example, 2-phenylethyl ester), fatty acids (for example, butyric acid), isoprene, lactic acid, lactones (for example, gamma-butyrolactone), methylketones (for example, acetone), monoterpenes (for example, farnesol), nitrogen compounds (for example, benzonitrile), sesquiterpenes (for example, pinene) and sulfur compounds (for example, dimethyl disulfide).

Ecological roles of microbial volatiles in antagonistic interactions

Microbial VOCs can have a significant role in antagonistic interactions between microorganisms occupying the same ecological niche. Here, we will focus on the antimicrobial activity of VOCs with specific emphasis on their antifungal and antibacterial activities.

Volatile-mediated antifungal activity

It is well known that germination of fungal spores as well as hyphal growth can be inhibited by bacterial VOCs (Herrington *et al.*, 1985; Herrington *et al.*, 1987). Furthermore, exposure to bacterial VOCs has been reported to change fungal morphology, enzyme activity and gene expression (Wheatley, 2002; Vespermann *et al.*, 2007; Minerdi *et al.*, 2008; Kai *et al.*, 2009; Minerdi *et al.*, 2009; Garbeva *et al.*, 2011; Garbeva *et al.*, 2014b). For example, activity of laccases and tyrosinases can be strongly affected by bacterial VOCs (Wheatley, 2002).

Fungal VOCs can also have inhibitory effects on other fungi. For example, the endophytic fungi *Muscodor albus* and *Oxysporus latemarginatus* strongly inhibited growth of several plant pathogenic fungi including *Botrytis cinerea* and *Rhizoctonia solani* (Strobel *et al.*, 2001). Moreover, *M. albus* VOCs were shown to kill the fungal human pathogens *Aspergillus fumigatus* and *Candida albicans* (Strobel et al., 2001). Fungi often live in symbiosis with bacteria. For *Fusarium oxysporum,* hyphae-associated bacteria were shown to produce the volatile sesquiterpene caryophyllene which repressed the expression of two virulence genes. When cured from the bacterial symbionts, caryophyllene was not detected and *F. oxysporum* became pathogenic (Minerdi *et al.*, 2008).

Sensitivity to VOCs can strongly differ between fungal species and the extent of inhibition depends on the individual bacteria-fungus or fungus-fungus interaction (Kai *et al.*, 2007; Vespermann *et al.*, 2007; Kai *et al.*, 2009; Garbeva *et al.*, 2014b). Several independent studies have reported that *Fusarium solani* is not much affected by bacterial VOCs, whereas *Pythium* species (oomycetes) are highly sensitive to bacterial VOCs (Kai *et al.*, 2009; Effmert *et al.*, 2012; Garbeva *et al.*, 2014b; Garbeva *et al.*, 2014a). *F. oxysporum* was also reported to be rather resistant to VOCs produced by the fungus *O. latemarginatus,* whereas *Magnaporthe grisea* was sensitive. High sensitivity to bacterial VOCs was recently reported for the late blight oomycete pathogen *Phytophthora infestans*. Two VOCs, hydrogen cyanide and 1-undecen, were indicated as the main compounds responsible for the growth inhibition (Hunziker *et al.*, 2015). The apparent high sensitivity of oomycetes to VOCs may be related to their cell wall composition and structure, which is different from that of fungi. To date, however, very little is known about fungal resistance to VOCs with the exception of resistance to azole-derived compounds (Lupetti *et al.*, 2002). Azole resistance commonly involves modifications of the *cyp51A*-gene, the target of antifungal azoles (Lupetti *et al.*, 2002; Seyedmousavi *et al.*, 2014). The resistance selection is believed to occur via exposure to azole compounds in the environment (Snelders *et al.*, 2009), released by humans via application of crop protection agents or by bacterial genera commonly found in soil, such as *Bacillus*, *Serratia*, *Pseudomonas* and *Burkholderia* (Lemfack *et al.*, 2014).

Volatile-mediated antibacterial activity

Relatively few studies have reported on VOCs with antibacterial activity. Screenings of commonly produced VOCs with antimicrobial activity often did not reveal antibacterial activity (Schulz *et al.*, 2010). Moreover, VOCs with strong antifungal activity (such as dimethyl disulfide, dimethyl trisulfide, S-methyl thioacetate, benzonitrile) did not exhibit antibacterial effects and even stimulated the growth of some bacteria (Garbeva *et al.*,

2014a). However, some specific VOCs produced by only a few microorganisms have been indicated as potential antibacterial agents. These include volatile lactones like γ-butyrolactones, which exhibit antibacterial activity against a broad range of Gram-positive and Gram-negative bacteria (Schulz *et al.*, 2010).

An odoriferous actinomycete isolate from corn seeds, identified as *Streptomyces albidoflavus,* was shown to produce a sesquiterpene, named albaflavenone with antibacterial properties (Gurtler *et al.*, 1994). More recently, albaflavenone was isolated from other *Streptomyces* species and fungi (Takamatsu *et al.*, 2011; Moody *et al.*, 2012). Another sesquiterpene compound with antibacterial activity, dihydro-β-agarofuran, is produced by *Streptomyces* sp. (Brana *et al.*, 2014). Recently, Dandurishvili *et al.* (2011) reported that VOCs emitted by *Pseudomonas fluorescens* and *Serratia plymuthica* have bacteriostatic effects against the bacterial plant pathogens *Agrobacterium tumefaciens* and *A. vitis* and inhibited the growth of these pathogens *in planta*. The major VOC emitted by *S. plymuthica* under the tested conditions was dimethyl disulfide, whereas *P. fluorescens* emitted a mix of 1-undecene, methanthiol, methanthiol acetate and dimethyl disulfide (Dandurishvili *et al.*, 2011).

VOC-producing endophytes have recently attracted great attention due to their strong antimicrobial activity. For example, *M. albus* (an endophytic fungus of tropical tree species) emitted a number of VOCs, such as tetrohydofuran, aciphyllene and an azulene derivate (Atmosukarto *et al.*, 2005). VOCs emitted by *M. albus* as well the artificial mixture of VOCs effectively inhibited or killed a range of plant- and human-pathogenic bacteria. Another recently described endophytic fungus *M. crispans,* isolated from wild pineapple, produced a mixture of VOCs with strong activity against a major bacterial pathogen of citrus, *Xanthomonas axonopodis* pv. *citri,* and the human pathogens *Yersinia pestis*, *Mycobacterium tuberculosis* and *Staphylococcus aureus* (Mitchell et al., 2010). Five classes of VOCs (acids, alcohols, esters, ketones and lipids) were identified in *Muscodor* species and although each class had some antimicrobial effect, their collective action was required to kill a broad range of bacterial pathogens.

In the past years, a group of pyrazine volatile compounds have been attracting wide interest due to their promising antitumor, antimicrobial and insecticidal activities (Rajini *et al.*, 2011). The production of pyrazines is widely distributed in plants, and only few bacteria have been reported so far to synthesize these VOCs (Rajini *et al.*, 2011; Brana *et al.*, 2014). *S. albus*, *Corynebacterium glutamicum* and *Bacillus* spp. produce tetramethylpyrazine (also known as ligustrazine), a compound that is used in traditional Chinese medicine against cystic fibrosis.

Although the mode of action of antibacterial VOCs has not been studied in detail, it is likely that hydrophobicity of some VOCs enables them to partition in the lipid layer of the cell membrane, rendering the membrane more permeable. Indeed, a study on the mechanisms of inhibitory action of three monoterpenes against *S. aureus* and *Escherichia coli* revealed a perturbation of the lipid fraction of microorganisms' plasma membrane, resulting in alteration of membrane permeability and a leakage of intracellular compounds (Trombetta *et al.*, 2005).

Finally, VOCs may have a synergistic effect when combined with antibiotics. For example, hydrophilic antibiotics such as vancomycin and β-lactam antibiotics, which have a marginal activity on the Gram-negative bacteria *E. coli* and *Listeria monocytogenes*, exhibited an enhanced antibacterial activity when pre-treated with the volatile eugenol (Hemaiswarya & Doble, 2010). Synergistic effects of terpenes and penicillin on multiresistant strains *S. aureus* and *E. coli* have also been reported (Gallucci *et al.*, 2009).

Ecological roles of microbial volatiles in interspecific interactions

Volatile organic compounds (VOCs) have an important role in interactions between physically separated microorganisms. Microarray analysis of *E. coli* exposed to VOCs emitted by *Bacillus subtilis* revealed that VOCs induce changes in gene expression and affect motility and biofilm formation of the exposed bacteria (Kim *et al.*, 2013). More recently, a study, using *P. putida* as a model organism, showed that indole has a role as an inter-specific signalling molecule (Molina-Santiago *et al.*, 2014). This compound influenced the expression pattern of *P. putida* genes involved in cell metabolism, cell wall biosynthesis and stress defense. In our research group, we have tested the effect of VOCs emitted by different soil bacteria grown in sand supplemented with artificial root exudates on the soil bacterium *P. fluorescens*. The *P. fluorescens* strain was grown on nutrient-limited agar while being exposed to VOCs produced by four phylogenetically different bacterial isolates (*Collimonas pratensis*, *S. plymuthica*, *Paenibacillus* sp. and *Pedobacter* sp.) as well as a mixture of all four bacteria. A genome-wide microarray-based analysis revealed that VOCs of each bacterial strain affected gene expression of *P. fluorescens*, but with a different pattern for each strain. Only a small core set of 22 genes was differentially expressed by all VOC-producing bacteria, including the mixture. These genes were mainly involved in amino acid transport and metabolism, energy production and conversion, signal transduction mechanisms, inorganic ion transport and metabolism, secretion and cell motility. Among these common, differentially expressed, genes was the Pfl_0064 catalase, an important enzyme that protects the cell against damage by reactive oxygen species (Lushchak, 2001).

Furthermore, the VOCs produced by *C. pratensis* triggered the production of antimicrobial secondary metabolites (Garbeva *et al.*, 2014a).

Antibiotic production triggered by VOCs in microbial interactions was also observed in *P. aeruginosa* during co-culture with *Enterobacter aerogenes* and this enhanced production was due to the volatile 2,3-butanediol emitted by *E. aerogenes* (Venkataraman *et al.*, 2014). Also for *Chromobacterium violaceum* and *P. aeruginosa,* several monoterpenes increased violacein and pyocyanin production, respectively (Ahmad *et al.*, 2014). The fact that the production of antibiotics in these bacteria is regulated by quorum sensing (QS) suggests that VOCs may interfere with bacterial cell-cell communication. Indeed, several studies revealed that VOCs can affect QS systems in bacteria, negatively or positively (Schulz *et al.*, 2010; Chernin *et al.*, 2011; Ahmad *et al.*, 2014). For example, VOCs produced by *S. plymuthica* can inhibit cell-cell communication mediated by acyl homoserine lactone (AHL) molecules in *Agrobacterium*, *Pectobacterium* and *Pseudomonas*. VOCs emitted by *S. plymuthica* decreased the amount of AHLs produced by these bacteria leading to significant suppression of transcription of AHL synthase genes (Chernin *et al.*, 2011).

Volatile organic compounds may also influence fungal QS as well as fungal development and virulence. *C. albicans* and *C. dubliniensis*, well-known human opportunistic pathogenic yeasts, produce large amounts of the QS molecule (E,E)-farnesol, a sesquiterpene, that is able to modulate morphogenesis of these species. Accumulation of farnesol blocked the yeast-to-mycelium morphology switch, mycelial development and biofilm formation, important traits for virulence of *Candida* (Hornby *et al.*, 2001; Martins *et al.*, 2007). Moreover, VOCs produced by *Trichoderma* were shown to function as signalling molecules regulating development and mediating intercolony communication: VOCs such as 1-octen-3-ol, 3-octanol and 3-octanone produced by conidiating colonies elicited conidiation in other colonies (Nemcovic *et al.*, 2008). The underlying mechanisms of the effects VOCs on fungal development remain largely unknown.

Recently, several studies reported on the effect of VOCs on bacterial antibiotic resistance or tolerance. For example, exposure of *E. coli* to VOCs emitted by *Burkholderia ambifaria* increased its resistance to gentamycin and kanamycin by yet unknown mechanisms (Groenhagen *et al.*, 2013). Exposure to the volatile compound trimethylamine (TMA) was shown to modify the antibiotic resistance profiles of several Gram-positive and Gram-negative bacteria (Létoffé *et al.*, 2014). In addition, indole, a VOC that has been proposed to act as signalling molecule, can also affect antibiotic resistance. For example, *P. putida* does not produce the volatile indole itself but recognizes indole produced by other bacteria (for example, *E. coli*) and activates the expression of the gene encoding the

TtgGHI efflux pump (Lee *et al.*, 2010; Molina-Santiago *et al.*, 2014). Biogenic ammonia, an inorganic volatile compound, was also reported to modify antibiotic resistance in physically separated bacteria (Bernier *et al.*, 2011). One of the underlying mechanisms proposed involves ammonia-induced synthesis of polyamines, which alters the permeability of bacterial membrane or helps the bacteria to cope with oxygen radicals. A recent study reported on ammonia-mediated growth promotion of ampicillin-sensitive bacteria by means of antibiotic inactivation (Cepl *et al.*, 2014). However, this phenomenon appeared to result from pH increase in the media caused by bacterial VOCs rather than by alteration of specific traits in the target bacterium. Another inorganic volatile compound, hydrogen sulfide, was suggested as a universal defense against antibiotics in bacteria as it seemed to trigger broad-spectrum antibiotic resistance, most probably due to alleviation of oxidative stress (Shatalin *et al.*, 2011).

As was shown for the above mentioned *Candida* species, also virulence and fitness of microorganisms can be affected by microbial VOCs. This was for instance observed for *Pectobacterium* species, bacterial pathogens responsible for soft rot disease in potato. Disruption of the biosynthesis of the volatile 2,3-butanediol coincided with reduced virulence (Marquez-Villavicencio *et al.*, 2011).

Volatile organic compounds can also have a role in the attraction of other microorganisms. During interaction between *X. perforans* and *Paenibacillus vortex,* VOCs produced by *X. perforans* were found to attract the proficient swarmer *P. vortex* (Hagai et al., 2014). Interestingly, the VOCs released by *X. perforans* did not only attract the swarmer but also increased its dispersal without affecting its growth rate. Using fluorescent stained *X. perforans*, Hagai *et al.* (2014) revealed that this hitch-hiking strategy also occurs on tomato leaves with different swarming bacterial species, suggesting that this might be a widespread and ecologically important phenomenon.

Conclusions and perspectives

Most studies to date have focused on the role of VOCs in plant-microbe interactions and their role in plant growth and health (Bitas *et al.*, 2013; Peñuelas *et al.*, 2014). However, the role of VOCs in microbe-microbe communication and competition in soils remains largely unknown. It is not completely clear why microorganisms produce VOCs and what their exact functions are. It has been proposed that VOCs represent waste material or a detoxification system of the producing microorganisms (Claeson *et al.*, 2007). However, from recent studies summarized in part 2 and 3 of this review, it is clear that microbial VOCs can have two major roles in a long-distance interactions in microbial communities as: (i) infochemical molecules affecting the behavior, population dynamic and gene

expression in the responding microorganism, and (ii) competitive tools directly exerting antimicrobial activity, providing an advantage by suppressing or eliminating potential enemies.

Currently, most studies on microbial VOCs are performed *in vitro* under nutrient rich conditions (Kai *et al.*, 2009; Weise *et al.*, 2012) and may not represent the conditions that prevail in the microbial environment. Furthermore, as indicated by Garbeva *et al.* (2014b); Garbeva *et al.* (2014a), the composition of VOCs produced by a mixture of bacterial species can differ from those produced by each bacterial monoculture.

Soil is a complex, highly diverse and heterogeneous environment; an important characteristic of most soils is the occurrence of air-filled pores. Hence, the gaseous phase forms an important part of the natural surroundings of soil microorganisms. It has been estimated that the area of soil particles covered by microorganisms is less than 1%, implying that the distance between microcolonies of microbial neighbors can be considerable (Young *et al.*, 2008). Compared to diffusible compounds, VOCs can travel faster and over longer distances through both the liquid and gaseous phase of the soil (Insam & Seewald, 2010; Effmert *et al.*, 2012), which facilitate the interactions between soil microorganisms. Therefore, VOCs have an important role in the communication and competitiveness between physically separated soil microorganisms (Kai *et al.*, 2009; Effmert *et al.*, 2012; Garbeva *et al.*, 2014a). It is plausible that in soil, dormant microorganisms can sense changes in their environments via emitted VOCs and change their behavior accordingly and in turn, influence the behavior of other soil microorganisms (Garbeva *et al.*, 2011). Although several studies have shown that VOCs can be used as signaling molecules in soil microbial communication, so far it is unclear how VOCs are perceived as signals by the microorganisms.

To date, little is still known about the regulatory pathways and genes involved in VOC biosynthesis as well as the possible role of quorum sensing in the production of VOCs. Because the production of VOCs is often reported to vary depending on cell density (Weise *et al.*, 2012; Groenhagen *et al.*, 2013), it is tempting to reason that VOCs are regulated by QS. However, there are only few and contradictory reports regarding QS regulation of VOC production. While for hydrogen cyanide in *Pseudomonas* and *Chromobacterium* species it was concluded to be QS regulated (Pessi & Haas, 2000; Blom *et al.*, 2011), for *B. ambifaria*, production of VOCs appeared not to be controlled by QS as the VOC profiles of the wild-type and the QS mutant were very similar (Groenhagen *et al.*, 2013). Future challenges are therefore to further elucidate the large chemical diversity of microbial VOCs, to discover regulatory pathways and genes involved in the biosynthesis of VOCs in soil bacteria and fungi, to determine biological relevant concentrations and to resolve the importance of

VOCs in ecosystem processes. Monitoring VOCs may be used as potential indicator of microbial activity, measuring shifts in community composition in the environment and ultimately for determining the soil health status of agricultural soils.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgments

This work is supported by the Netherlands Organization for Scientific Research (NWO) VIDI personal grant to PG (864.11.015). This is publication 5808 of the Netherlands Institute of Ecology (NIOO-KNAW). The authors wish to thank the anonymous reviewers for their helpful comments.

Chapter 3

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

Viviane Cordovez, Victor J. Carrion, Desalegn W. Etalo, Roland Mumm, Hua Zhu, Gilles P. van Wezel, Jos M. Raaijmakers

Frontiers in Microbiology (6) 1081 | doi: 10.3389/fmicb.2015.01081

Abstract

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 analogues 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.*, 2016). 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.*, 2016). PhyloChip-based metagenomics detected more than 33.000 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 10.000 secondary metabolites, including volatile organic compounds (VOCs) (Bérdy, 2005; Hopwood, 2007; van Wezel *et al.*, 2009). Approximately 1.000 microbial VOCs have been identified to date (Piechulla & Degenhardt, 2014). Although the production of VOCs by microorganisms is known for many years (Zoller & Clark, 1921; Stotzky & 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 sulphur-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 & 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; Ryu *et al.*, 2004; Vespermann *et al.*, 2007; Kai *et al.*, 2009; Verhulst *et al.*, 2009; Bailly & 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 µmol 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 three 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 (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 °C 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'- GAGTTTGATCCTGGCTCAG - 3') and 1492R (5'- ACCTTGTTACGACGACTT- 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 13.000 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 & Bork, 2011) (http://itol.embl.de/). A Neighbor-joining consensus tree (Saitou & Nei, 1987) with 1000 bootstrap replicates (Felsenstein, 1985) was constructed using Tamura-Nei model (Tamura & 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 eleven *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 1.439 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 fibre (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 33-300 *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 & 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. 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. Linear retention indices (RI) of VOCs were calculated as previously described (Strehmel *et al.*, 2008) and compared with those in the literature. 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 $1/10^{th}$ 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 x 1.5 cm) on the bottom. 20 µL 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 = 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 & 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 Fig. 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 (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* (Fig. 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 (Fig. 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 (Fig. 2). This comparison led to the selection of 11 isolates (Fig. 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 (Fig. S1A). Additionally, we sequenced the house-keeping genes *atpD* and *recA* (Fig. S1B). Concatenation of *atpD*, *recA* and 16S sequences allowed a better resolution of the different *Streptomyces* isolates than based on 16S sequences only. However, closely related but phenotypically different isolates, like *Streptomyces* strains W75.5 and W126 (Fig. 3), could not be distinguished based on these three molecular markers.

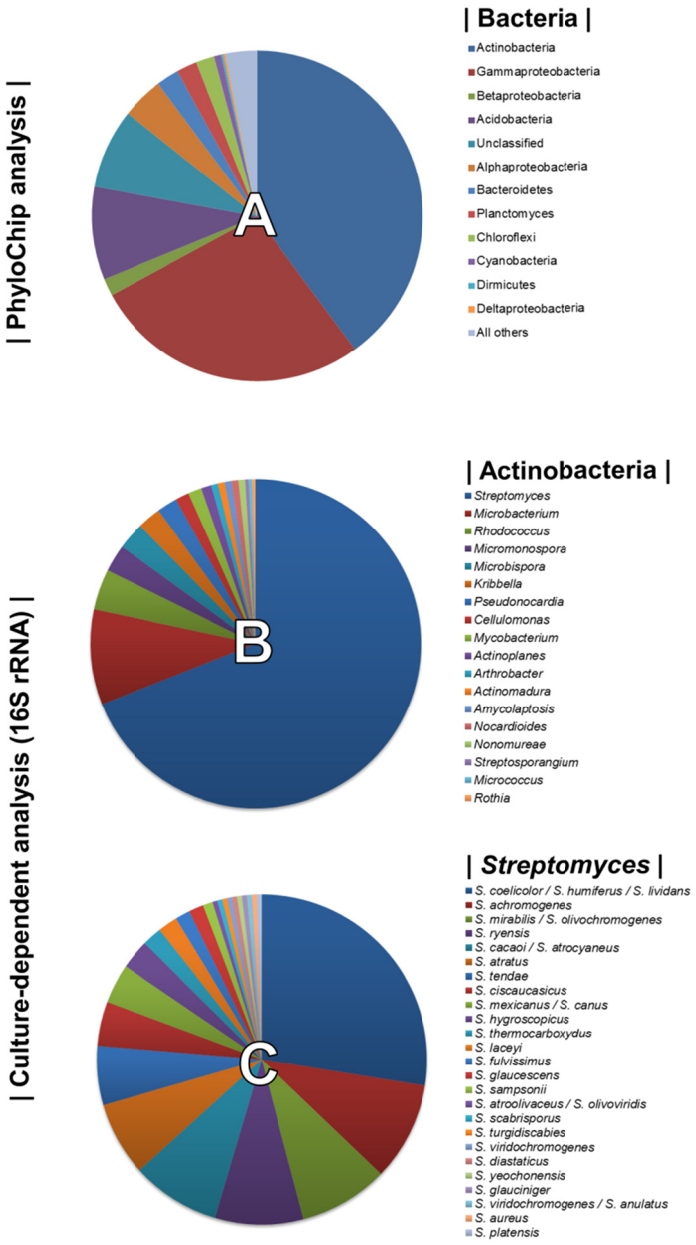


Fig. 1 | Top 10% most dynamic bacterial (and archaeal) phyla detected by PhyloChip analysis of the rhizosphere microbiome of sugar beet seedlings grown in *Rhizoctonia*-suppressive soil (pie chart A, adapted from Mendes et al. 2011). Diversity of Actinobacteria (pie chart B) and of *Streptomyces* species (pie chart C) isolated from the rhizosphere of sugar beet seedlings grown in *Rhizoctonia*-suppressive soil (this study).

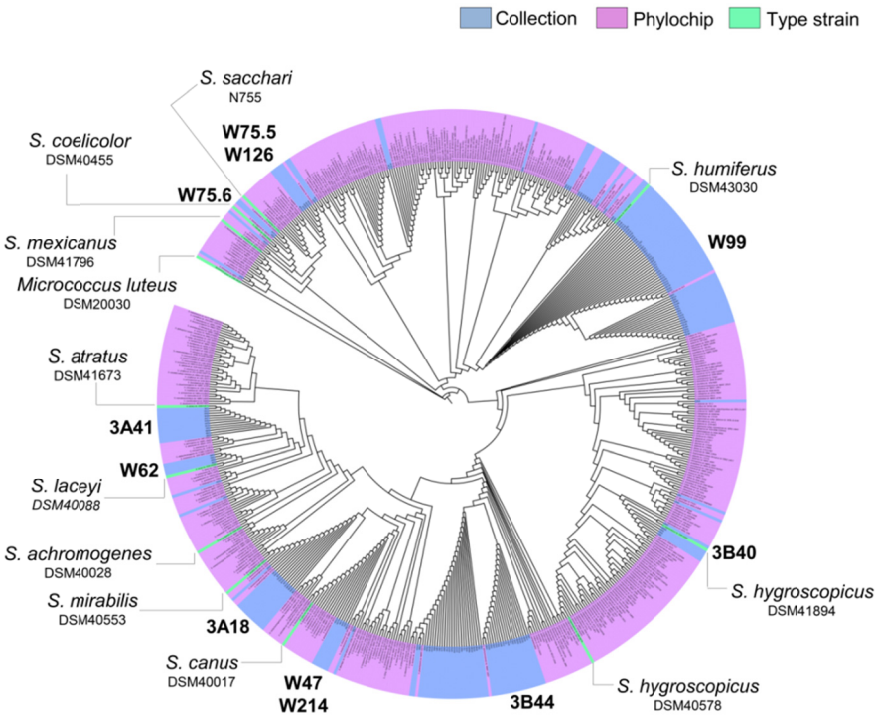


Fig. 2 | Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences of the *Streptomyces* collection obtained in this study (in blue), *Streptomyces* detected by Phylochip analysis (in pink) and *Streptomyces* type strains (in green). *Streptomyces* isolates selected for VOC analysis are indicated in bold.

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 analyses. The diversity of VOCs produced by the different *Streptomyces* isolates is shown in Table S2 and highlighted in the heat-map (Fig. 4). The VOCs detected belong to diverse classes of compounds such as alcohols, aldehydes, carboxylic acids, esters, ketones, sulphur compounds, and several terpenes (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; Table S2) was one of these common VOCs. Hierarchical cluster analysis of the VOC profiles resulted in a

similar clustering of the 12 *Streptomyces* isolates as the clustering based on the different molecular markers (Fig. 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.

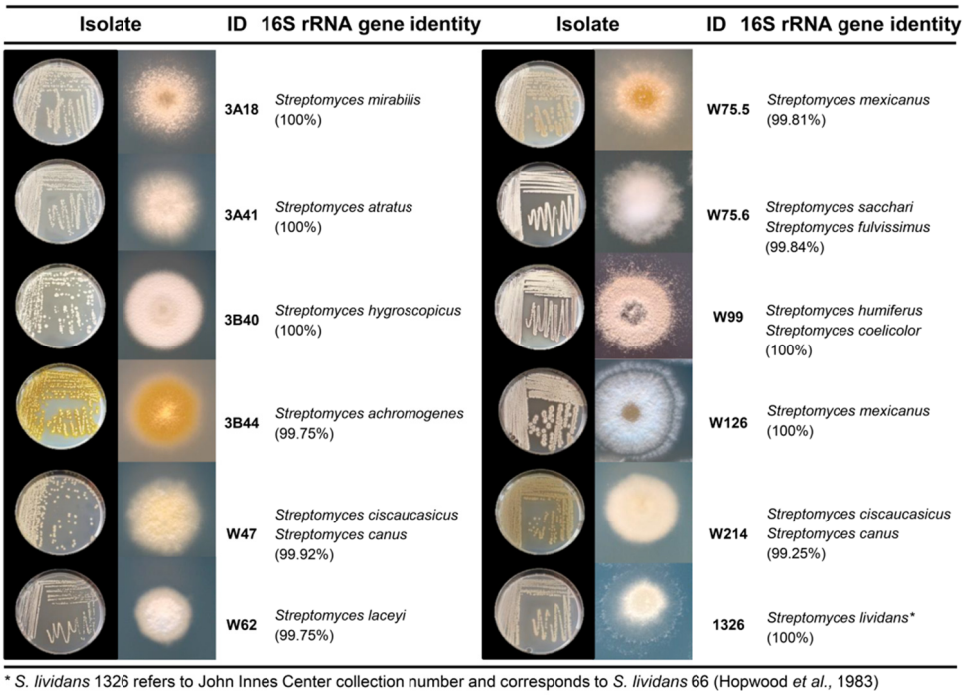


Fig. 3 | Characterization of *Streptomyces* isolates used in this study. Species names are based on 16S rRNA gene sequence comparison using the Greengenes database. Pictures depict 4-7 day-old isolates grown on GA medium.

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 (Fig. 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 (Fig. 6B). *S. lividans* 1326, and *Streptomyces* strains W47 and W62 led to the largest increase in plant biomass, whereas *Streptomyces* strains W214 and 3A41did 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 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 (Fig. 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 (Fig. 7A and 7B). 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.* The VOC 1,3,5-trichloro-2-methoxy benzene completely inhibited radial hyphal growth of *R. solani* at concentrations of 1M and 100mM (Fig. 7D). Exposure to this VOC led to melanization of *R. solani* hyphae (Fig. 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 (Fig. 7F, 7G).

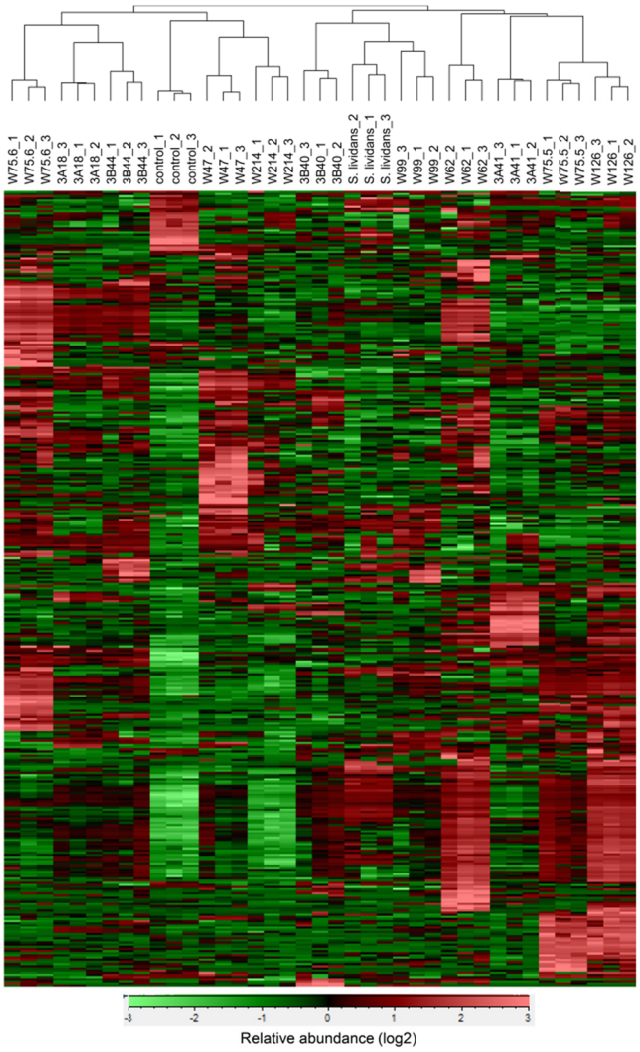


Fig. 4 | Hierarchical cluster and heat-map analyses of VOC profiles of the selected *Streptomyces* isolates. Columns represent three replicate VOC measurements of each of the 12 isolates and the medium alone (control). Rows represent the different VOCs (green: low abundance, red: high abundance), several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

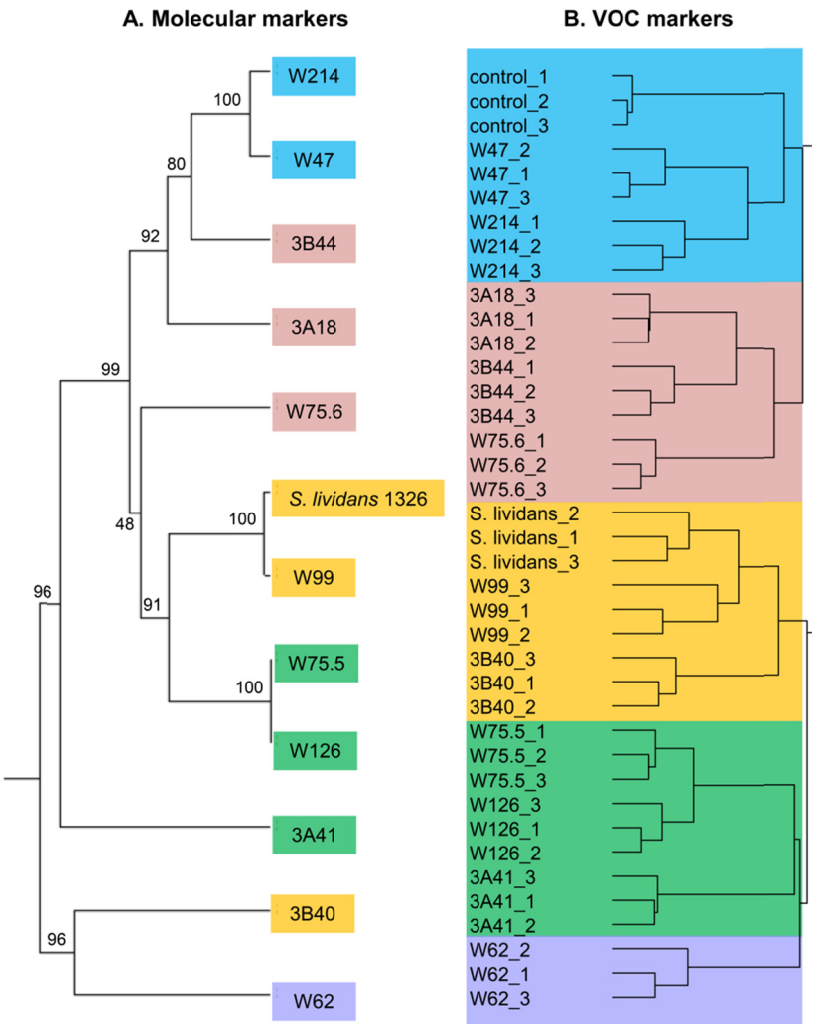


Fig. 5 | (A) Phylogenetic tree of concatenated partial sequences of 16S *rRNA*, *atpD* and *recA* genes of 11 *Streptomyces* isolates from the *Rhizoctonia*-suppressive soil and the reference strain *S. lividans* 1326. The tree was constructed using UPGMA method and Tamura-3 parameter calculation model with gamma distribution and 1.000 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.

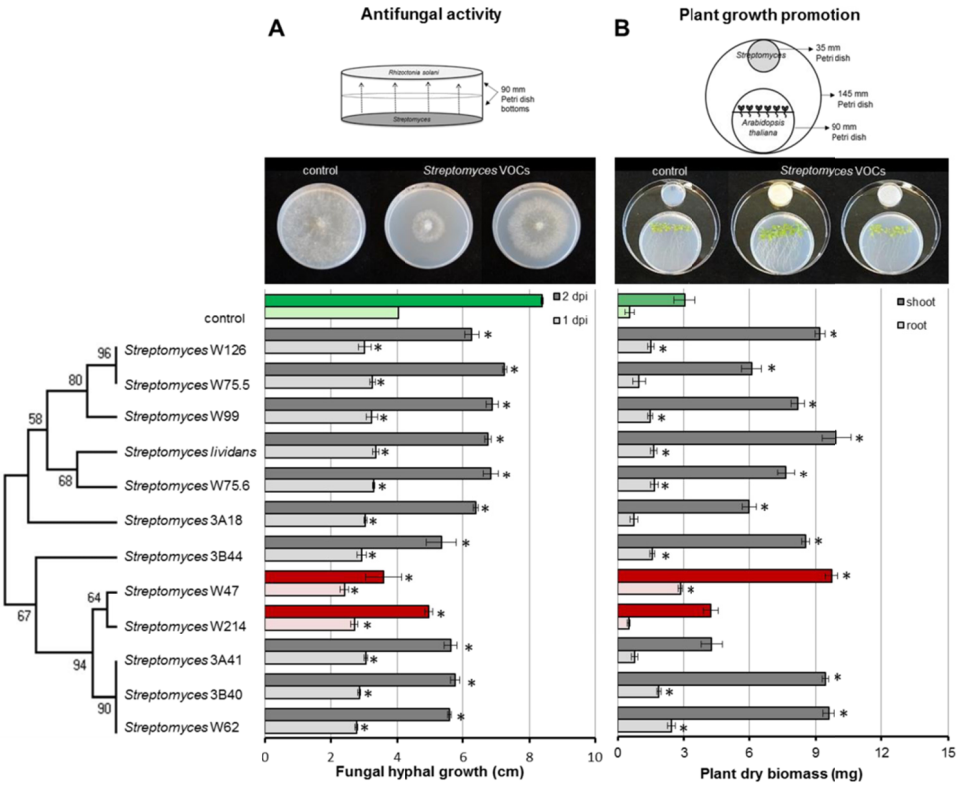


Fig. 6 | Inhibition of fungal growth after 1 and 2 days of exposure to *Streptomyces* VOCs **(A)** and growth of *Arabidopsis thaliana* seedlings after 2 weeks of exposure to *Streptomyces* VOCs **(B)** The controls are displayed in green and isolates with the strongest antifungal activity in red. Bars represent standard errors of the mean of 3 independent biological replicates. Asterisks indicate a statistical differences 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.

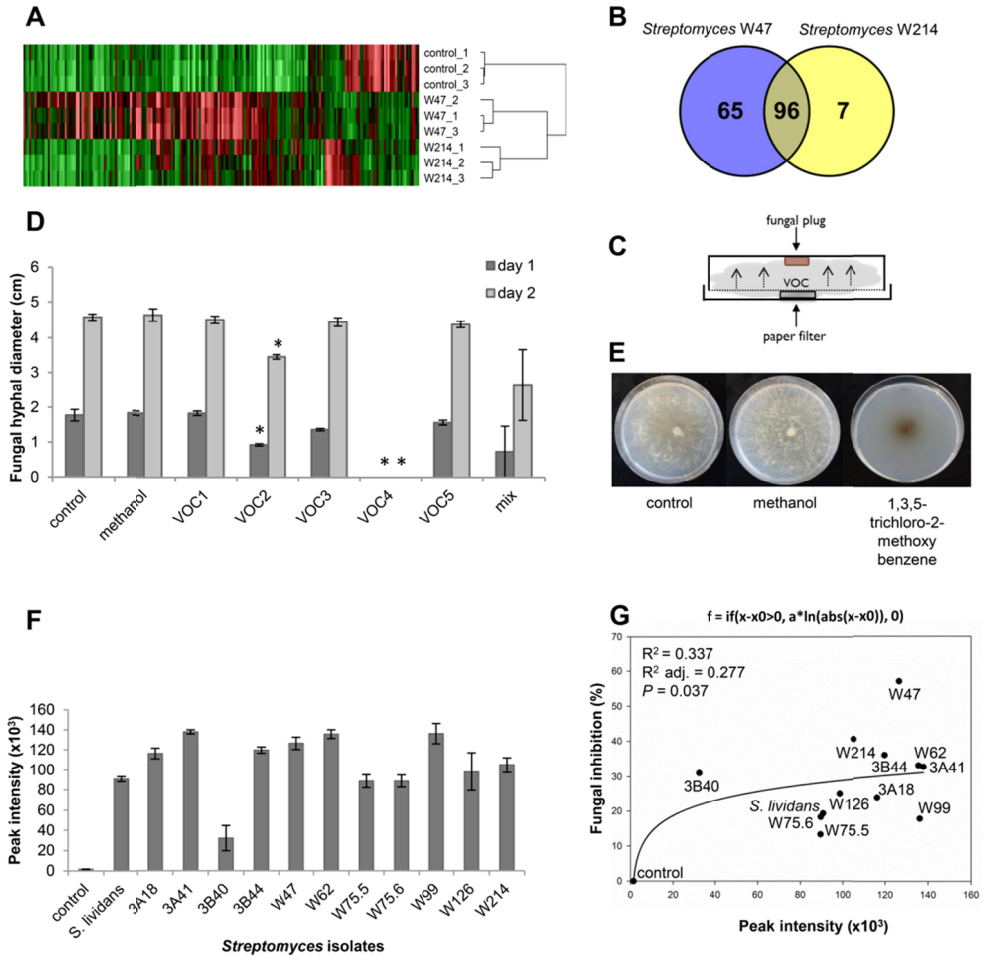


Fig. 7 | (A) VOC profiles of *Streptomyces* strains W47 and W214 compared to control (medium only). (B) Venn diagram for common and unique VOCs produced by *Streptomyces* strains W47 and W214. (C) Experimental set-up for *in vitro* antifungal activity assay with synthetic VOCs. (D) *In vitro* antifungal activity with synthetic VOCs at 1M (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 = 5). (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.

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 & Herbert, 2009; Muller *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 8 isolates. Members of the *Streptomyces* genus differ greatly in their morphology, physiology, and biochemical characteristics (Anderson & 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, 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 & 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 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 & 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 & 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 biomass.

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.

Acknowledgments

We thank Jacques Davis for assistance with the GC-MS. This manuscript is publication number 5937 of Netherlands Institute of Ecology (NIOO-KNAW). The authors also acknowledge funding support from the Netherlands Organization for Scientific Research (NWO) and the Consortium of Improved Plant Yield which is part of the Netherlands Genomics Initiative (NGI).

Supplementary materials

Table S1 | Selective treatments and media used for the isolation of Actinobacteria from suppressive soil.

Pre-treatment	Medium	Incubation	Reference
6% yeast extract (30 °C, 120 min)	HA, GCA, SFM, MM 50 µg/ml nystatin 10 µg/ml nalidixic acid	1-3 weeks, 30 °C	Hayakawa & Nonomura, 1987 Hayakawa & Nonomura, 1989 Kuester & Williams, 1964 Zhang, 1985 Kiezer et al., 2000 Zhu et al., 2015
none	HV 20 µg/ml nalidixic acid 20 µg/ml trimethoprim 100 µg/ml delvocid*	3 weeks, 30 °C	Hayakawa et al., 1991 Tamura et al. 1997 Zhu et al., 2015
rehydration (30 °C, 90 min) centrifugation	HV 20 µg/ml nalidixic acid 20 µg/ml trimethoprim 100 µg/ml delvocid*	3 weeks, 30 °C	Hayakawa et al., 2000
none	CN 100 µg/ml delvocid*	2-3 weeks, 25-30 °C	Gavrish et al., 2010
none	TSAYE 100 µg/ml delvocid*	10 days, 25 °C	Hagedorn and Holt, 1975
1.5% phenol (30 °C, 30 min)	HV	3 weeks, 30 °C	Hayakawa et al., 2004
wet-heating (70 °C, 15 min)	HHVA 50 µg/ml nystatin 20 µg/ml nalidixic acid	4 weeks, 25 °C	Seong et al., 2001

*Cycloheximide was replaced by delvocid

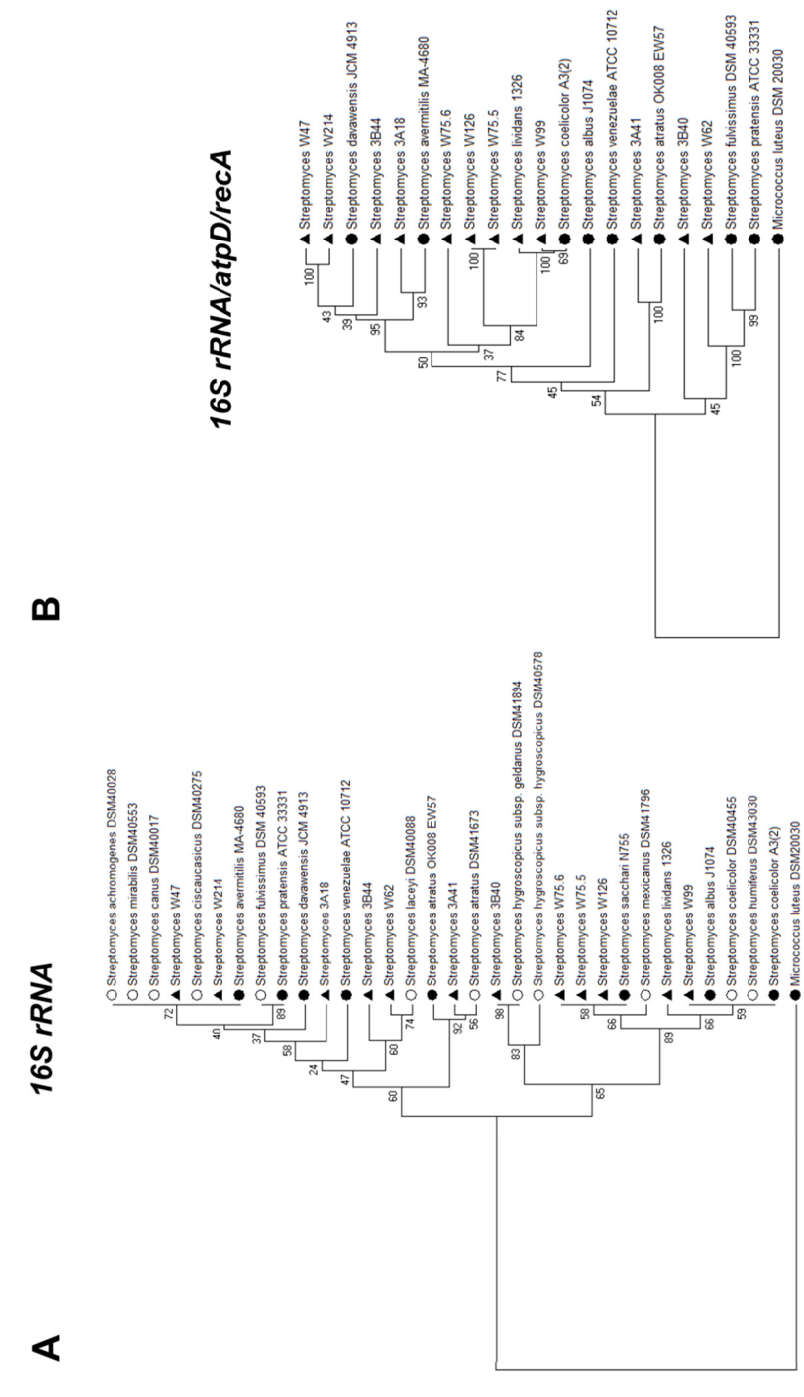


Fig. S1 | Phylogenetic tree of *Streptomyces* partial sequences of 16S rRNA gene **(A)** and concatenated sequences of 16S *rRNA*, *atpD* and *recA* genes **(B)**. The tree was constructed using UPGMA method and Tamura-3 parameter calculation model with gamma distribution and 1000 bootstrap replicates. *Micrococcus luteus* DSM200330 was used as the outgroup. (▲) isolates described in this study, (○) type strains, (●) strains with sequenced genome available on NCBI database).

Table S2 | List of putative VOCs produced by the 11 Streptomyces isolated from a *Rhizoctonia*-suppressive soil and the reference strain *S. lividans* 1326. VOCs displayed are significantly different ($P < 0.05$), detected 2 times or more than in the control (medium only) and with a match factor higher than 800. Compounds were putatively annotated by comparing their mass spectra with those of NIST and in-house mass spectral libraries.

RI	Putative compound	3A18	3A41	3B40	3B44	W47	W62	W75.5	W75.6	W99	W126	W214	*S. lividans*
737	3-Hydroxy-2-butanone	X	X		X	X			X	X		X	
743	Methyl butanoate		X	X	X	X	X		X	X	X	X	X
745	Butanoic acid, methyl ester	X		X	X	X				X			X
749	3-Methyl-3-buten-1-ol	X	X	X	X	X	X	X	X	X	X	X	X
751	1-Butanol, 3-methyl	X	X	X	X	X		X	X	X	X	X	X
754	1-Butanol, 2-methyl-, (S)	X	X	X	X	X		X	X	X	X	X	X
760	Dimethyl disulfide	X	X	X	X	X	X	X	X	X	X	X	X
774	2-Methylpropanoic acid					X			X				
792	Butanoic acid		X	X	X	X			X	X		X	
796	Furan, 2-propyl								X				
799	Butanoic acid, 2-methyl-, methyl ester	X	X	X	X	X	X		X	X	X	X	X
805	meso-3,4-Hexanediol	X	X	X	X	X			X	X		X	X
807	Propanoic acid, 2-hydroxy-2-methyl-, methyl ester	X	X	X	X	X			X	X		X	X
812	Oxirane, (methoxymethyl)	X	X	X	X	X	X		X	X		X	X
817	Acetic acid, butyl ester		X	X			X						X
818	Butanoic acid, 3,3-dimethyl-, methyl ester		X	X	X	X	X		X		X	X	
850	Butanethioic acid, S-methyl ester	X	X	X	X	X	X	X	X	X	X	X	X
856	2-Methylbutanoic acid			X		X			X	X		X	X
858	2-Hexanone, 5-methyl	X	X		X	X	X	X	X	X	X	X	X
861	Butanoic acid, 2-ethyl-, methyl ester			X	X	X			X	X		X	X
867	2-Butenoic acid, 2-methyl-, methyl ester, (E)-		X	X		X	X	X	X	X	X		X
869	Pentanoic acid, 2-methyl-, methyl ester			X	X	X	X	X	X	X	X	X	X
886	1-Propanone, 1-(2-furanyl)							X			X		
888	Butanethioic acid, S-methyl ester	X	X	X	X	X			X		X	X	
890	Pentanoic acid, 4-methyl-, methyl ester			X		X	X	X	X		X		
894	5-Hydroxy-4-octanone	X	X	X	X	X	X		X	X	X	X	X
899	2-Heptanol	X				X			X			X	
921	Pentanoic acid, 2,4-dimethyl-, methyl ester	X	X	X	X	X	X	X	X	X	X	X	X
925	Hexanoic acid, methyl ester			X		X	X	X	X		X	X	X
927	alpha-Phellandrene	X		X	X				X		X		
929	2-Pentenoic acid, 4-methyl-, methyl ester						X				X		
935	alpha-Pinene	X			X	X	X		X				
935	5-Methylhexane-2,4-dione, enol	X	X	X	X	X	X	X	X	X	X	X	X
939	3-Pentenoic acid, 4-methyl-, methyl ester	X	X	X	X	X	X	X	X	X	X	X	
940	S-Methyl 3-methylbutanethioate	X	X	X	X	X	X	X	X	X	X	X	X
946	Pentanoic acid, 4-methyl	X	X	X	X	X		X	X		X	X	X
948	Pentanoic acid, 2-ethyl-, methyl ester			X	X	X	X		X	X		X	X
951	Camphene	X		X	X	X	X		X				
962	Benzaldehyde												X
965	Pentanoic acid, 2,4-dimethyl-, methyl ester	X	X		X	X	X	X	X	X	X	X	X
970	1-Heptanol				X			X					
973	5-Methylhexane-2,4-dione, enol	X	X	X	X	X	X	X	X	X	X	X	X

RI	Putative compound	3A18	3A41	3B40	3B44	W47	W62	W75.5	W75.6	W99	W126	W214	*S. lividans*
973	Dimethyl trisulfide	X	X	X	X	X	X	X	X	X	X	X	X
974	2,4-Heptanedione	X	X		X	X		X			X	X	
976	Methyl 2-furoate			X			X						
980	beta-Pinene	X			X		X		X				
982	1,3- Cyclopentadiene, 1,3-bis(1-methylethyl)	X		X	X	X	X	X	X	X	X		X
984	Pentane, 3-bromo							X			X		
987	3-Octanone	X	X		X	X	X	X	X		X	X	
989	2,4- Cycloheptadien-1-one, 2,6,6-trimethyl	X		X	X	X	X		X	X			X
989	Hexanoic acid, 5-methyl-, methyl ester	X	X	X	X	X	X	X	X	X	X	X	X
991	3(2H)-Thiophenone, dihydro-2-methyl							X			X		
998	4-Methylhexanoic acid, methyl ester	X	X	X		X	X	X	X		X	X	
1019	Hexanoic acid, 2,4-dimethyl-, methyl ester, (2DL,4L)	X		X	X	X	X	X	X	X	X	X	X
1023	p-Menth-8-ene, 3-methylene	X	X	X	X	X	X	X	X	X	X	X	X
1026	Methyl heptanoate	X	X	X	X	X	X	X	X	X	X	X	X
1028	p- Cymene								X				
1029	1-Hexanol, 2-ethyl					X							
1032	Limonene	X					X	X	X		X		
1037	1-Heptene, 6-methyl				X			X			X		
1044	Hexanoic acid, 2-ethyl-, methyl ester				X	X			X				X
1044	Cyclohexanol, 3,3,5-trimethyl-, trans						X	X	X		X		
1054	(R)-(-)-4-Methylhexanoic acid	X	X	X	X	X	X	X	X		X	X	X
1055	Thiophene, 2-methyl-5-propyl	X				X		X	X		X		
1061	7-Methyl-1,6-dioxaspiro[4.5]decane			X	X			X		X	X		X
1063	gamma-Terpinene	X		X	X	X	X	X	X	X	X		X
1072	1-Octanol					X		X					
1089	Methyl 6-methyl heptanoate					X						X	
1094	Terpinolene	X		X	X	X	X	X	X	X	X		X
1095	2-Nonanone	X	X		X	X			X				
1101	Benzoic acid, methyl ester		X	X	X	X	X		X	X			X
1102	2-Nonanol					X							
1103	1,6-Octadien-3-ol, 3,7-dimethyl		X		X	X	X				X		
1125	Heptanoic acid, 2,6-dimethyl-, methyl ester			X	X	X	X		X	X	X	X	X
1127	Octanoic acid, methyl ester			X		X	X		X	X		X	
1132	Cyclohexane, 4-methyl-2-methylene-1-(1-methylethylidene)	X		X	X	X	X		X				
1138	1-Octene, 7-methyl				X			X			X		
1139	4-Nonanone, 8-methyl		X	X	X			X		X	X		X
1142	cis-Ocimene, 8-oxo	X		X	X	X	X		X				
1150	Benzene, 2,4-dimethyl-1-(1-methylethyl)	X					X		X				
1157	6-Nonen-1-ol, (Z)	X	X	X		X		X		X	X	X	
1163	Octanoic acid, 2-methyl-, methyl ester			X	X	X	X		X			X	
1167	1,3- Cyclopentadiene, 1,3-bis(1-methylethyl)	X	X		X	X	X		X				
1184	Benzeneacetic acid, methyl ester	X	X	X	X	X	X	X	X	X	X	X	
1194	2-Methylisoborneol	X		X	X	X	X		X	X			X
1208	Benzene, 2,4-dichloro-1-methoxy	X	X			X		X	X		X		
1210	Benzene, isothiocyanato												X
1221	Benzoic acid, 3-methyl-, methyl ester	X	X	X	X	X	X	X	X	X	X	X	X

RI	Putative compound	3A18	3A41	3B40	3B44	W47	W62	W75.5	W75.6	W99	W126	W214	*S. lividans*
1241	11,12,13-Trinor-5-eudesmene	X	X	X	X	X	X	X	X	X	X	X	X
1274	1-Decanol					X					X		
1275	Bicyclo[4.3.0]non-3-ene, 3,4,7-trimethyl	X		X	X	X	X	X	X	X	X	X	X
1286	2,6-Octadienoic acid, 3,7-dimethyl-, methyl ester								X				
1290	Methyl 8-methyl-nonanoate		X	X	X	X	X	X		X	X	X	X
1296	Cogeijerene	X	X	X	X		X	X	X	X	X		X
1297	2-Undecanone	X	X		X	X	X						
1297	2-Bromo-4-chloroanisole	X	X			X		X	X		X		
1314	(E)-Methyl cinnamate			X			X		X	X			
1328	2,6-Octadienoic acid, 3,7-dimethyl-, methyl ester				X	X			X				
1339	Azulene, 1,2,3,3a,4,5,6,7-octahydro-1,4-dimethyl-7-(1-methylethenyl)-, [1R-(1**α**,3a**β**,4**α**,7**β**)]-	X	X	X	X	X	X	X	X	X	X		X
1341	Benzene, 1,3,5-trichloro-2-methoxy	X	X	X	X	X	X	X	X	X	X	X	X
1358	Benzoic acid, 3,5-dimethyl-, methyl ester	X	X	X	X	X	X	X	X	X	X	X	
1361	alpha- Cubebene	X	X	X	X	X	X	X	X	X	X	X	X
1367	African-1-ene		X	X	X	X	X		X	X	X	X	X
1372	Tridecane, 3-methyl										X		
1385	alpha-Ylangene	X	X	X	X	X	X	X	X	X	X	X	X
1390	alpha- Copaene	X	X	X	X	X	X	X	X	X	X		X
1393	2-Propenoic acid, 3-phenyl-, methyl ester, (E)-						X				X		
1397	Methyl 8-methyl-decanoate		X	X	X	X	X	X	X	X	X	X	X
1405	beta-Elemene	X	X	X	X	X	X	X	X	X	X	X	X
1409	2-Undecanone, 6,10-dimethyl		X				X		X	X			X
1415	10-Undecenoic acid, methyl ester		X	X		X	X	X	X	X		X	X
1423	Undecanoic acid, 2-methyl-, methyl ester	X		X	X	X	X	X	X	X	X		X
1423	trans-1,10-Dimethyl-trans-9-decalol	X	X	X	X	X	X	X	X	X	X	X	X
1432	alpha- Cedrene	X	X	X	X	X	X	X	X	X	X	X	X
1435	beta-Ylangene	X	X	X	X	X	X	X	X	X	X	X	X
1442	beta- Cedrene	X	X	X	X	X	X	X	X	X	X	X	X
1447	1H-Isoindole-1,3(2H)-dione, 2-phenyl					X	X		X			X	X
1451	beta-Gurjunene	X	X	X	X	X	X	X	X	X	X		X
1466	4,7-Methanoazulene, 1,2,3,4,5,6,7,8-octahydro-1,4,9,9-tetramethyl-, [1S-(1**α**,4**α**,7**α**)]-	X	X	X	X	X	X	X	X	X	X	X	X
1471	1,2-Benzenediol, O-(4-butylbenzoyl)-O'-(isobutoxycarbonyl)	X		X	X	X	X	X		X	X		X
1474	beta-Selinene	X	X	X	X	X	X	X	X	X	X	X	X
1477	Cadina-1(10),6,8-triene	X	X	X	X	X	X	X	X	X	X		X

RI	Putative compound	3A18	3A41	3B40	3B44	W47	W62	W75.5	W75.6	W99	W126	W214	*S. lividans*
1481	Zizaene	X	X	X	X	X	X	X	X	X	X		X
1482	4,11-Epoxy-cis-eudesmane	X	X	X	X	X	X	X	X	X	X	X	X
1489	Methyl 10-methyl-undecanoate	X	X	X	X	X	X	X	X	X	X		X
1491	Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)-, (1**α**,4a**β**,8a**α**)-	X	X	X	X	X	X	X	X	X	X	X	X
1495	alpha-Muurolene	X		X	X	X	X	X		X	X		X
1499	Germacrene D	X		X	X	X	X	X		X	X		X
1505	beta-Selinene	X	X	X	X	X	X	X	X	X	X	X	X
1510	Naphthalene, 1,2,3,5,6,7,8,8a-octahydro-1,8a-dimethyl-7-(1-methylethenyl)-, [1R-(1**α**,7**β**,8a**α**)]-	X	X	X	X	X	X	X	X	X	X	X	X
1514	Cubeb-11-ene		X			X	X	X		X	X		
1521	1H-Benzocyclohepten-7-ol, 2,3,4,4a,5,6,7,8-octahydro-1,1,4a,7-tetramethyl-, cis	X	X	X	X	X	X	X	X	X	X		X
1523	Benzene, 1-methoxy-2-methyl		X			X					X		
1526	Dodecanoic acid, methyl ester			X						X			
1526	gamma-Bisabolene, (E)	X	X	X	X	X	X	X	X	X	X	X	X
1528	Dihydroagarofuran, trans	X	X	X	X	X	X	X	X	X	X	X	X
1530	4-Epicubebol	X	X	X	X	X	X	X	X	X	X	X	X
1537	delta-Amorphene	X	X	X	X	X	X	X	X	X	X	X	X
1541	Zonarene	X	X	X	X	X	X	X	X	X	X	X	X
1548	Cedrene	X	X	X	X	X	X	X		X	X	X	X
1553	alpha- Cadinene	X	X	X	X	X	X	X	X	X	X		X
1560	alpha- Calacorene	X	X	X	X	X	X	X	X	X	X		X
1563	2-Tetradecanone	X	X	X	X	X	X	X		X	X	X	X
1568	alpha-Agarofuran	X	X	X	X	X	X	X		X	X	X	X
1571	2-Tetradecanone	X	X	X	X	X	X	X	X	X	X	X	X
1592	7- Caryophyllen-6-ol		X	X			X			X	X		
1596	Methyl 10-methyl-dodecanoate	X	X	X	X	X	X	X		X	X		X
1606	2-Naphthalenemethanol, 2,3,4,4a,5,6,7,8-octahydro-**α**,**α**,4a,8-tetramethyl-, [2R-(2**α**,4a**β**,8**β**)]-	X	X	X	X	X	X	X		X	X		X
1646	Cubenol		X				X	X			X	X	X
1658	2-Naphthalenemethanol, decahydro-**α**,**α**,4a-trimethyl-8-methylene-, [2R-(2**α**,4a**α**,8a**β**)]-	X	X	X	X	X	X	X	X	X	X		X
1664	2-Pentadecanone	X	X	X	X	X	X	X	X	X	X	X	X
1682	7-epi-alpha-Selinene	X	X	X			X	X	X	X	X		X
1699	Heptadecane						X						

RI	Putative compound	3A18	3A41	3B40	3B44	W47	W62	W75.5	W75.6	W99	W126	W214	*S. lividans*
1699	Phenol, 2,4-di-t-butyl-6-nitro	X		X		X	X	X		X	X		X
1717	2,2,7,7-Tetramethyltricyclo[6.2.1.0(1,6)]undec-4-en-3-one	X			X		X	X	X	X	X	X	X
1890	Acetic acid, methoxy-, methyl ester		X	X						X	X	X	X
1940	Cembrene			X						X			
2038	Bicyclo[9.3.1]pentadeca-3,7-dien-12-ol, 4,8,12,15,15-pentamethyl-, [1R-(1R*,3E,7E,11R*,12R*)]		X	X	X		X	X		X			X

Chapter 4

Genomic and functional analyses of rhizospheric and endophytic *Microbacterium* species

Viviane Cordovez, Victor J. Carrion, Victor de Jager, Jos M. Raaijmakers

Abstract

The rhizosphere microbiome of sugar beet plants grown in a soil suppressive to the root pathogen *Rhizoctonia solani* harbours diverse consortia of bacterial genera consistently associated with the disease-suppressive state. Among the Actinobacteria, *Streptomyces* and *Microbacterium* species were the most abundant genera found in the disease-suppressive soil. For *Streptomyces,* we previously showed that they inhibit hyphal growth of *R. solani* and enhanced plant biomass *via* the production of volatile organic compounds (VOCs). Here, we isolated and characterized *Microbacterium* species from the sugar beet rhizosphere and endosphere, and investigated their effects on plant and fungal growth. We showed that also *Microbacterium* species inhibit *R. solani* and promote plant growth *via* VOCs. Chemical profiling and genome analysis of endophytic *Microbacterium* sp. EC8 revealed common and rare sulfur-containing VOCs as well as genes involved in sulfur metabolism. Genome-wide transcriptome analysis of *Arabidopsis thaliana* seedlings exposed to the VOCs from EC8 showed up-regulation of genes involved in assimilation and transport of sulfate and nitrate. Also genes involved in the biosynthesis of jasmonic acid and genes responsive to ethylene and cytokinin were significantly up-regulated. Collectively, these findings suggest that VOCs from EC8 impact sulfur metabolism and pathogen resistance in *Arabidopsis*. The results on the genome sequence and VOC-profile of EC8 combined with the plant transcriptome data provide a strong basis to further unravel the molecular mechanisms underlying VOC-mediated interactions between the endophytic *Microbacterium* sp. EC8, the plant and the pathogenic fungi.

Introduction

In disease-suppressive soils, plants are effectively protected from infections by specific root pathogens due to the combined antagonistic activities of different soil and rhizosphere microorganisms (Weller *et al.*, 2002; Raaijmakers & Mazzola, 2016). This phenomenon has been described worldwide but, for most suppressive soils, the responsible consortia of microorganisms and underlying mechanisms are largely unknown. In recent studies, we characterized the microbiome composition of a soil suppressive to *Rhizoctonia solani*, an economically important fungal root pathogen of many crops including sugar beet, wheat, potato, and rice (Mendes *et al.*, 2011; Chapelle *et al.*, 2015). PhyloChip-based analyses of the rhizosphere microbiome of sugar beet plants grown in the disease-suppressive soil revealed bacterial groups consistently associated with the disease-suppressive state. Actinobacteria were significantly more abundant in the rhizosphere of sugar beet seedlings grown in the suppressive than in the non-suppressive (conducive) soil. Among the Actinobacteria, *Streptomyces* and *Microbacterium* species were the most abundant genera (Mendes *et al.*, 2011; Cordovez *et al.*, 2015). We recently showed that several *Streptomyces* isolates inhibited hyphal growth of *R. solani* and enhanced plant shoot and root biomass by the production of volatile organic compounds (VOCs) (Cordovez *et al.*, 2015). However, the antifungal and plant growth-promoting activities as well as the VOC profile of the *Microbacterium* species are yet unknown.

The genus *Microbacterium* comprises Gram-positive bacteria of the Microbacteriaceae family within the Actinobacteria phylum (Stackebrandt *et al.*, 1997). This genus currently comprises 97 species (http://www.bacterio.cict.fr) isolated from terrestrial and aquatic ecosystems, but also from clinical and food samples (Lee *et al.*, 2006; Anand *et al.*, 2012; Karojet *et al.*, 2012; Sharma *et al.*, 2013; Soto-Rodriguez *et al.*, 2013; Cogan *et al.*, 2014). The majority of functional studies on *Microbacterium* relate to their ability to degrade hydrocarbons and complex polysaccharides of economic importance (Qian *et al.*, 2007; Sheng *et al.*, 2009; Kim *et al.*, 2013; Corretto *et al.*, 2015). Their effects on plant and fungal growth have received little attention to date. Madhaiyan *et al.* (2010) reported that some *Microbacterium* isolates produce the plant growth hormone indole acetic acid, solubilize phosphate or exhibit ACC deaminase activity. Barnett *et al.* (2006) further showed that *Microbacterium* isolated from a soil suppressive to *Rhizoctonia* root rot of wheat, enhanced, together with *Pantoea* and *Exiguobacterium*, growth of wheat seedlings and reduced root infections by *R. solani*. However, the underlying mechanisms of plant growth promotion and antifungal activity of *Microbacterium* have, to our knowledge, not yet been investigated.

Here, we isolated and characterized *Microbacterium* species from the rhizosphere and endosphere of sugar beet plants grown in the *Rhizoctonia*-suppressive soil and tested their effects on plant and fungal growth. We specifically focused on the role of VOCs from the different *Microbacterium* species in plant growth promotion and activity against *R. solani*. We then zoomed in on endophytic *Microbacterium* sp. strain EC8 for comparative genomics, VOC profiling and VOC-mediated transcriptional responses induced in *Arabidopsis thaliana* seedlings.

Materials and Methods

Isolation of *Microbacterium*

Microbacterium isolates were obtained from the rhizosphere (roots with adhering soil) of sugar beet plants (*Beta vulgaris* cv. Aligator) grown in a soil suppressive to *R. solani* as previously described (Cordovez *et al.*, 2015). An additional isolation method based on Chapelle *et al.* (2015) with modifications was used to isolate *Microbacterium* endophytes. For that, roots of sugar beet seedlings were rinsed with 10 ml of 10 mM MgSO4.7H2O (hereafter: buffer) to remove the rhizospheric soil and washed three times with buffer supplemented with 0.01% Tween 20 (v/v). Subsequently, roots were immersed for 2 min under slow agitation in 1% (v/v) sodium hypochlorite solution supplemented with 0.01% (v/v) Tween 20 and then rinsed five times with buffer. To confirm that the roots were sterile, surface-sterilized roots were spread on Luria-Bertani (LB; Oxoid Thermo Scientific, Lenexa, USA) and 1/10th strength Tryptic Soy Agar (1/10th TSA; Difco, BD Laboratories, Houston, USA) agar plates. In addition, 100 µl of the last rinsing step were also plated. To separate plant from microbial cells, root tissue in buffer was disrupted using a blender. The homogenate was filtered consecutively through 25 µm and 10 µm-mesh cheesecloth to remove plant tissue. The flow through was further cleaned by centrifugation at 500 X g for 10 min. Bacterial cells were collected by centrifuging the supernatant at 9500 rpm for 15 min. The pellet, consisting of endophytic microorganisms and plant material, was suspended in 3.5 ml buffer supplemented with Nycodenz® resin (PROGEN Biotechnik, Germany) to a final concentration of 50% (w/v). A Nycodenz density gradient was mounted above the sample by slowly depositing various layers of Nycodenz (3 ml of 35% Nycodenz, 2 ml of 20% Nycodenz, 2 ml of 10% Nycodenz) and the gradient was centrifuged for 45 min at 8500 rpm (Sorvall HB-6). Endophytic microbiome, visualized as a whitish band, was recovered by pipetting. The recovered cells were washed five times with buffer and centrifuged at 13 000 g for 5 min in order to remove the Nycodenz resin. Finally, bacterial cells were suspended in 500 µl of buffer, recovered by quick centrifugation at 16000 X g.

Samples were frozen in liquid nitrogen and stored at -80°C. For the isolation of single cells, 100 µl were plated on 1/10th TSA medium.

Phylogeny of *Microbacterium*

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'- GAGTTTGATCCTGGCTCAG - 3') and 1492R (5'- ACCTTGTTACGACGACTT- 3') (Lane, 1991; DeAngelis *et al.*, 2009). For obtaining DNA, cell suspensions were prepared in TE buffer and centrifuged at 13.000 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 (http://greengenes.lbl.gov/) (McDonald *et al.*, 2012). The 16S *rRNA* gene sequences of the *Microbacterium* isolates were compared with the 16S rRNA gene sequences of *Microbacterium* OTUs previously identified by PhyloChip-based metagenomic analysis (the top 10% of most abundant taxa associated with disease suppressiveness) and *Microbacterium* type strains (Mendes *et al.*, 2011; Cordovez *et al.*, 2015). Phylogenetic analysis using partial sequences of 16S *rRNA* gene, resulted from alignment of 665 sites, was performed with Muscle (Edgar, 2004) in MEGA6 (Tamura *et al.*, 2013) and visualized with iTOL (Letunic & Bork, 2011; Letunic & Bork, 2016) (http://itol.embl.de/). A neighbor-joining consensus tree (Saitou & Nei, 1987) was constructed using Tamura-Nei model (Tamura & Nei, 1993) with the optimal model parameters and the option of complete deletion of gaps and gamma distribution. Confidence levels for the branching points were determined using 1,000 bootstrap replicates (Felsenstein, 1985).

VOC-mediated antifungal activity by *Microbacterium*

To investigate the effect of *Microbacterium* VOCs on the growth of the fungus *R. solani* (AG2-2 IIIB), two-compartment Petri dishes were used allowing physical separation between the bacteria and the fungus. Bacterial cells (50 µl of OD_{600} = 1 previously at 30 °C) were plated on one compartment containing TSA medium and, after 3 days, *R. solani* mycelium (∅ 5 mm-plug) of 5-7 days old culture) was added to the other compartment containing 1/10th TSA medium. Petri dishes were sealed and incubated at 25°C. As a control, *R. solani* was exposed to a Petri dish containing 1/10th TSA medium only. A total of 5-10 replicates were used per treatment. Student's t-Test was performed to determine statistically significant differences between *R. solani* culture exposed to *Microbacterium* VOCs and control.

To further test whether EC8 could also protect sugar beet plants against *R. solani*, we conducted soil bioassays. The bacterial culture (initial cell density: 10^9 cfu ml^{-1}) was inoculated into 250 g of conducive soil placed in plastic trays (19.5 × 6 × 3.5 cm). The soil moisture was brought to 20% using the bacterial culture and tap water to reach concentrations of 10^6 cfu g^{-1} soil. Sugar beet seeds (cv. Rhino coated with thiram, hymexazol and poncho-beta) were sown in a row, 1 cm apart. Eight replicates were prepared per treatment and non-inoculated conducive soil was used as control. Trays were closed and placed in a growth chamber (24 °C; 180 µmol light m^{-2} s^{-1} at plant level; 16 h : 8 h, light : dark; 70% R.H.). Five days after sowing, trays were open and *R. solani* was inoculated in soil. The first seedling was inoculated with the mycelial plug (∅ 5 mm from a 5-day-old 1/5th PDA culture) and disease incidence was monitored. Student's t-Test was performed to determine statistically significant differences between EC8-inoculated and non-inoculated soils.

VOC-mediated plant growth promotion by *Microbacterium*

To determine whether VOCs emitted by *Microbacterium* isolates had an effect on plant growth, *Arabidopsis thaliana* seedlings Col-0 (collection of the Department of Phytopathology at Wageningen University, the Netherlands) were exposed to the VOCs from the different isolates. Seeds were surface sterilized as previously described (van de Mortel *et al.*, 2012) and kept in the dark at 4 °C for 3-4 days. Sterile *Arabidopsis* seeds sown on Petri dishes (∅ 90 mm) containing 25 ml of 0.5X Murashige and Skoog medium (Murashige & Skoog, 1962) supplemented with 0.5% (w/v) sucrose. Petri dishes were placed inside a larger Petri dish (∅ 145 mm), sealed and incubated in a climate cabinet (21 °C; 180 µmol light m^{-2} s^{-1} at plant level; 16 h : 8 h, light : dark; 60-70% R.H.). After 7 days, seedlings were exposure to the VOCs emitted by the *Microbacterium* isolates. For that, Petri dishes (∅ 35 mm) containing *Microbacterium* cultures, previously incubated at 30 °C for 3 days (10 µl; OD_{600} = 1), were placed in the larger Petri dish (∅ 145 mm) containing the Petri dish (∅ 90 mm), with the seedlings. Plates were sealed and returned to growth cabinet. Plant fresh weight was determined after 14 days and plant dry weight was determined after overnight incubation at 65 °C. A total of 4-5 replicates were used per treatment. Student's t-Test was performed to determine statistically significant differences compared to the control treatment (plants exposed to medium only).

VOC profiling of *Microbacterium*

For the global VOC profile of *Microbacterium* isolates, solid phase microextraction (SPME) with a 65-mm polydimethylsiloxane-divinylbenzene fibre (Supelco, Bellefonte, USA) was

used. Isolates were inoculated (10 µl OD_{600} = 1) individually in 10 ml sterile glass vials containing 2.5 ml of TSA medium. A total of 3 replicates per treatment were used and vials containing medium only served as control. All vials were closed and incubated at 30°C. VOCs from the headspace of each vial were collected after 7 days. VOCs were analyzed by Gas Chromatography-Mass Spectrometry (GC-MS) and raw data was processed as previously described (Cordovez *et al.*, 2015). Hierarchical cluster analysis (HCA) using Pearson's correlation coefficient with UPGMA algorithm was performed with GeneMaths XT Version 2.11 (Applied Maths, Belgium).

Based on the results from the antifungal and plant growth-promotion assays, we decided to study the VOC profile of *Microbacterium* sp. EC8 in detail. *Microbacterium* cells (100 µl OD_{600} = 0.1) were plated on sterile glass Petri dishes (∅ 90 mm) containing 20 µl of TSA medium. Petri dishes were sealed and incubated at 30 °C for 6 days. VOC collection started right after plating, and for that, the lids of these Petri dishes were designed with an outlet where the Tenax tubes were connected. Trapped compounds were subjected to Gas Chromatography-Quadrupole Time of Flight-Mass Spectrometry (GC-QTF-MS). Compounds were desorbed from the Tenax tubes in a thermodesorption unit (model UnityTD-100, Markes International Ltd., Llantrisant, UK) at 210 °C for 12 min (Helium flow 50 ml min^{-1}) using 1:20 split ratio. Released compounds were focused on a cold trap at - 10 ° C and introduced into the GC-QTF-MS (Agilent 7890B GC and the Agilent 7200A QTOF, Santa Clara, USA). Compounds were transferred to the analytical column (30 m × 0.25 mm ID RXI-5MS, film thickness 0.25 µm; Restek 13424-6850, Bellefonte, PA, USA) by heating the cold trap to 250 °C for 12 min. Temperature program of the GC oven was: 39 ° C for 2 min, from 39° C to 95 ° C at 3.5 ° C min^{-1}, from 95 ° C to 165 ° C at 6 ° C min^{-1}, 165 ° C to 250 ° C at 15 ° C min^{-1} and finally, from 250 ° C to 300 ° C at 40 ° C min^{-1} and 20 min-hold at a constant gas flow of 1.2 ml min^{-1}. Mass spectra were acquired by electron impact ionization (70 eV) with a scanning from m/z 30-400 with a scan rate of 4 scans s^{-1}.

Mass-spectra were analyzed with MassHunter Qualitative Analysis Software B.07.00 (Agilent Technologies, Santa Clara, USA) using the GC-Q-TOF qualitative analysis module. VOCs were selected based on three criteria: peak intensity of at least 10^4, $P < 0.05$ (t-Test), and fold change (FC) > 2. Selected VOCs were tentatively identified by comparison of the mass spectra with those of NIST (National Institute of Standards and Technology, USA) and Wiley libraries and by comparing the experimentally calculated LRI with the literature values.

Genome sequencing and annotation of *Microbacterium* sp. EC8

For the DNA isolation, a single colony of *Microbacterium* sp. EC8 was inoculated in LB medium and incubated for 2 days at 25 °C. Prior to DNA isolation, bacterial pellet was collected by centrifugation and mixed with 0.9 % NaCl. Suspension was centrifuged and the pellet was washed with 0.9 % NaCl three times. Total genomic DNA was extracted using the MasterPure™ Complete DNA and RNA Purification Kit (Epicentre Biotechnologies, Madison, WI, USA) according to manufacturer's instructions with minor modifications. DNA sequencing of *Microbacterium* sp. EC8 was performed with PacBio sequencing and subsequent *de novo* assembly using the Hierarchical Genome Assembly Process (HGAP v3) in the SMRTPortal software v3.0 (Chin *et al.*, 2013). A library of approximately 10 KB was sequenced on the PacBio RS II in two SMRT cells. Reads were filtered for a minimal polymerase read length of 500 bp, a read quality of 0.8 and a minimal subread length of 500 bp. After assembly the remaining InDel and base substation errors in the draft assembly were reduced with two rounds of the Quiver algorithm. The resulting singular contig was checked using a custom script and overlapping ends trimmed. There was no evidence of plasmids in the sequence data.

Annotation was performed with a modified version of Prokka v 1.11 (Seemann, 2014). COG annotations for the predicted proteins were determined using KOGnitor (Snel *et al.*, 2002). Gene clusters encoding antibiotics and secondary metabolites were predicted using the online tool antiSMASH 3.0.5 with the ClusterFinder algorithm (Medema *et al.*, 2011; Weber *et al.*, 2015).

Comparative genomics of *Microbacterium*

A total of 24 reference genomes from the *Microbacteriaceae* family were used to perform whole-genome, core and housekeeping genes phylogeny (Table S1). Genome sequences with complete, scaffold and/or contig level sequences were downloaded from GenBank. Whole genome comparisons were conducted in Gegenees (Ågren *et al.*, 2012). In this program, the genome sequences were fragmented in 200 bp fragments using overlap size of 100 bp, and all-against-all BLASTn comparison with a 20% cutoff threshold for non-conserved material was conducted. A core phylogenetic tree was constructed using a set of 210 gene clusters shared by all *Microbaterium* spp. used in this study (Table S1). The presence of 31 well-conserved, single-copy, bacterial genes was established in each *Microbacterium* species proteome using AMPHORA (Wu & Eisen, 2008). Subsequently, a concatenated alignment and phylogenetic analyses of these marker genes was performed using RAxML v.8.0.2 (Stamatakis, 2014). From the generated distance matrices, whole genome, core and housekeeping genes, phylogenetic trees were made and visualized with

Splitstree4 (Huson & Bryant, 2006). Pan-core genome analyses were performed by clustering protein coding genes from closely related genomes used in the whole-genome phylogeny using CD-HIT (Huang *et al.*, 2010) with word length 3 (-n 3), global identity (-G 1 and a minimal alignment coverage of 60% for the shortest protein (-aS 0.6). CD-HIT clusters were parsed into a presence-absence matrix from which the pan, core and variable genomes were parsed using custom scripts. Protein coding genes of *Microbacterium* sp. EC8, *Microbacterium* sp. XT11, *Microbacterium oxydans* BEL162RN51 and *Microbacterium* sp. CGR1were used to construct a Venn diagram with Venny web-tool (http://bioinfogp.cnb.csic.es/tools/venny/).

Plant transcriptome analysis

Total RNA was extracted from shoot and root tissues *A. thaliana* seedlings exposed to the VOCs from *Microbacterium* sp. EC8 for one week. Seedlings exposed to TSA medium only were used as control. For the sequencing of plant RNA, total RNA was extracted from roots and shoots. A total of 4 replicates were used, and each replicate consisted of 4 plates with 6 seedlings each in order to obtain enough biomass. RNA was obtained from frozen tissues with Trizol reagent (Invitrogen). The RNA samples were further purified using the NucleoSpin RNA II kit (Macherey-Nagel) and kept at -80°C until sequencing. For RNA sequencing, samples were processed using the NEBNext Ultra Directional RNA Library Prep Kit for Illumina at ServiceXS (GenomeScan B.V., Leiden, The Netherlands). Briefly, mRNA was isolated from the total RNA suing the oligo-dT magnetic beads. After fragmentation of the mRNA, cDNA was synthesized, ligated with sequencing adapters and amplified by PCR in order to obtain cDNA libraries. Each cDNA library was individually analyzed for quality and yield using a Fragment Analyzer. cDNA was then clustered and a concentration of 1.6 pM was sequenced with an Illumina NextSeq 500 sequencer.

Illumina sequences were trimmed and filtered with FASTQC with a threshold of 25 (Q > 25). Quality-trimmed reads were counted using RSEM software package (Li & Dewey, 2011) transformed into RPKM (Reads Per Kilobases per Million reads). Reads were mapped to the *Arabidopsis* reference genes using the software Bowtie2 v.2.1.0 (Langmead & Salzberg, 2012). The Bioconductor package DESeq2 (Love *et al.*, 2014) was used for normalization and differential expression analyses. The *P*-value was obtained from the differential gene expression test. FDR (False Discovery Rate) manipulation was used to determine the P-value threshold in multiple tests and analyses. Significant differentially expressed genes (DEGs) were selected using FDR < 0.05 and the absolute value of the log2Ratio ≥ 0.585 (at least 1.5x higher than the expression level in control) as thresholds.

Biological interpretation of the DEGs was performed using Cytoscape software with ClueGO plugin (Bindea *et al.*, 2009).

Results

Isolation, diversity and activities of *Microbacterium*

Microbacterium isolates were obtained from the rhizosphere of sugar beet plants grown in a soil suppressive to *R. solani* as described previously (Cordovez *et al.*, 2015). Additionally, we isolated *Microbacterium* from the endosphere of sugar beet roots. For the phylogenetic characterization and functional analyses, a total of 26 isolates (20 from rhizosphere and 6 from endosphere) were selected. Subsequent analysis of the 16S *rRNA* sequences from these 26 isolates together with isolates previously detected in the sugar beet rhizosphere by PhyloChip (n = 40) and type strains (n = 17), showed that the rhizospheric and endospheric isolates (n = 26) were phylogenetically diverse and closely related to *M. paraoxydans*, *M. esteraromaticum*, *M. hydrothermale*, *M. oleivorans*, *M. marinum*, *M. aerolatum*, *M. invictum* and *M. schleiferi* (Fig. 1A). Several of the *Microbacterium* isolates obtained from the sugar beet roots by culture-based approaches matched phylogenetically to the isolates detected initially by the culture-independent PhyloChip-based analysis (Mendes *et al.*, 2011; Cordovez *et al.*, 2015). From the total of 26 *Microbacterium* isolates, 2 endophytic and 6 rhizospheric isolates were selected for a more detailed analysis of their VOC-mediated effects on fungal and plant growth.

To test the antifungal activity of *Microbacterium* VOCs, the fungal root pathogen *R. solani* was exposed to the VOCs emitted by each of the *Microbacterium* isolates. Five out of the eight *Microbacterium* isolates inhibited hyphal growth (Fig. 1B). VOCs emitted by the endophytic strain *Microbacterium* sp. EC8 (hereafter: EC8) showed the strongest antifungal effects with 45% (t-Test, $P < 0.001$) and 51% (t-Test, $P = 0.008$) hyphal growth inhibition after 2 and 3 days of exposure, respectively (Fig. 2A,B,C). Inhibition was still observed after several weeks of VOC exposure (data not shown). VOCs from EC8 caused melanization of the fungal hyphae and were fungicidal, as the fungus was not able to re-grow when transferred to and cultivated on a fresh agar plate in the absence of the bacterial VOCs (Fig. 2B). Subsequent soil bioassays with EC8 revealed that this strain is able to protect sugar beet seedlings from infection by *R. solani*. In EC8-inoculated soil, disease incidence was significantly reduced at 10 and 14 days after fungal inoculation (Fig. 2D).

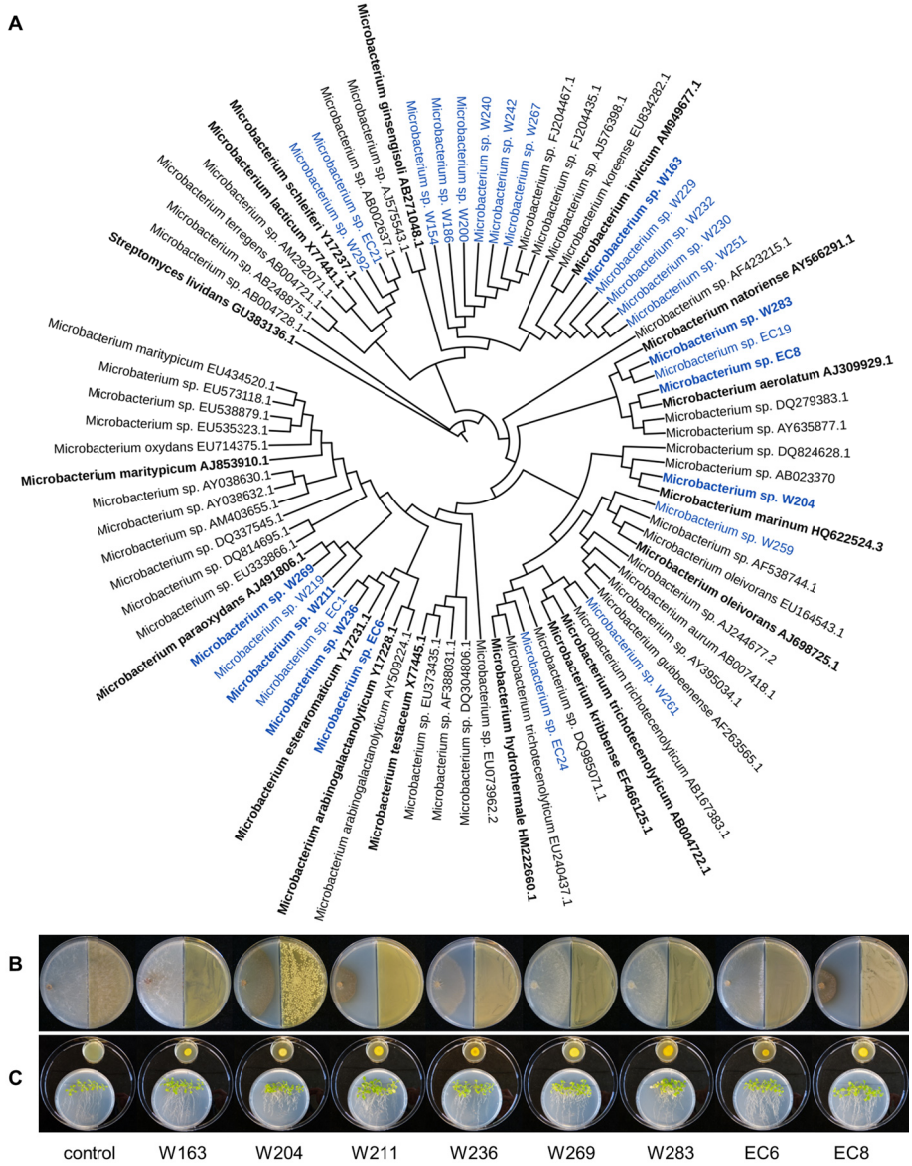


Fig. 1 | Diversity and activities of *Microbacterium* species. **(A)** Phylogenetic analysis of the 26 *Microbacterium* isolates used in this study. Neighbor-joining phylogenetic tree based on 16S *rRNA* sequences. Phylogenetic tree was constructed with MEGA6 (Tamura *et al.* 2013) and visualized with iTOL (Letunic and Bork 2016). The *Microbacterium* isolates described in this study are highlighted in blue, *Microbacterium* isolates detected by Phylochip analysis (Mendes *et al.* 2011) in black, *Microbacterium* type strains in bold. Isolates selected for plant growth promotion, antifungal and VOC profiling analyses are indicated in bold blue. **(B)** *In vitro* activity of *Microbacterium* VOCs against the fungal root pathogen *Rhizoctonia solani*. Pictures were taken 7 days after exposure. **(C)** Effects of *Microbacterium* VOCs on growth and root architecture of *Arabidopsis* seedlings. Pictures were taken 14 days after exposure.

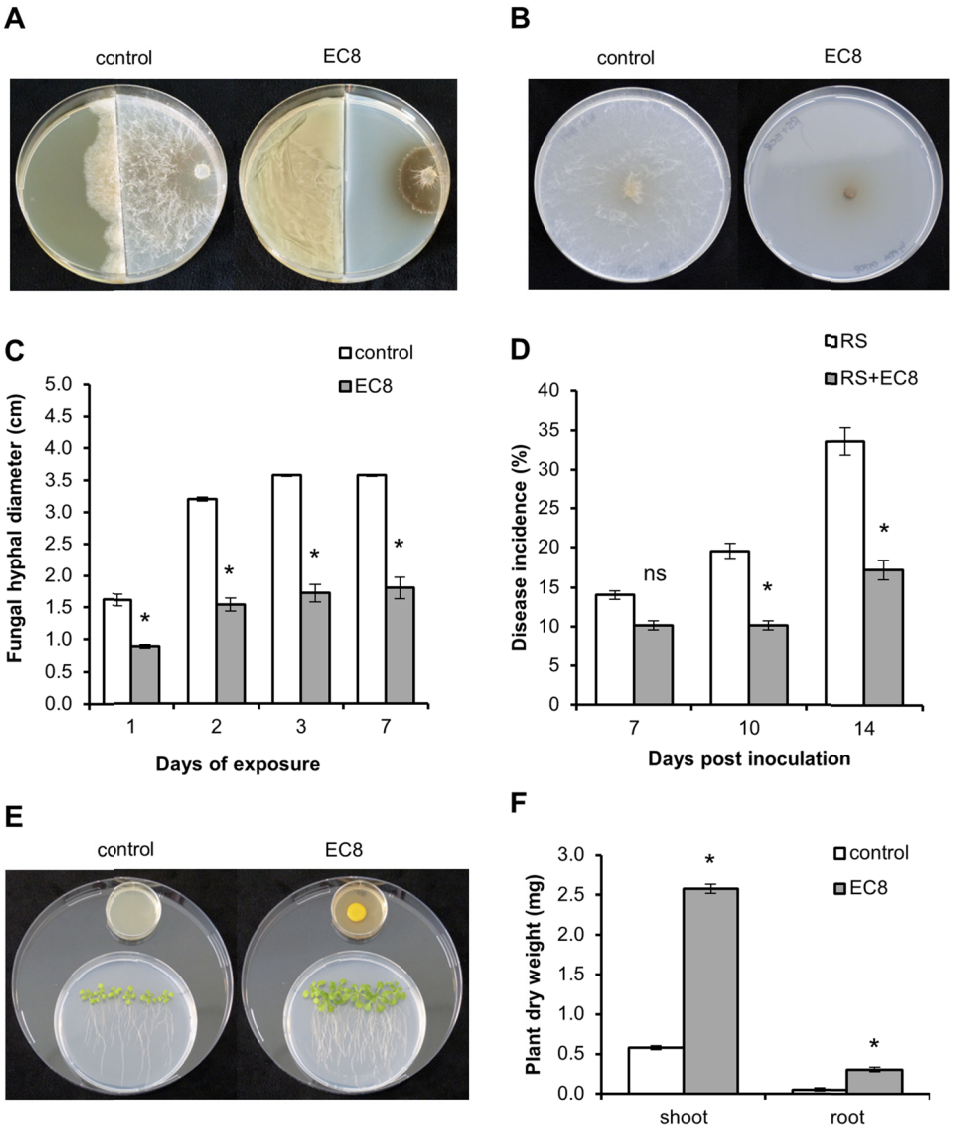


Fig. 2 | Effects of volatile organic compounds (VOCs) emitted by *Microbacterium* sp. EC8 on fungal and plant growth. **(A)** *Rhizoctonia solani* growth inhibition after 7 days of exposure to VOCs from EC8. **(B)** Fungicidal effect of VOCs from EC8. **(C)** Hyphal growth (mean ± SE, n = 10) of *R. solani* after 1, 2, 3 and 7 days of exposure to VOCs from EC8. **(D)** Effects of EC8 on damping-off disease incidence of sugar beet plants caused by *R. solani* (RS). **(E)** Phenotypic changes of *Arabidopsis* seedlings after 14 days of exposure to VOCs from EC8. **(F)** Biomass (mean ± SE, n = 4-5) of shoots and roots of VOC-exposed and control seedlings (exposed to the agar medium only). Asterisks indicate statistically significant differences in pairwise comparisons between VOC-exposed and control seedlings (Student's t-Test, $P < 0.05$).

To test for plant growth-promotion, 7-day-old *Arabidopsis* seedlings were exposed to the VOCs emitted by each of the *Microbacterium* isolates. All *Microbacterium* isolates tested promoted the growth of *Arabidopsis* seedlings *in vitro* (Fig. 1C, S1). VOCs of the eight *Microbacterium* isolates induced significant increases in shoot and root biomass of *Arabidopsis*, but differed in their effects on root architecture (Fig. 1C). Seedlings exposed to the VOCs from EC8 showed an increase of 345% ($P < 0.001$) and 472% (t-Test, $P < 0.001$) in shoot and root biomass, respectively (Fig. 2E,F). Effects of VOCs from this strain were the strongest on root tissue compared to the other isolates (Fig. S1). Exposure of *Arabidopsis* seedlings to different initial cell densities of EC8 (ranging from 10^2 to 10^9 cfu/ml) did not result in significant differences in shoot and root biomass increases. However, when cell suspensions of EC8 were spread onto the entire surface of the agar medium (10 µl of 10^9 cfu/ml culture) instead of spot-inoculated, substantial adverse effects on plant growth were observed (Fig. S2). Given its exceptionally strong antifungal and plant growth-promoting effects, we selected the endophytic *Microbacterium* strain EC8 for further in-depth analysis.

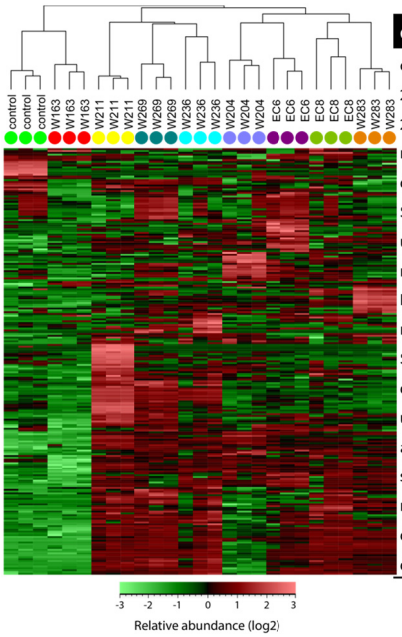
VOC profiling and genomics of *Microbacterium* sp. EC8

Analysis of the headspace of cultures of the 8 different *Microbacterium* isolates provided a global profile of the VOCs emitted by the different isolates. Hierarchical cluster analysis showed that the VOC profiles of the different isolates were diverse (Fig. 3A). To study the diversity of the VOCs in more detail, headspace VOCs of cultures of *Microbacterium* sp. EC8 were collected for 6 days and analyzed by GC-QTOF-MS. A total of 18 VOCs were detected that were not found in the control (agar medium only) or were detected with peak areas significantly different (t-Test, $P < 0.05$) and at least 2-fold larger than those in the control (Table S2). The vast majority of VOCs that met these criteria were identified as sulfur-containing compounds (Fig. 3B). These included sulfur-containing compounds commonly found for other bacterial genera such as dimethyl-disulfide and dimethyl trisulfide, but also more rare compounds such as S-methyl 2-methylpropanethioate and S-methyl pentanethioate. In addition, four ketones, including 3,3,6-trimethylhepta-1,5-dien-4-one (also known as Artemisia ketone), were found in the headspace of EC8 culture.

To identify the genetic basis of VOC production in *Microbacterium* strain EC8, we sequenced and analyzed its genome. The assembled EC8 genome consists of a single chromosome of 3.18 Mb with G+C content of 67.5% and 3,101 coding DNA sequences (CDSs) (Table S1). A total of 2,127 CDSs (68.6%) could be assigned to putative functions, while the remaining 974 CDSs (31.4%) were annotated as hypothetical proteins of unknown function. The three most abundant COG classes are ‘amino acid transport and

metabolism' (7.9%), 'carbohydrate transport and metabolism' (7.9%) and 'transcription' (6.4%) (Fig.S3). Putative genes associated with the production of VOCs include those involved in sulfur metabolism and hormone (IAA) biosynthesis (Fig. 4). Using antiSMASH, we further identified 35 gene clusters encoding for secondary metabolite including the terpene biosynthesis and type III polyketide synthase gene clusters (T3pks) (Fig. S4). Twenty-seven (77%) out of these 35 identified gene clusters did not have a hit to known clusters, highlighting the potential of *Microbacterium* sp. EC8 as a source of new secondary metabolites. Phylogenetic analyses using the whole genome sequence showed that EC8 is closely related to *Microbacterium* sp. XT11, a soil isolate with xanthan-degrading properties (Qian *et al.* 2007) (Fig. S5). Pan-core genome analysis performed with EC8 and three closely related *Microbacterium* isolates further showed a total of 1195 genes (16.4%) common for all 4 genomes, and 1163 unique genes (16%) for EC8 (Fig. 5).

A



B

Compound	IUPAC name	RI
cyclopentene	cyclopentene	543
2-butanone	butan-2-one	600
2-pentanone	pentan-2-one	688
methyl thiolacetate*	S-methyl ethanethioate	704
dimethyl disulfide*	(methyldisulfanyl)methane	750
S-methyl propanethioate*	S-methyl propanethioate	800
unknown		806
methyl isobutanethioate	S-methyl 2-methylpropanethioate	847
bis(methylthio)methane	2,4-dithiapentane	886
methyl propyl disulfide	1-(methyldisulfanyl)propane	888
S-methyl pentanethioate	S-methyl pentanethioate	940
dimethyl trisulfide*	(methyltrisulfanyl)methane	973
unknown ketone		1036
artemisia ketone	3,3,6-trimethylhepta-1,5-dien-4-one	1051
sulfur-containing compound		1059
methyl (methylthio)methyl disulfide	disulfide, methyl (methylthio)methyl	1122
dimethyl tetrasulfide	(methyltetrasulfanyl)methane	1212
dimethyl pentasulfide	(methylpentasulfanyl)methane	1460

Fig. 3 | Profiling of volatile organic compounds (VOCs) emitted by *Microbacterium*. **(A)** Hierarchical cluster and heat-map analyses of VOC profiles of Microbacterium isolates. Columns represent three replicate VOC measurements of each of the 8 isolates and the medium alone (control). Rows represent the different VOCs (green: low abundance, red: high abundance). **(B)** List of VOCs emitted by *Microbacterium* sp. EC8. VOCs displayed were detected only for strain EC8 or were significantly different (Student's t-Test, $P < 0.05$, $n = 3$) and detected at peak intensities at least twice as high as in the control (medium only) (Table S2). Compounds were putatively annotated by comparing their mass spectra (MS) and calculated retention indices (RI) with those of NIST and in-house mass spectral libraries and standard (*).

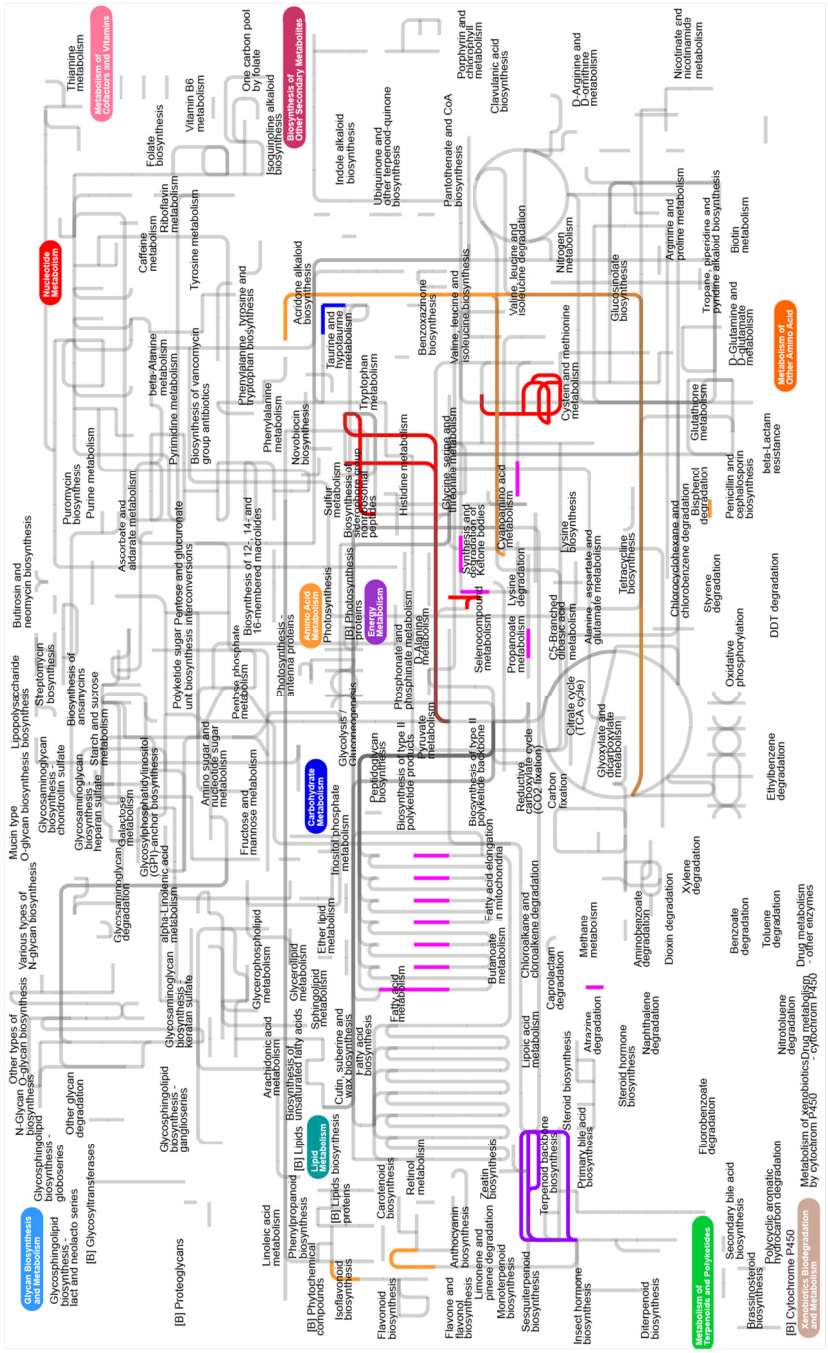


Fig. 4 | *Microbacterium* sp. EC8 genes involved in sulfur metabolism (red), and the biosynthesis of secondary metabolites (orange), terpenoids (purple), and indole-3-acetic acid (IAA) (blue). The metabolic map was generated with the web-based tool iPath (http://pathways.embl.de) (Letunic *et al.*, 2008).

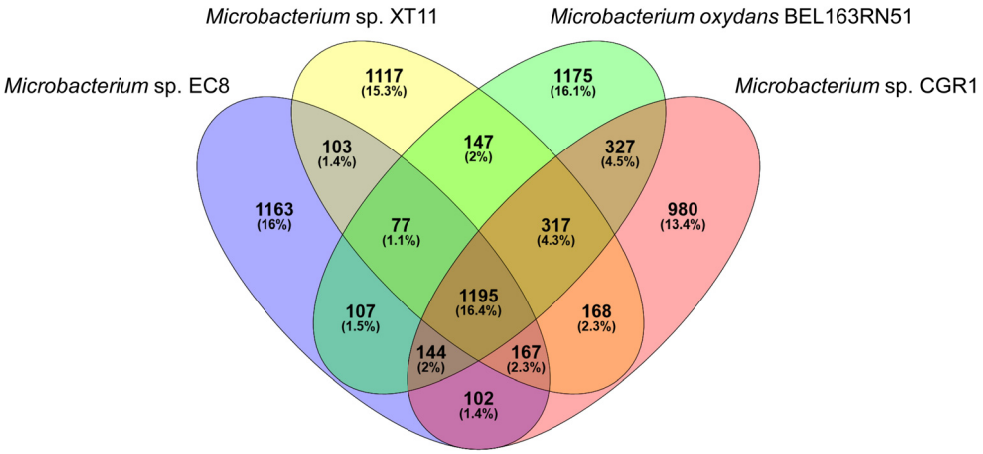


Fig. 5 | Pan-core genome analysis of *four Microbacterium* isolates. Venn diagram shows numbers of unique and shared genes found in each of the four *Microbacterium* genomes. The core orthologous gene number is depicted in the center of the diagram.

Plant transcriptional changes induced by VOCs emitted by *Microbacterium* EC8

To understand the molecular mechanisms underlying VOC-mediated plant growth promotion by EC8, RNAseq analysis was performed for *Arabidopsis* seedlings exposed for one week to the bacterial VOCs. Genes of shoot and root tissues with an adjusted $P < 0.05$ and with a value of log2|Ratio| ≥ 0.585 or ≤ 0.585 (1.5-fold change) were considered as differentially expressed from the non-exposed (control) seedlings. A total of 946 (545 up- and 401 down-regulated) and 1361 (698 up- and 663 down-regulated) differentially expressed genes (DEGs) were identified in shoot and root tissues, respectively. *Arabidopsis* root tissue displayed a higher number of transcriptional changes compared to shoot tissue. GO terms associated with shoot DEGs were grouped into 23 functional clusters including 'purine ribonucleoside metabolic process', 'response to cytokinin', 'response to ethylene', 'response to oxidative stress' and several processes related to sulfur metabolism including 'sulfate assimilation', 'sulfur compound catabolic process', 'sulfur compound metabolic process' and 'S-adenosylmethionine metabolic process' (Fig. 6A, Fig. S6). Down-regulated shoot DEGs were grouped into 13 functional clusters and included 'cellular carbohydrate metabolic process', 'regulation of post-embryonic development', 'plastic organization' and 'movement of cell or subcellular component' (Fig. 6A, Fig. S6). GO terms associated with up-regulated DEGs in root tissue were grouped into 31 functional clusters including 'nitrate assimilation', 'small molecule catabolic process', 'jasmonic acid metabolic process', 'regulation of actin filament polymerization', acetyl-CoA metabolic

process' and 'response to oxidative stress' (Fig. 6B, Fig. S7). Down-regulated root DEGs were grouped into 6 functional clusters including 'anion transport', 'response to herbicide', 'transmembrane transport' and 'syncytium formation' (Fig. 6B, Fig. S7).

A total of 20 genes involved in sulfur metabolism and transport were found to be differentially expressed in shoot and root tissues upon exposure to VOCs from EC8. DEGs involved in sulfur metabolism were mostly up-regulated in shoots, but down-regulated in root tissue. Genes encoding for the S-adenosylmethionine synthases SAM-1 (AT1G02500.1) and SAM-2 (AT4G01850.2), adenosylhomocysteinase MEE58 (AT4G13940.1) and SAHH2 (AT3G23810.1) were specifically up-regulated in shoot tissue, whereas genes encoding for the phosphosulfate reductases APR1 (AT4G04610.1) and APR3 (AT4G21990.1) were specifically down-regulated in root tissues. DEGs involved in sulfur transport such as the genes encoding for the sulfate transporters SULTR1.2 (AT1G78000.2), SULTR3.2 (AT4G02700.1) and SULTR4.2 (AT3G12520.2) were down-regulated in root tissue. Furthermore, several genes encoding glutathione transferases were up-regulated in shoot tissue, such as GSTU13 (AT1G27130.1), GSTU19 (AT1G78380.1), GSTF9 (AT2G30860.1) and GSTF10 (AT2G30870.1), whereas GSTU1 (AT2G29490.1), GSTU7 (AT2G29420.1), GSTU8 (AT3G09270.1), GSTU10 (AT1G74590.1) and GSTU19 (AT1G78380.1) were down-regulated in root tissue. The methionine gamma-lyase MGL (AT1G64660.1), which is involved in methionine homeostasis, and the beta-thioglucoside glucohydrolase TGG2 (AT5G25980.2), which catalyzes the hydrolysis of glucosinolates, were 3.5 and 2.9-fold up-regulated in shoot and root tissues, respectively. Collectively these results showed that VOCs from EC8 have a significant impact on sulfur metabolism and transport in *Arabidopsis*.

Transcriptome analysis further showed an enrichment of genes involved in nitrate-related processes in VOC-exposed root tissue. DEGs of root tissue involved in nitrate assimilation were up-regulated, whereas DEGs involved in nitrate reduction were down-regulated. Expression of genes encoding the three nitrate transporters NRT2.1 (AT1G08090.1), NRT2.6 (AT3G45060.1) and NRT2.7 (AT5G14570.1), and a chlorine channel CLC-A was up-regulated, whereas the genes for nitrate reductases NIA1 (AT1G77760.1) and NIA2 (AT1G37130.1) were down-regulated. Shoot genes involved in nitrate-related processes were not found to be differentially expressed. Nitrate has been reported to not only serve as a nutrient for plants, but also to act as a signal in the regulation of carbon and nitrogen metabolism (Scheible *et al.*, 1997). Thus, our results indicate that VOCs from EC8 may impact on the transport of nitrate and that they may affect carbon and nitrogen metabolism in roots of *Arabidopsis*.

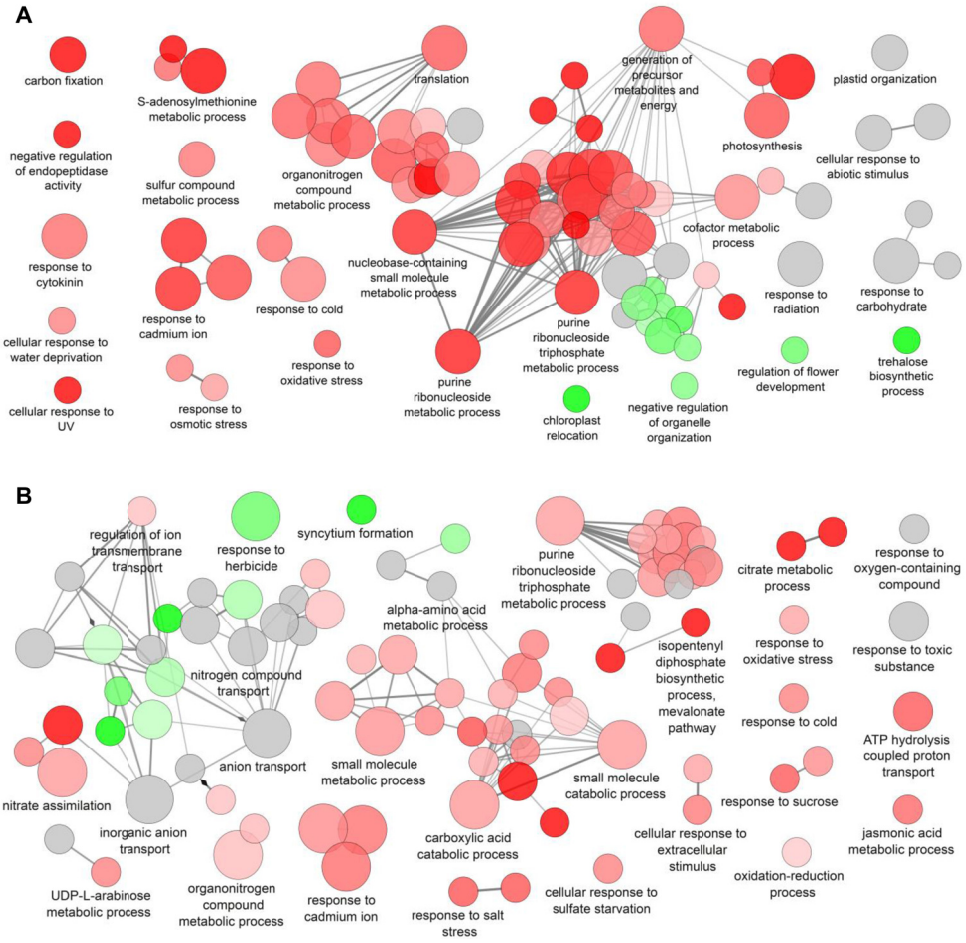


Fig. 6 | Enrichment analysis of differentially expressed genes (DEGs) of *Arabidopsis thaliana* shoots **(A)** and roots **(B)** exposed to volatile organic compounds (VOCs) emitted by *Microbacterium* sp. EC8. Single cluster analysis was performed using Cytoscape software with ClueGO plugin. Fusion option was used to reduce redundancy of GO terms. Networks with terms functionally grouped with GO pathways are indicated as nodes (Two-sided hypergeometric test corrected with Benjamini-Hochberg $P < 0.05$) linked by their kappa score level (≥ 0.4), with only the label of the most significant term per group shown. Functional groups of up-regulated DEGs are shown in red whereas down-regulated DEGs are shown in green. Functional groups with up- and down-regulated DEGs are shown in grey.

Several other genes involved in plant growth and development were enriched among the up-regulated DEGs. For example, genes involved in regulation of actin assembly were up-regulated in root tissue exposed to VOCs from EC8 and included genes encoding for the proteins villin PFN2 (AT4G29350.1) and profilin VLN3 (AT3G57410.1). Villins regulate directional organ growth (van der Honing *et al.*, 2012), whereas profilins play a role in cell elongation, cell shape maintenance, polarized growth of root hair, and flowering time (Ramachandran *et al.*, 2000). In addition, the nitrate transporter protein NRT2.1 (AT1G08090.1), proposed to coordinate the development of the root system with nutritional cues (Little *et al.*, 2005), was also up-regulated in root tissue. Down-regulated DEGs included genes involved in reproduction such as flowering and seed development. These included genes encoding the transcription factor MAF3 (AT5G65060.1), a floral repressor, and the early flowering protein ELF3 (AT2G25930.1) and ELF6 (AT5G04240.1) both involved in the photoperiodic induction of flowering (Zagotta *et al.*, 1996; Noh *et al.*, 2004). These findings suggest that *Arabidopsis* seedlings invest in growth instead of reproduction upon exposure to VOCs from EC8.

Furthermore, genes involved in hormone signaling were also differentially expressed in seedlings exposed to VOCs from strain EC8. DEGs involved in response to ethylene and cytokinin were up-regulated in shoot tissue and DEGs involved in jasmonic acid metabolism were up-regulated in root tissue. VOC-exposed shoot tissue showed an up-regulation of three ethylene-responsive transcription factors, CRF4 (AT4G27950.1), ERF3 (AT1G50640.1) and ERF8 (AT1G53170.1). Genes involved in plant resistance, in particular the genes encoding for plant defensin PDF1.2 (AT5G44420.1) and pathogenesis-related protein PR4 (AT3G04720.1) were 27.7- and 8.8-fold up-regulated in shoot tissue. The latter was also 7-fold up-regulated in root tissue. Also, the genes encoding for the map kinase kinase MKK9 (AT1G73500.1), involved in ethylene and camalexin biosynthesis (Xu et al. 2008), and for the cytochrome P450 protein CYP94B3 (AT3G48520.1), described to be involved in jasmonate-mediated signaling pathway (Kitaoka *et al.*, 2011; Koo *et al.*, 2011), were up-regulated. Collectively these results indicate that EC8 VOCs modulate the expression of genes in signal transduction pathways associated with induced resistance.

Table 1 | Differentially expressed genes (DEGs) of *Arabidopsis thaliana* seedlings exposed to volatile organic compounds (VOCs) emitted by *Microbacterium* sp. EC8. One-week-old seedlings were exposed to VOCs for one week. DEGs involved in metabolism and transport of sulfur and nitrate, response to ethylene and cytokinin, and biosynthesis of jasmonic acid are indicated in bold. Fold change (FC) was calculated using the log_2FC (VOC-exposed seedlings/control).

Code	Name	Description	FC shoots	FC roots
Sulfur metabolism and transport				
AT1G02500.1	SAM-1	S-adenosylmethionine synthase	**1.7**	1.1
AT4G01850.2	SAM-2	S-adenosylmethionine synthase	**1.7**	-1.1
AT4G13940.1	MEE58	S-adenosyl-L-homocysteine hydrolase	**2.5**	2.1
AT3G23810.1	SAHH2	S-adenosyl-L-homocysteine hydrolase	**2.1**	1.6
AT1G64660.1	MGL	methionine gamma-lyase	**3.5**	**2.7**
AT5G25980.2	TGG2	thioglucoside glucohydrolase	**2.9**	1.0
AT1G27130.1	GSTU13	glutathione transferase	**2.8**	1.3
AT1G78380.1	GSTU19	glutathione transferase	**1.7**	-1.7
AT2G30860.1	GSTF9	glutathione transferase	**2.1**	1.1
AT2G30870.1	GSTF10	glutathione transferase	**2.3**	**2.2**
AT2G29490.1	GSTU1	glutathione transferase	-1.0	**-1.7**
AT2G29420.1	GSTU7	glutathione transferase	-1.3	**-3.0**
AT3G09270.1	GSTU8	glutathione transferase	-1.2	**-2.2**
AT1G74590.1	GSTU10	glutathione transferase	**-2.9**	**-3.9**
AT1G78380.1	GSTU19	glutathione transferase	1.7	**-1.7**
AT4G04610.1	APR1	phosphosulfate reductase	-1.1	**-3.6**
AT4G21990.1	APR3	phosphosulfate reductase	-1.2	**-1.9**
AT1G78000.2	SULTR1.2	sulfate transporter	-1.9	**-6.9**
AT4G02700.1	SULTR3.2	sulfate transporter	1.0	**-2.0**
AT3G12520.2	SULTR4.2	sulfate transporter	-1.6	**-3.4**
Nitrogen metabolism and transport				
AT1G08090.1	NRT2.1	nitrate transporter	1.0	**14.0**
AT3G45060.1	NRT2.6	nitrate transporter	-1.5	**2.4**
AT5G14570.1	NRT2.7	nitrate transporter	-1.7	**1.5**
AT1G77760.1	NIA1	nitrate reductase	1.0	**1.6**
AT1G37130.1	NIA2	nitrate reductase	-1.5	**-7.3**
AT5G40890.1	CLC-A	chloride channel	-1.7	**6.1**
Response to ethylene				
AT4G27950.1	CRF4	ethylene-responsive transcription factor subfamily	**2.2**	-1.3
AT1G50640.1	ERF3	ethylene-responsive transcriptional factor	**1.7**	**-1.8**
AT1G53170.1	ERF8	ethylene-responsive transcriptional factor	**2.2**	1.5
AT2G05520.5	GRP-3	glycine-rich protein	**5.0**	1.1
AT3G50480.1	HR4	homolog of RPW8	**2.0**	1.2
AT5G44420.1	PDF1.2	plant defensin	**27.7**	1.0
AT3G04720.1	PR4	pathogenesis-related protein	**8.8**	**7.0**
AT1G75950.1	SKP1	SCF family of E3 ubiquitin ligases	**1.7**	1.6
AT1G25560.1	TEM1	RAV transcription factor family	**1.7**	2.0
AT3G62980.1	TIR1	auxin receptor	**1.8**	**2.3**
AT5G54810.1	TSB1	tryptophan synthase beta subunit	**1.5**	1.9
AT1G73500.1	MKK9	MAP kinase kinase family	**2.2**	-2.0

Code	Name	Description	FC shoots	FC roots
Response to cytokinin				
AT5G09810.1	ACT7	actin gene family	**1.5**	1.4
AT3G22890.1	APS1	ATP sulfurylase	**1.8**	-1.4
AT1G07890.3	APX1	cytosolic ascorbate peroxidase	**2.1**	1.0
AT4G09650.1	ATPD	chloroplast ATPase delta-subunit	**1.6**	1.8
AT3G25520.1	ATL5	ribosomal protein	**1.9**	**3.1**
AT4G27950.1	CRF4	ethylene response factor subfamily	**2.2**	-1.3
AT5G10360.1	EMB3010	ribosomal protein small subunit	**1.7**	1.2
AT2G40490.1	HEME2	uroporphyrinogen decarboxylase	**1.7**	2.1
AT1G56070.1	LOS1	translation elongation factor 2-like protein	**1.7**	**2.5**
AT3G09260.1	PYK10	beta-glucosidase	**1.7**	-1.1
AT4G38630.1	RPN10	non-ATPase subunit of the 26S proteasome	**1.6**	**2.1**
AT3G11940.2	RPS5A	ribosomal protein	**1.6**	1.3
AT4G01850.2	SAM-2	S-adenosylmethionine synthase	**1.7**	-1.1
Jasmonic acid biosynthesis				
AT4G16760.1	ACX1	acyl-CoA oxidase	1.0	**13.9**
AT4G29010.1	AIM1	L-3-hydroxyacyl-CoA hydrolyase	-1.4	**2.9**
AT3G48520.1	CYP94B3	jasmonoyl-isoleucine-12-hydroxylase	1.0	**4.3**
AT4G03560.1	TPC1	depolarization-activated Ca(2+) channel	-1.3	**2.2**

Discussion

Bacterial VOCs have been reported to positively and negatively affect plant performance. However, most studies on VOC-mediated interactions have focused on species and strains belonging to the bacterial genera *Pseudomonas* and *Bacillus* (Zhang *et al.*, 2007; Meldau *et al.*, 2013; Hernández-León *et al.*, 2015; Park *et al.*, 2015; Ryu, 2015). Although members of the *Microbacterium* genus are widespread in nature, their VOC-mediated effects on plant growth and development as well as on plant pathogens are currently unknown. Here, we showed that VOCs emitted by different *Microbacterium* isolates promoted the growth of *Arabidopsis* and inhibited plant infection by the root pathogen *R. solani*.

Profiling of VOCs from EC8 identified an array of sulfur-containing compounds, including dimethyl disulfide and dimethyl trisulfide. In addition, genome analysis of EC8 identified several genes involved in sulfur metabolism, including cystathionine-γ-lyase that encodes an enzyme involved in dimethyl disulfide production (Meldau *et al.*, 2013). The VOCs dimethyl disulfide and dimethyl trisulfide are produced by several bacterial genera and known to inhibit mycelial growth of fungal pathogens such as *Alternaria*, *Sclerotinia* and *Fusarium* (Fernando *et al.*, 2005; Groenhagen *et al.*, 2013; Wu *et al.*, 2015). In addition to these sulfur-containing VOCs, we also detected ketones, including the putatively identified 3,3,6-trimethylhepta-1,5-dien-4-one (also known as Artemisia ketone) in the VOC profile of EC8. This compound is a major component of the essential oils of several *Artemisia* plants, which possess antibacterial and antifungal activities (Kazemi, 2014; Stappen *et al.*, 2014). Soylu *et al.* (2005) previously showed that volatile components of the essential oil from *Artemisia annua* inhibit the growth of *Sclerotinia sclerotiorum*, *Botrytis cinerea*, *Phytophthora infestans* and *Verticillium dahliae.* Although these VOCs have antifungal activities, further investigation is still needed to determine if these VOCs are indeed responsible for the antifungal effects against *R. solani* observed *in vitro* and *in situ*. Plant protection by VOCs from EC8 could also be a result of induced systemic resistance (ISR) as was previously demonstrated for VOCs from other bacterial genera (Ryu *et al.*, 2004). More specifically, application of dimethyl disulfide as a soil drench protected corn and tobacco plants against *Cochliobolus heterostrophus* and *Botrytis cinerea*, respectively (Huang *et al.*, 2012).

Our genome-wide transcriptome analyses of *Arabidopsis* exposed to VOCs from EC8 revealed that ethylene/jasmonic acid and cytokinin signaling pathways as well as sulfur and nitrogen metabolism were affected. VOC exposure strongly induced the up-regulation of the plant basic chitinase PR4 and the defensin protein PDF1.2. These proteins have been shown to be induced by pathogen attack and by ethylene in *Arabidopsis* and peach plants (Gu *et al.*, 2002; Ruperti *et al.*, 2002). *PDF1.2* is also a marker for jasmonate-

dependent defense responses. These genes have been reported to be regulated by ethylene response factors (ERFs). Although the proteins PDF1.2 and PR4, together with NPR1, are associated with systemic acquired resistance (SAR), we have not detected an up-regulation of *NPR1* in VOC-exposed plants. In fact, *NPR1* was 2-fold down-regulated in root tissue. Several ERF proteins have been suggested to play a role in plant growth and development as well as to function as activators or repressors of pathogen-related genes. Here, we found an up-regulation of ERF3 and ERF8 in shoot tissue, which were suggested to be involved in the regulation of gene expression by stress factors and to be a transcriptional inhibitor of other ERFs (Fujimoto *et al.*, 2000; Ohta *et al.*, 2001). However, these transcriptional changes were not enough to repress the expression of the pathogen-related (PR) genes. Also, the genes *TGG2*, *ACX1* and *AIM1*, previously reported to be involved in plant resistance to herbivores, and genes involved in the biosynthesis of jasmonic acid were found to be up-regulated in VOC-exposed seedlings (Castillo *et al.*, 2004; Barth & Jander, 2006). Taken together, these results suggest that VOCs from EC8 modulate the expression of genes involved in SAR as well as of genes involved in ISR.

Previous studies have shown that VOCs produced by different rhizobacteria and soil-borne fungi can promote plant growth and change plant development by affecting auxin signaling and transport in the plant (Zhang *et al.*, 2007; Bailly *et al.*, 2014; Bitas *et al.*, 2015). Ethylene regulates auxin synthesis and a cross-talk between these two plant hormones is known to regulate root growth (Růžička *et al.*, 2007; Negi *et al.*, 2008; Robles *et al.*, 2013). Here we found that exposure of *Arabidopsis* to VOCs from EC8 up-regulated the auxin receptor TIR1 in both shoot and root tissues, which mediates the degradation of Aux/IAA proteins and auxin-regulated transcription. Together with the SCF ubiquitin ligase proteins, TIR1 regulates root and hypocotyl growth, lateral root formation and cell elongation (Dharmasiri *et al.*, 2005). However, we did not identify an enrichment of genes involved in auxin signaling in VOC-exposed *Arabidopsis* seedlings, suggesting that EC8-mediated plant growth promotion may involve other mechanisms. Possible mechanisms may be modulation of sulfur and nitrogen metabolism and transport or direct assimilation of sulfur-containing VOCs from EC8. In nature, inorganic sulfur is taken up by roots in the form of sulfate. However, 95% of the sulfur present in soils is bound to organic molecules (organosulfur) and is not directly available to plants. Soil microorganisms play a critical role by catalyzing organosulfur compounds and allowing access to the plants (Kertesz & Mirleau, 2004). For example, the VOC dimethyl disulfide emitted by *Bacillus* sp. BG55 promotes growth of *Nicotiana attenuata* plants (Meldau *et al.*, 2013). Meldau *et al.* (2013) further showed that tobacco seedlings absorb and assimilate dimethyl disulfide.

Our genome-wide transcriptome revealed that VOCs from EC8 alter sulfur and also nitrogen metabolism of exposed seedlings. Genes involved in nitrate transport and assimilation were up-regulated in VOC-exposed root tissue, whereas genes involved in sulfur assimilation and biosynthesis were up-regulated in shoot tissue, but down-regulated in root tissue. Assimilation of nitrogen and sulfur by plants are well coordinated processes and are involved in the synthesis of cysteine, an important structural and functional component of proteins and enzymes. However, the molecular mechanisms, sensors and signals involved in this regulation are largely unknown (Kruse *et al.*, 2007). Three nitrogen transporters, NRT2.1, NRT2.6 and NRT2.7, were up-regulated in root tissue, with NTR2.1 showing a 14-fold up-regulation. The latter has been reported to be regulated by nitrate and to function as a negative regulator of lateral root initiation under high sucrose and low nitrate condition, whereas NRT2.6 has been reported to be involved in growth promotion of *Arabidopsis* by the rhizobacterium *Phyllobacterium brassicacearum* STM196 (Little *et al.*, 2005; Kechid *et al.*, 2013). Kechid *et al.* (2013) showed that NRT2.6 together with NRT2.5 are up-regulated in *Arabidopsis* leaves inoculated with the bacteria, suggesting that these genes might be part of the regulation of the nitrogen control of root development. We showed that VOC-exposure induced an up-regulation of nitrogen transporters in root tissue, however, no differential expression was found in shoot tissue. Among the genes involved in sulfur metabolism and transport, different members of the glutathione S-transferases family (GSTs) were found to be differentially expressed in shoot and root tissue. GSTs are ubiquitous in plants and have been suggested to be involved in herbicide detoxification and stress response (Wagner *et al.*, 2002). However, little is known about their roles in normal plant physiology, during biotic and abiotic stress response (Nutricati *et al.*, 2006) and in bacteria-plant interactions.

In conclusion, this study explored the genome and volatome of endophytic *Microbacterium* sp. EC8 as well as plant transcriptional changes induced in *Arabidopsis* mediated by VOCs from EC8. Further exploration of the molecular mechanisms underlying VOC-mediated interactions between EC8 and plants will be needed to translate the findings of this study into strategies to increase plant growth and resistance and ultimately, help to minimize fertilizer and pesticide inputs.

Acknowledgments

We thank Hans Zweers for running the GC-MS samples and Dr Kees Hordijk for assisting with the identification of volatile organic compounds.

Supplementary Materials

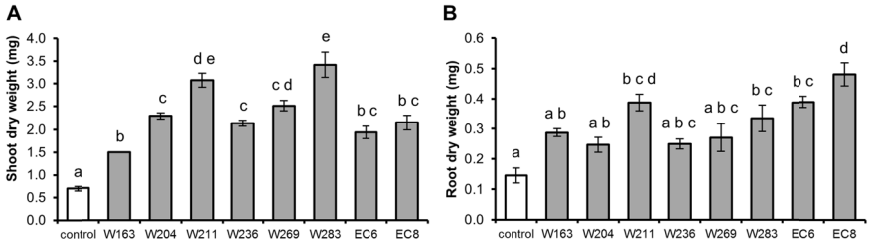


Fig. S1 | Plant growth-promoting effects of VOCs emitted by *Microbacterium* isolates in vitro. Biomass (mean ± SE, n = 4-5) of shoots (A) and roots (B) of VOC-exposed and control seedlings (exposed to the medium only). Different letters indicate statistically significant differences between the treatments (One-way ANOVA, Tukey HSD post-hoc Test, $P < 0.05$)

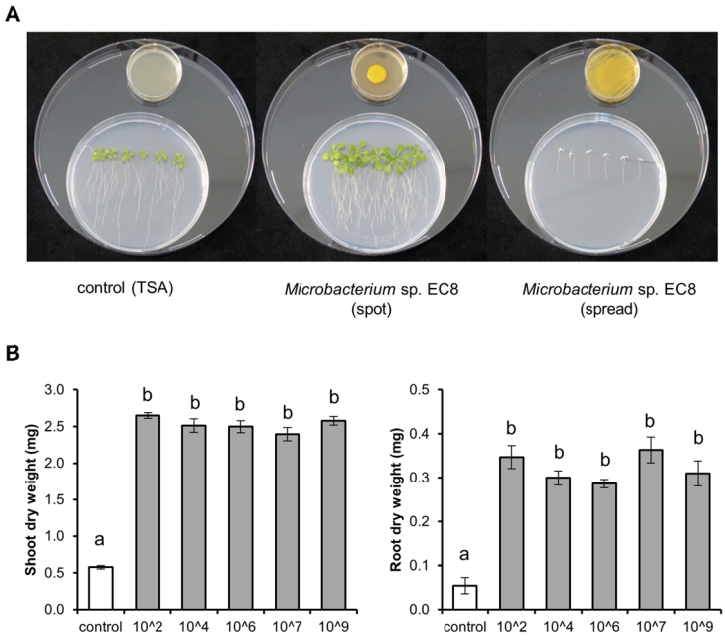


Fig. S2 | Density-dependent effects of *Microbacterium* sp. EC8 on plant growth. (A) *Microbacterium* sp. EC8 was spot-inoculated at different initial densities (10^2 to 10^9 cfu/ml) or spread over the surface of the entire agar medium (10 µl of 10^9 cfu/ml culture) and incubated at 30 °C. After three days, 7-day-old *Arabidopsis* seedlings were exposed to the bacterial VOCs for 14 days. (B) Shoot and root dry biomass (mean ± SE, n = 5) of *Arabidopsis* seedlings exposed to different initial cell densities of EC8. Different letters indicate statistically significant differences between the treatments (One-way ANOVA, Tukey HSD post-hoc Test, $P < 0.05$).

Table S1 | List of isolates used for comparative genome analysis of *Microbacterium* species. Genome sequences were downloaded from GeneBank.

Organism	Assembly	Size (Mb)	GC%	Scaffolds	Proteins	Level	Source	Reference
Frondihabitants sp. PB247	*to be submitted*	3.40	70.0	1	3212	Complete	animal	Liu et al. 2016 *in prep.*
Microbacterium arborescens ND21	GCA_001662775.1	3.43	70.2	22	3190	Contig	animal	unpublished
Microbacterium azadirachtae ARN176	GCA_000956505.1	4.24	70.1	40	3758	Contig	soil	Corretto *et al.* (2015)
Microbacterium azadirachtae DSM23848	GCA_000956545.1	4.04	70.5	86	3580	Contig	plant	Corretto *et al.* (2015)
Microbacterium chocolatum SIT101	GCA_001652465.1	3.17	70.0	4	2885	Complete	soil	unpublished
Microbacterium foliorum DSM12966	GCA_000956415.1	3.56	68.7	46	3217	Contig	plant	Corretto *et al.* (2015)
Microbacterium ginsengisoli DSM18659	GCA_000956535.1	3.05	70.2	80	2850	Contig	soil	Corretto *et al.* (2015) Park *et al.* (2008)
Microbacterium hominis TPW29	GCA_000813805.1	2.89	69.4	28	2646	Contig	water	Adrian *et al.* (2016)
Microbacterium ketosireducens DSM12510	GCA_000956575.1	3.92	70.3	57	3370	Contig	soil	Corretto *et al.* (2015)
Microbacterium laevaniformans OR221	GCA_000255595.2	3.43	68.0	535	3054	Contig	sediment	Brown *et al.* (2012)
Microbacterium mangrovi MUSC115	GCA_000802305.1	4.42	70.0	55	3918	Contig	soil	Lee *et al.* (2014)
Microbacterium maritypicum MF109	GCA_000455825.1	4.00	68.2	260	3718	Contig	animal	Chauhan *et al.* (2013)
Microbacterium oleivorans RIT293	GCA_000632065.1	2.90	69.0	11	2732	Contig	plant	Gan *et al.* (2014)
Microbacterium oxydans BEL163	GCA_000956405.1	3.69	68.0	30	3455	Contig	plant	Corretto *et al.* (2015) Midha *et al.* (2015)
Microbacterium paraoxydans DH1b	GCA_000633215.1	3.62	70.2	47	3413	Contig	plant	Chan *et al.* (2016)
Microbacterium paraoxydans NBRC103076	GCA_001552495.1	3.47	70.0	265	3172	Contig	human	Laffineur *et al.* (2003)
Microbacterium sp. CGR1	GCA_001266755.1	3.63	68.0	1	3364	Complete	soil	Mandakovic *et al.* (2015)
Microbacterium sp. EC8	*to be submitted*	3.18	67.5	1	3101	Complete	plant	This study
Microbacterium sp. No. 7	GCA_001314225.1	4.83	71.0	3	4304	Complete	water	Ohtsubo *et al.* (2015)
Microbacterium sp. HSR44	GCA_001558975.1	3.54	70.4	1	3276	Complete	lichen	Han *et al.* (2016)
Microbacterium sp. XT11	CA_001513675.1	3.48	69.4	1	1927	Complete	soil	Yang *et al.* (2014)
Microbacterium testaceum StLB037	CA_000202635.1	3.98	70.3	1	3532	Complete	plant	Morohoshi *et al.* (2011)
Microbacterium trichothecenolyticum DSM8608	CA_000956465.1	4.52	70.2	41	3927	Contig	soil	Corretto *et al.* (2015)
Microbacterium xylanilyticum JCM13591	CA_001313345.1	2.56	68.3	1774	-	Contig	water	Kim *et al.* (2005)

Table S2 | List of volatile organic compounds (VOCs) detected in the headspace of *Microbacterium* sp. EC8 (EC8) and control (agar medium only; TSA). Compounds with peak area 2-fold larger and significantly different than the control (t-Test, $P < 0.05$) were selected and are highlighted in bold. Compounds were putatively annotated by comparing their mass spectra (MS) and calculated retention indices (RI) with those of NIST and in-house mass spectral libraries.

RI	Library hit	EC8_1	EC8_2	EC8_3	Average	TSA_1	TSA_2	TSA_3	Average	T-test	FC
543	**Cyclopentene**	**12991535**	**16192304**	**16278659**	**15154166**	**0**	**0**	**0**	**0**	**n.d.**	
572	unknown	0	0	0	0	5686743	8600004	11871927	8719558	n.d.	
600	**2- Butanone**	**42886289**	**38860381**	**41633313**	**41126661**	**5688257**	**8932674**	**10532849**	**8384593**	**0.000**	**4.9**
605	Furan, 3-methyl	2411160	2268630	3037531	2572440	709848	1338875	2438104	1495609	0.154	1.7
625	Trichloromethane	0	0	0	0	12355072	18145474	23434255	17978267	0.030	n.d.
659	Butanal, 3-methyl	30157530	35084049	33115153	32785577	55374948	81798506	89995959	75723138	0.052	0.4
664	Benzene	3440029	3351325	2345989	3045781	1819594	1984765	2527470	2110610	0.099	1.4
668	Butanal, 2-methyl-	11725936	12903024	12512506	12380489	16641265	26902231	33323837	25622444	0.112	0.5
688	**2-Pentanone**	**3055249**	**2803373**	**3157662**	**3005428**	**0**	**0**	**0**	**0**	**n.d.**	
704	**Methyl thiolacetate**	**52246846**	**65210061**	**51686797**	**56381235**	**0**	**0**	**0**	**0**	**n.d.**	
718	Methane, bromodichloro	0	0	0	0	1581709	2627385	3793935	2667676	n.d.	
738	Pyrazine	0	0	0	0	6219671	10203392	8914056	8445706	n.d.	
750	**Dimethyl disulfide**	**1455814333**	**2479066770**	**1808473575**	**1914451559**	**39789054**	**64750082**	**88315462**	**64284866**	**0.025**	**29.8**
770	Toluene	0	0	0	0	1520324	1535026	2191936	1749095	n.d.	
800	**S-Methyl propanethioate**	**79394199**	**116987552**	**112161335**	**102847695**	**0**	**0**	**0**	**0**	**n.d.**	
806	**unknown**	**8875414**	**26542055**	**13125667**	**16181045**	**0**	**0**	**0**	**0**	**n.d.**	
823	Methylpyrazine	5421239	6550979	6687309	6219842	5089568	6955007	7403533	6482703	0.767	1.0
829	Furfural	0	0	0	0	2010158	2865548	2863547	2579751	n.d.	
847	**Butanethioic acid, S-methyl ester**	**23195204**	**52313194**	**30133792**	**35214063**	**0**	**0**	**0**	**0**	**n.d.**	
859	2-Furanmethanol/3-Methylbutanoic acid	997458	1144777	3459798	1867344	11915105	14213611	29584584	18571100	0.092	0.1
886	**2,4-Dithiapentane**	**3660944**	**3359530**	**4284242**	**3768239**	**0**	**0**	**0**	**0**	**n.d.**	
888	**Methyl propyl disulfide**	**5487769**	**4085487**	**6556257**	**5376504**	**0**	**0**	**0**	**0**	**n.d.**	
913	Ethylpyrazine	0	0	0	0	1235915	3952562	2085236	2424571	n.d.	

RI	Library hit	EC8_1	EC8_2	EC8_3	Average	TSA_1	TSA_2	TSA_3	Average	T-test	FC
932	alpha-Pinene	0	0	0	0	1377488	3399446	2783812	2520249	n.d.	
940	**S-Methyl pentanethioate**	**71085271**	**12**	**81278285**	**50787856**	**0**	**0**	**0**	**0**	**n.d.**	
965	Benzaldehyde	13185862	512120	11881164	8526382	69357836	92096006	74183799	78545880	0.002	0.1
973	**Trisulfide, dimethyl-**	**919975423**	**1598734218**	**1030198975**	**1182969539**	**43599855**	**50584399**	**55635092**	**49939782**	**0.033**	**23.7**
980	Phenol	0	0	0	0	3284803	1757327	1389422	2143851	n.d.	
1005	Pyrazine, 2-ethyl-5-methyl-	0	0	0	0	3791534	4203545	4021182	4005420	n.d.	
1036	**unknown**	**27158101**	**63657342**	**34887158**	**41900867**	**0**	**0**	**0**	**0**	**n.d.**	
1051	**Artemisia ketone**	**12182602**	**18956586**	**17918754**	**16352647**	**0**	**0**	**0**	**0**	**n.d.**	
1059	**S-Methyl methanethiosulphonate**	**1336605**	**6046364**	**10815578**	**6066182**	**0**	**0**	**0**	**0**	**n.d.**	
1076	3-Ethyl-2,5-dimethylpyrazine	2507065	2493611	4744059	3248245	2547341	2901377	3061906	2836875	0.640	1.1
1095	1,2,4-Trithiolane	11733508	22124304	14588740	16148851	4558738	7602677	10882162	7681192	0.094	2.1
1122	**Disulfide, methyl (methylthio)methyl**	**25885591**	**46028244**	**28970264**	**33628033**	**768466**	**650855**	**3750707**	**1723343**	**0.034**	**19.5**
1142	2-Isobutyl-4-methylpyridine	0	0	0	0	1289020	562740	222102	691287	n.d.	
1150	Benzoic acid	0	0	0	0	4547703	1984269	2234433	2922135	n.d.	
1202	3-Decen-1-ol	0	0	0	0	3244527	1903320	2183948	2443932	n.d.	
1212	**Tetrasulfide, dimethyl-**	**126676749**	**344353644**	**168387676**	**213139356**	**0**	**0**	**0**	**0**	**n.d.**	
1218	Furan, 3-phenyl	0	0	0	0	2151370	1528324	2282184	1987293	n.d.	
1354	Phenol, 2-(1,1-dimethylethyl)-4-methyl-	0	0	0	0	1909637	1775980	2319915	2001844	n.d.	
1460	**Pentasulfide, dimethyl-**	**2569509**	**9991124**	**2781544**	**5114059**	**0**	**0**	**0**	**0**	**n.d.**	
1467	2-(2-Methylpropyl)-3,5-di(1-methylethyl)pyridine	0	0	0	0	6276889	5634681	4538454	5483341	n.d.	
1497	Butylated Hydroxytoluene	30547157	25853685	37838222	31413021	20241372	13530127	22863885	18878461	0.051	1.7

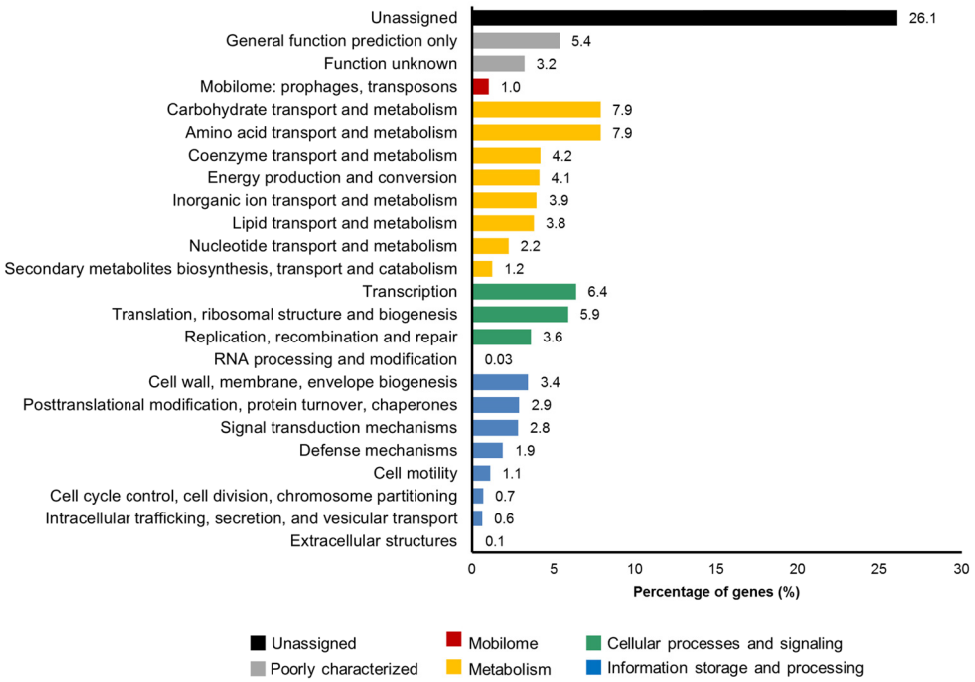
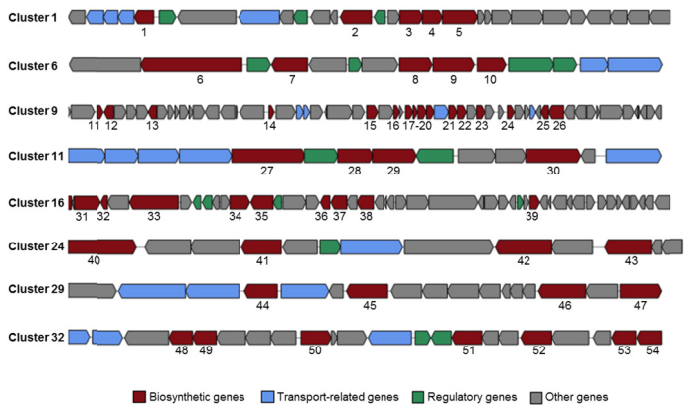


Fig. S3 | Functional annotation of the *Microbacterium* sp. EC8 genome. Coding sequences were assigned to clusters of orthologous groups (COGs) and specific functional descriptions were grouped into more general category associations represented by the different colors.



Cluster	Type	Location (nt)	Identity (%)[a]	ORF	Biosynthetic gene description[b]	Size (aa)
1	Terpene	55710 to 82771	Carotenoid (35%)	1	ectoine/hydroxyectoine ABC transporter	297
				2	argininosuccinate synthase	476
				3	geranylgeranyl pyrophosphate synthase	351
				4	phytoene synthase	305
				5	phytoene dehydrogenase	535
6	Putative	578940 to 595171	Actagardine (6%)	6	MMPL-domain containg trasnporter	925
				7	O-succinylbenzoate synthase	325
				8	1,4-dihydroxy-2-naphthoyl-CoA syrthase	299
				9	acyl-CoA synthetase	378
				10	methyltransferase type 11	263
9	Putative Saccharide	1052866 to 126870	Phosphonoglycans (9%)	11	hydrolase	263
				12	aminotransferase	400
				13	glycosyl transferase family 2	357
				14	glycosyl transferase family 1	224
				15	2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase	466
				16	dolichyl-phosphate mannose synthase	242
				17	glycosyltransferase	310
				18	acetylglucosamine-1-phosphate uridylyltransferase	203
				19	aminotransferase DegT	365
				20	dehydrogenase	327
				21	unknown	362
				22	glycosyl transferase	380
				23	glycosyl transferase family 2	352
				24	nuclectide sugar dehydrogenase	295
				25	glycosyl transferase	317
				26	glycosyl transferase	636
11	Unknown	1325435 to1338082	Becillomycin (20%)	27	acid-CoA ligase	520
				28	3-hydroxybutyrate dehydrogenase	250
				29	alpha/beta hydrolase	315
				30	acetyl-CoA acetyltransferase	392
16	Type III PKS	1913590 to 1954741	Lomaiviticin (3%)	31	long-chain-fatty-acid-CoA ligase	608
				32	peptide deformylase	163
				33	pyruvate carboxylase	1135
				34	dihydrolipoyl dehydrogenase	465
				35	glycerol-3-phosphate dehydrogenase	533
				36	short-chain dehydrogenase	221
				37	unknown	369
				38	naringenin-chalcone synthase	383
				39	NADP oxidoreductase	232
24	Putative	2791327 to 2804058	Salinomycin (4%)	40	phosphogluconate dehydrogenase	489
				41	lysophospholipase	284
				42	ornithine-oxo-acid transaminase	405
				43	unknown	332
29	Putative	2883537 to 2902578	Pimaricin (11%)	44	3-hydroxyisobutyryl-CoA hydrolase	353
				45	aspartate aminotransferase	442
				46	protoporphyrinogen oxidase	509
				47	glutamyl-tRNA reductase	441
32	Putative	3030932 to 3054047	Amphotericin (23%)	48	MBL fold metallo-hydrolase	307
				49	alpha/beta hydrolase	309
				50	cytochrome P450	390
				51	beta-ketoadipyl CoA thiolase	389
				52	acyl-CoA dehydrogenase	405
				53	aldo/keto reductase	314
				54	oxidoreductase	321

Fig. S4 | Mining of the *Microbacterium* sp. EC8 genome for genes encoding bioactive compounds. Gene clusters predicted for secondary metabolites and antibiotics were identified in the genome of EC8 using the web-tool antiSMASH (Medema *et al*., 2011; Weber *et al*., 2015). Numbers represent specific open reading frames (ORFs) of biosynthetic genes with similarity to known clusters and are described in the table. Biosynthetic gene description was based on NCBI BlastP.

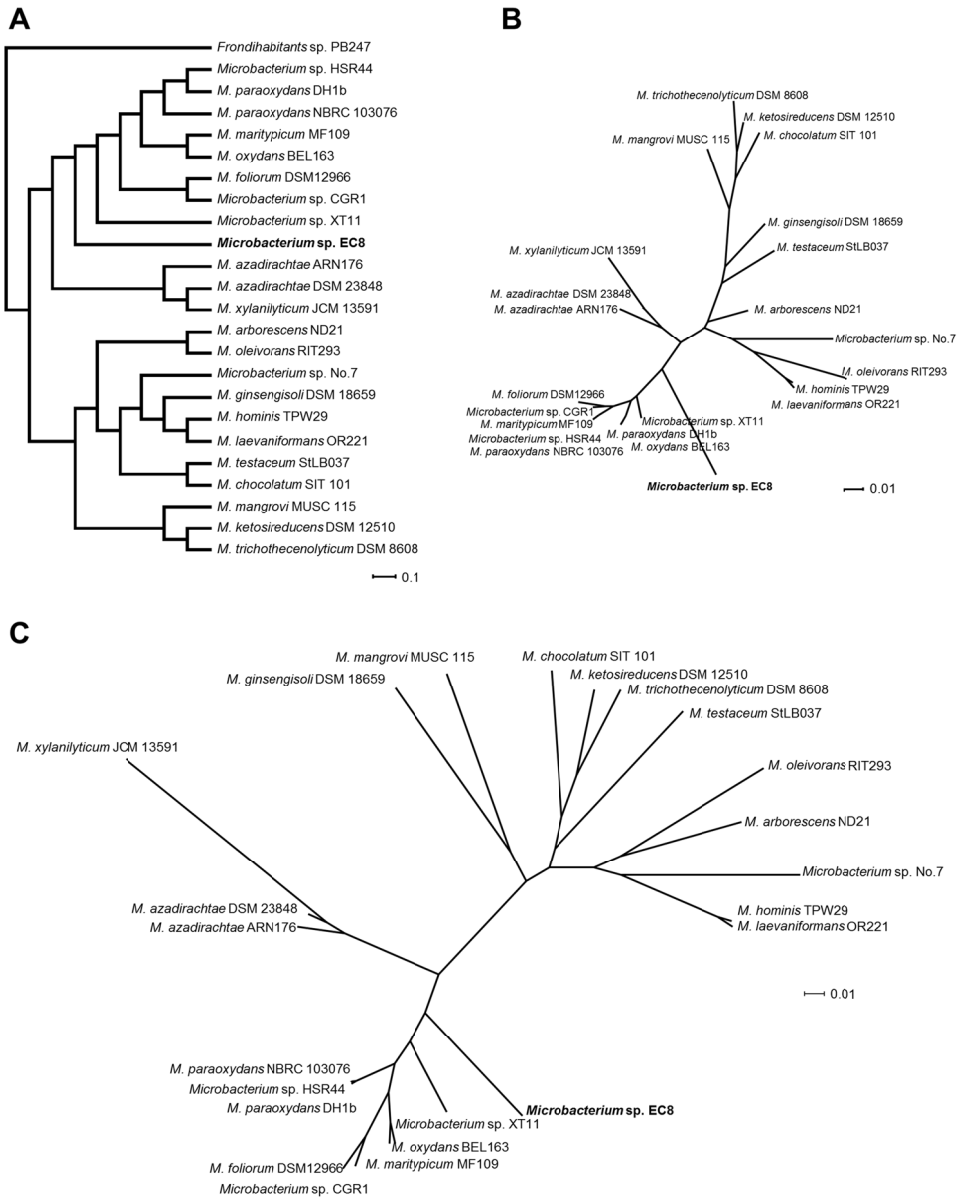


Fig. S5 | Phylogeny of *Microbacterium* species isolated from different environments. Phylogenetic trees are based whole genome (A), housekeeping genes (B) and core genes (C). A total of 23 *Microbacterium* genomes were used for the phylogenetic analysis (Table S1) with *Frondhabitans* sp. PB247 as outgroup.

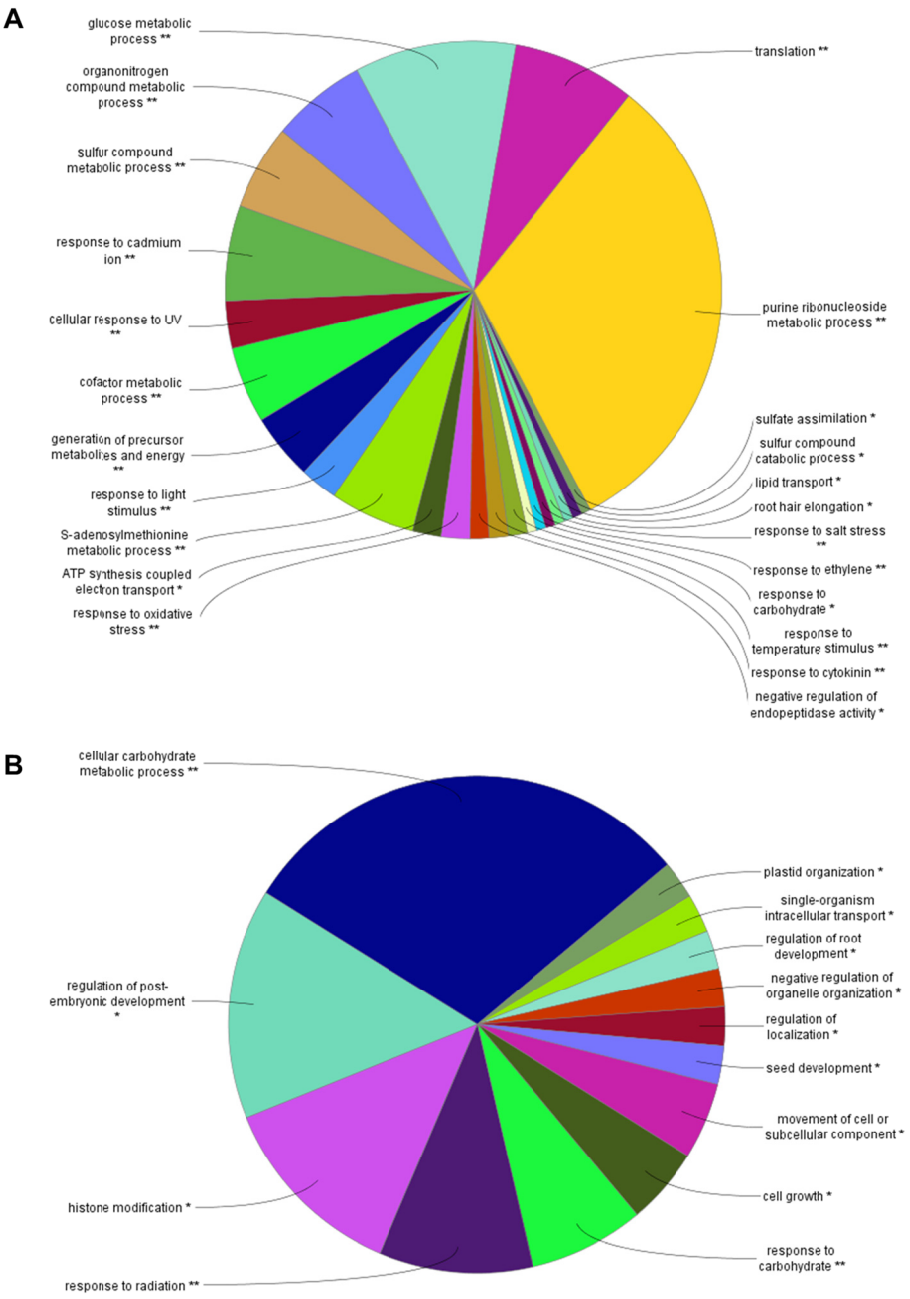


Fig. S6 | Functional anlaysis of differentially expressed genes (DEGs) of *Arabidopsis thaliana* shoots exposed to VOCs emitted by *Microbacterium* sp. EC8. Pie charts display the distribution of Gene Ontology (GO) terms grouped by a representative molecular function enriched among the up-regulated **(A)** and down-regulated **(B)** in shoot tissue exposed to *Microbacterium* VOCs.

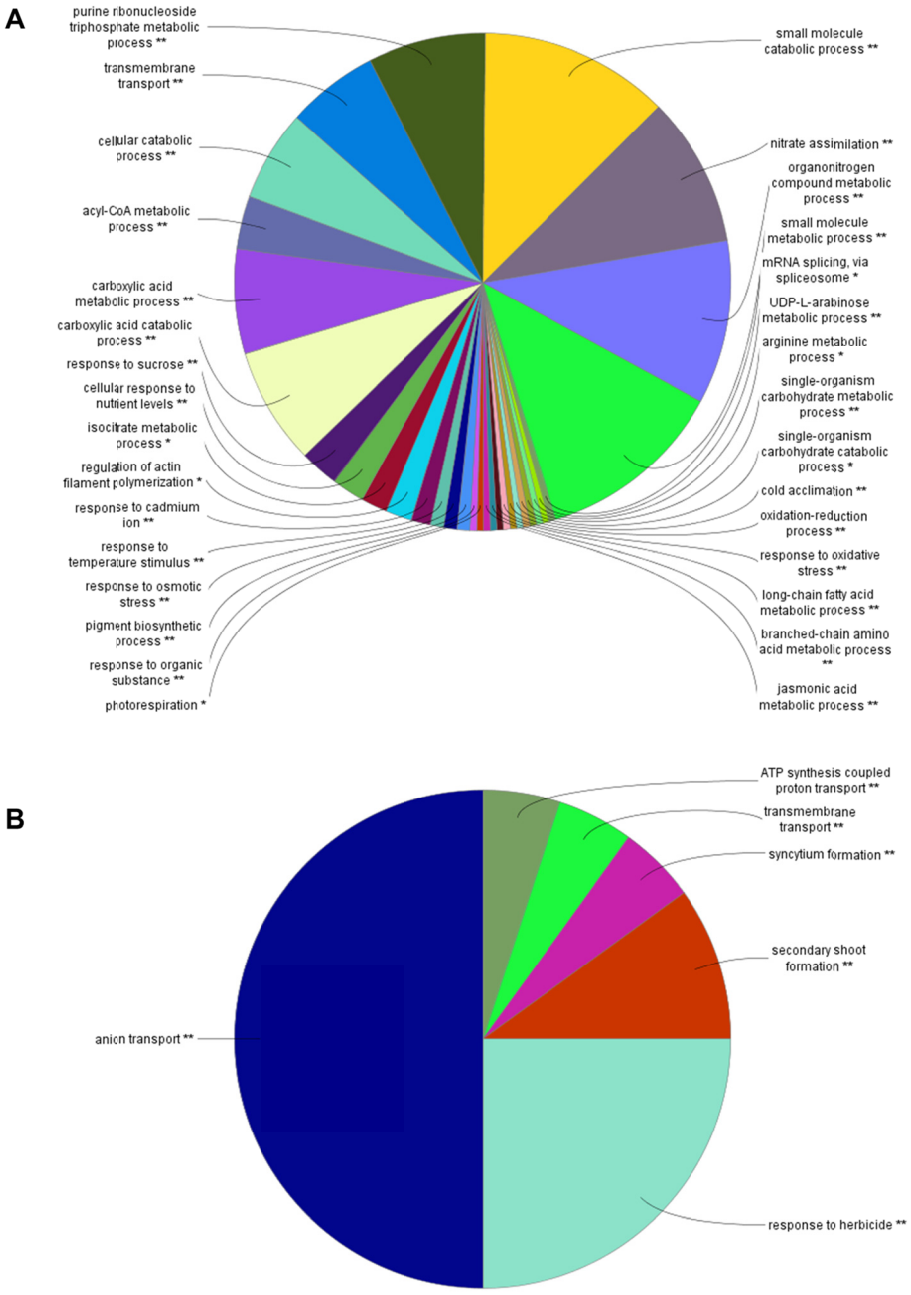


Fig. S7 | Functional anlaysis of differentially expressed genes (DEGs) of *Arabidopsis thaliana* roots exposed to VOCs emitted by *Microbacterium* sp. EC8. Pie charts display the distribution of Gene Ontology (GO) terms grouped by a representative molecular function enriched among the up-regulated **(A)** and down-regulated **(B)** in root tissue exposed to *Microbacterium* VOCs.

Chapter 5

Exploring volatile organic compounds of *Microbacterium* for plant growth promotion and biocontrol

Viviane Cordovez, Sharella Schop, Victor J. Carrion, Hans Jacobs,
Filip Coppens, Inge Hanssen, Jos M. Raaijmakers

Abstract

Volatile organic compounds (VOCs) produced by plant-associated bacteria represent a potential source for new measures to promote plant growth and to control infections by plant pathogens. Here we showed that *Microbacterium* sp. EC8 (hereafter referred as 'EC8') stimulated the growth of *Arabidopsis*, lettuce and tomato, but did not control damping-off disease of lettuce caused by the fungal root pathogen *Rhizoctonia solani*. Significant biomass increases were also observed for plants exposed only shortly to the bacterial VOCs prior to transplantation of the seedlings to soil. These results indicate that VOCs from EC8 can prime plants for growth promotion without direct and prolonged contact. We further showed that the induction of the plant growth-promoting effects are plant tissue specific; VOC exposure of roots led to an increase in plant biomass whereas shoot exposure resulted in no or less growth promotion. Subsequent VOC profiling by Gas Chromatography-Mass Spectrometry (GC-MS) showed that EC8 emits VOCs in soil and that these VOCs were structurally diverse and different from those produced on agar medium. Although the VOCs responsible for plant growth promotion were not identified here, our results show that VOC-producing EC8 has substantial potential as a new biostimulant of different crop plants.

Introduction

Plant-associated bacteria produce an array of secondary metabolites including a wide range of volatile organic compounds (VOCs). VOCs are organic molecules with low molecular weight and high vapor pressure that can travel through the soil matrix, facilitating long-distance interactions between microorganisms and plants without direct contact (Wheatley, 2002).

The production of VOCs by soil and plant-associated microorganisms has long been recognized (Zoller & Clark, 1921). Their effects on soil-borne fungi were already reported since the early 1950s (Dobbs & Hinson, 1953; Hora & Baker, 1972; Epstein & Lockwood, 1984; Fernando *et al.*, 2005; Kai *et al.*, 2007), but their impact on plant growth and health has only been recognized in the past decade. The VOCs 2,3-butanediol and 3-hydroxy-2-butanone, emitted by *Bacillus* species, enhanced growth of *Arabidopsis thaliana* seedlings (Ryu *et al.*, 2003). Seedlings exposed to 2,3-butanediol also showed reduced disease symptoms caused by a bacterial leaf pathogen (Ryu *et al.*, 2004). Since then, an increasing number of studies have shown the promising effects of bacterial VOCs in the inhibition of plant pathogens and the promotion of plant growth (Vespermann *et al.*, 2007; Zhang *et al.*, 2007; Meldau *et al.*, 2013; Bailly *et al.*, 2014; Cordero *et al.*, 2014; Garbeva *et al.*, 2014).

To date, the majority of plant growth-promoting and biocontrol effects by VOC-producing bacteria have been demonstrated *in vitro* on nutrient-rich media in sealed petri dishes (Blom *et al.*, 2011; Cordovez *et al.*, 2015; Hernández-León *et al.*, 2015). Little is still known on the potential of VOCs in agriculture and horticulture. For successful implementation, the microbial strain should colonize the plant rhizosphere and endosphere at sufficient densities, and produce the bioactive VOCs at the right time, the right place and at biologically relevant concentrations. The production of VOCs by bacteria *in situ* is not well-studied due to technical limitations. In soils, VOC production levels by bacteria are presumed to be low and strongly dependent on nutrient and oxygen availability as well as on the physiological state of the bacteria (Insam & Seewald, 2010). Variations in soil physico-chemical characteristics can lead to a rapid and uneven evaporation of VOCs resulting in inconsistent outcomes (Ryu, 2015). Furthermore, plant exudates can affect bacterial densities in the rhizosphere (Lemanceau *et al.*, 1995; Grayston *et al.*, 1998) which in turn could impact on the quantity and diversity of VOCs produced *in situ*.

We have previously shown that *Microbacterium* species, isolated from the sugar beet rhizosphere and endosphere, inhibited the growth of the fungal root pathogen *Rhizoctonia solani* and promoted the growth of *Arabidopsis thaliana via* the production of

VOCs (Chapter 4). Here, we investigated if and how these observations for *Arabidopsis* could be translated to crop plants. Using both *in vitro* and soil bioassays, we profiled VOCs produced by *Microbacterium* sp. strain EC8 (hereafter referred as 'EC8') in soil and evaluated the potential of these VOCs for growth promotion of lettuce and tomato plants.

Materials and Methods

Bacterial strain

Microbacterium sp. strain EC8 was grown on Tryptone Soy Broth (Oxoid Thermo Scientific, Lenexa, USA) supplemented with 18 g of technical agar (Oxoid Thermo Scientific, Lenexa, USA) for 3 days at 21 °C. Cells were obtained from agar plate and mixed with 10mM $MgSO_4.7H_20$ buffer. Cell density was measured and adjusted to OD_{600} = 1 (~10^9 cfu ml^{-1}).

Plant material

For the *in vitro* assays, seeds of *Arabidopsis* (*Arabidopsis thaliana* Col-0) were surface-sterilized for 3 h by placing seeds in Eppendorf tubes open in a desiccator jar. Two beakers, each containing 50 ml of sodium hypochlorite solution were placed inside, and 1.5 ml of 37% hydrochloric acid was added to each beaker. The desiccator jar was closed, and the seeds were sterilized by chlorine gas. Eppendorf tubes containing the sterile seeds was kept open in the flow cabinet for 30 min and after that placed on a wet paper filter in a Petri dish. Petri dish was sealed and wrapped in tin foil and kept at 4°C for 3-4 days. Seeds of lettuce (*Lactuca sativa*) and tomato (*Solanum lycopersicum* L.) were surface-sterilized by soaking in 70% ethanol for 2 min, followed by soaking in 1% sodium hypochlorite solution for 20 min. After soaking, seeds were rinsed three times in sterile demi-water. Plants were kept in climate cabinets at 21 °C; 180 µmol light m^{-2} s^{-1} at plant level; 16 h : 8 h, light : dark; 70% R.H..

In vitro plant growth promotion assay

Sterile seeds of *Arabidopsis*, lettuce and tomato were sown on Petri dishes (∅ 90 mm) containing 25 ml of 0.5X Murashige and Skoog medium (Murashige & Skoog, 1962) supplemented with 0.5% sucrose. These Petri dishes (without lids) were kept inside a larger Petri dish (∅ 145 mm) which were sealed and kept in the climate cabinet. After four days, seedlings were exposed to the bacterial VOCs or to agar medium by introducing a small Petri dish (∅ 35 mm) containing a 3-day-old bacterial culture or the agar medium (control). Petri dishes (∅ 145 mm) were sealed and kept in the climate cabinet. Plant shoot and root

biomass was determined after 12, 10 and 7 days for *Arabidopsis*, tomato and lettuce seedlings, respectively.

To test if a short exposure to the bacterial VOCs had an effect on plant growth, seedlings were exposed *in vitro* using the three-compartment set-up described above. Seven-day-old *Arabidopsis* and three-day-old lettuce seedlings were exposed for five and three days, respectively, and then transplanted to soil. Plants were kept in plastic pots containing 130 g of potting soil with 40% moisture. A total of 5-9 replicates were used per treatment. *Arabidopsis* shoot biomass, number of flower and length of flower stem were determined 21 days after soil transplantation. Lettuce biomass was determined 13 days after soil transplantation.

Soil inoculation with *Microbacterium*

To test the plant-growth promoting effects of *Microbacterium* EC8 when directly inoculated in soil, a first trial was performed with *Arabidopsis* plants. The bacterial culture (initial cell density: 10^9 cfu ml^{-1}) was inoculated into a potting soil-sand (1:2 v/v) and the soil moisture was brought to 10% using the bacterial culture and tap water to reach concentrations of 10^6 and 10^7 cfu g^{-1} soil. Five *Arabidopsis* seeds were sown per pot and a total of 10 replicates per treatment were prepared. Shoot biomass, flower number and length of flower stem of *Arabidopsis* plants were determined 21 days after sowing. For the lettuce plants, bacterial cultures at the same concentrations described for *Arabidopsis* plants were added to 100% potting soil. A total of 10 replicates containing five seeds each were used per treatment. A second inoculation was performed 7 days after sowing lettuce plants, by adding in each pot 100 ml of the bacterial culture at 10^6 and 10^7 cfu ml^{-1}. Lettuce shoot biomass was determined 15 days after sowing.

Biocontrol assay

Biocontrol potential of *Microbacterium* sp. EC8 against the fungal pathogen *Rhizoctonia solani* AG 1-1B was investigated using two different experimental set-ups. In the first set-up, the bacterial culture was directly inoculated in soil as described above for *in vivo* plant growth promotion of lettuce plants. After the second bacterial inoculation (7 days after sowing), the fungal pathogen was added to soil. A mycelial plug (5 mm of a 6-day-old fungal culture maintained on $1/5^{th}$ Potato Dextrose Agar) was used as inoculum, which was placed close to the plant in the center of the pot. In the second set-up, four-day-old lettuce seedlings were *in vitro* exposed to the bacterial VOCs for three days and after that, transplanted to soil. After three days, the *R. solani* mycelial plug was placed close to the

roots of the plant in the center of the pot. Disease incidence was scored every two days as follow: [(number of diseased plants x 100) / total number of plants per pot].

Shoot and root exposure to *Microbacterium* VOCs

To expose plant shoots and roots to the bacterial VOCs, two different experimental set-ups were used. For the exposure of plant shoots, a closed sterile container (OS140box, Duchefa Biochemie, Haarlem, the Netherlands) was used. Seedlings were sown in pots (inner diameter: 6.5 cm, height: 5 cm) containing potting soil and kept in the climate chamber. *Microbacterium* sp. EC8 was inoculated on Petri dishes containing TSA medium and incubated for 6 days at 21 °C. Ten holes were made on the walls of these Petri dishes to allow diffusion of the bacterial VOCs as displayed in Fig. 3. *Arabidopsis*, lettuce and tomato seedlings were exposed to the bacterial VOCs seven, four and six days after sowing, respectively. After one week of co-cultivation, pots were kept open in the flow cabinet for 30 min to remove excess of condensation on the pot walls. Plants were exposed three more days and allowed to grow for four days in the absence of the bacterial VOCs. After that, shoot biomass was determined.

For the exposure of plant roots, two-compartment pots were used. Top and bottom compartments were separated by a polyester membrane (5 µm, Nedfilter, Lelystad, The Netherlands). Upper compartment (inner diameter: 5.5 cm, height: 8 cm) was filled with potting soil-sand mixture (1:2 v/v; 25% moisture) where one *Arabidopsis* or lettuce seed was sown. Bottom compartment (inner diameter: 6.5 cm, height: 4.5 cm) was filled with the soil-sand mixture mixed with the bacterial culture (10^7 cfu g^{-1} soil) or a Petri dish (∅ 35 mm) containing a three-day-old bacterial culture on TSA medium (initial concentration 10^9 cfu ml^{-1}) previously incubated at 21 °C. Shoot and root biomass were determined three weeks after sowing.

Soil VOC profiling

To identify the VOCs produced by *Microbacterium* sp. EC8 in soil, VOCs were collected from a Petri dish with an inlet for the Tenax TA tubes which contained 8 g of non-inoculated soil (control) and 8 g soil inoculated with the bacterial culture (10^7 cfu g^{-1} soil adjusted with tap water to 40% soil moisture). Headspace VOCs were collected for three, seven and 10 days at 21 °C. Trapped compounds were subjected to Gas Chromatography-Quadrupole Time of Flight-Mass Spectrometry (GC-QTF-MS). Compounds were desorbed from the Tenax tubes in a thermodesorption unit (model UnityTD-100, Markes International Ltd., Llantrisant, UK) at 210 °C for 12 min (Helium flow 50 ml min^{-1}) using 1:10 split ratio. Released compounds were focused on a cold trap at - 10 ° C and introduced into the GC-QTF-MS (Agilent 7890B

GC and the Agilent 7200A QTOF, Santa Clara, USA). Compounds were transferred to the analytical column (30 m × 0.25 mm ID RXI-5MS, film thickness 0.25 µm; Restek 13424-6850, Bellefonte, PA, USA) by heating the cold trap to 250 °C for 12 min. Temperature program of the GC oven was: 39 °C for 2 min, from 39 °C to 95 °C at 3.5 °C min^{-1}, from 95 °C to 165 °C at 4 °C min^{-1}, from 165 °C to 280 °C at 15 °C min^{-1} and finally, from 250 °C to 320 °C at 30 °C min^{-1} and 7 min-hold at a constant gas flow of 1.2 ml min^{-1}. Mass spectra were acquired by electron impact ionization (70 eV) with a scanning from m/z 30-400 with a scan rate of 5 scans s^{-1}.

Chromatograms were integrated using MassHunter Qualitative Analysis Software V B.07.00 Build 6.0.633.0 (Agilent Technologies, Santa Clara, USA). VOCs were tentatively identified by comparison of the mass spectra with those of NIST and Wiley libraries and the Wageningen Mass Spectral Database of Natural Products and by comparing the experimentally calculated LRI with the literature. Compounds were considered detected for the soil inoculated with *Microbacterium* if they presented a relative peak area at least twice bigger than non-inoculated soil and $P < 0.05$ (t-Test).

Statistical analysis

Data from the *in vitro* and *in vivo* plant growth promotion and biocontrol were analyzed by Student's t-Test and ANOVA with Tukey HSD test ($P < 0.05$).

Results

VOC-mediated plant growth promotion by *Microbacterium in vitro*

Previous studies showed that VOCs emitted by EC8 increased root and shoot biomass and induced lateral root formation of *Arabidopsis* seedlings grown *in vitro* (Chapter 4). To investigate if EC8 can also promote growth of different crop plants, we exposed *Arabidopsis* (used as a positive control), lettuce and tomato seedlings to VOCs emitted by EC8. Upon exposure to the bacterial VOCs, lettuce seedlings showed an increase of 178% in shoot biomass (t-Test, $P < 0.001$), 253% in root biomass (t-Test, $P < 0.001$) and 217% in lateral root density (t-Test, $P < 0.001$); tomato seedlings showed an increase of 44% in shoot biomass (t-Test, $P < 0.001$), 27% in root biomass (t-Test, $P = 0.038$) and 54% in lateral root density (t-Test, $P < 0.001$) (Fig. 1A,B,C, S1) compared to control seedlings (exposed to agar medium only).

To test if VOCs emitted by EC8 could prime plant growth and development, *Arabidopsis* and lettuce seedlings were exposed *in vitro* to the bacterial VOCs and then transplanted to soil. Short VOC-exposure (four days) promoted the growth of *Arabidopsis*

and lettuce plants transplanted to and grown in soil. *Arabidopsis* plants showed a significant increase of 35% in shoot biomass (Fig. 2A). We also observed increases of 27% in length of the flower stem and of 51% in number of flowers, although these increases were not statistically significant (Fig. 2B,C). Lettuce plants showed a significant 12% increase in shoot biomass (t-Test, P = 0.038; Fig. 2D). These results demonstrate that a four-day-exposure of *Arabidopsis* and lettuce to VOCs from EC8 is sufficient to trigger plant growth promotion without direct and prolonged contact between the plants and the bacterial strain.

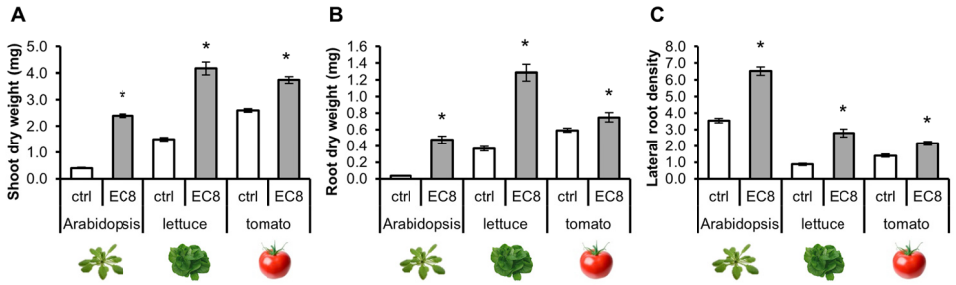


Fig. 1 | Plant growth-promoting effects of volatile organic compounds (VOCs) emitted by *Microbacterium* sp. EC8 *in vitro*. Dry biomass (mean ± SE, n = 6-8) of shoots **(A)** and roots **(B)** and lateral root density (number of lateral roots/length (cm) of primary root)**(C)** of *Arabidopsis*, lettuce and tomato seedlings after exposure to the bacterial VOCs for 12, 7 and 10 days, respectively. Control seedlings are referred as 'ctrl' and were exposed to agar medium only; seedlings exposed to VOCs emitted by EC8 are referred as 'EC8'; asterisks indicate statistically significant differences between VOC-exposed and control seedlings (Student's t-Test, $P < 0.05$).

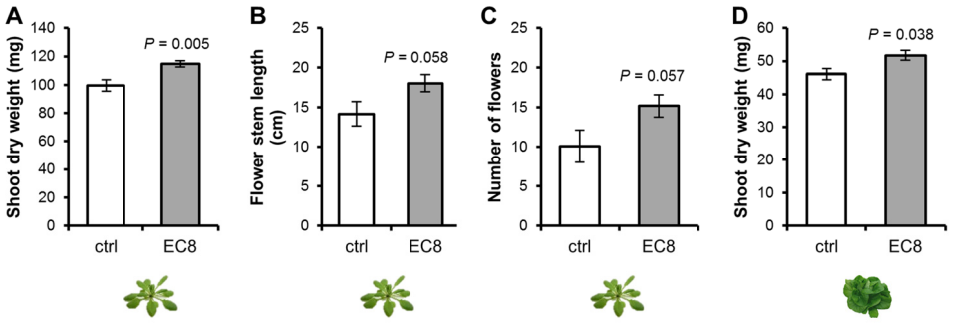


Fig. 2 | Growth-promoting effects of short *in vitro* exposure of *Arabidopsis* and lettuce seedlings to volatile organic compounds (VOCs) emitted by *Microbacterium* sp. EC8. **(A)** Shoot dry biomass, **(B)** flower stem length and **(C)** number of flowers of *Arabidopsis* plants (mean ± SE, n = 9); **(D)** shoot dry biomass of lettuce plants (mean ± SE, n = 4-5). Control plants are referred as 'ctrl' and were exposed to agar medium only; seedlings exposed to VOCs emitted by EC8 are referred as 'EC8'. Statistically significant differences between VOC-exposed and control seedlings were determined with Student's t-Test.

VOC-mediated plant growth promotion by *Microbacterium in situ*

To test the effects of VOCs emitted by *Microbacterium* EC8 on the growth of plants in soil, two different experimental approaches were used. In the first set-up, plants and the bacterial culture were kept inside a sterile container for one week allowing exposure of the plant shoots to the bacterial VOCs (Fig. 3A). Exposure to the VOCs emitted by EC8 resulted in a 45% increase of shoot biomass of *Arabidopsis* plants (t-Test, $P = 0.002$). However, no significant increases in shoot biomass were observed for lettuce plants (t-Test, $P = 0.336$) and tomato (t-Test, $P = 0.837$) (Fig. 3B). In the second experimental set-up, plant roots were exposed to VOCs emitted by EC8 either inoculated in a soil-sand mixture or inoculated onto agar medium. To expose only the roots to the bacterial VOCs, we used a pot with two compartments separated by a membrane (Fig. 4A,B). VOCs emitted by EC8 from the soil-sand mixture promoted the growth of *Arabidopsis* roots (Fig. 4C, t-Test, $P = 0.004$) but not of lettuce (Fig. 4D, t-Test, $P = 0.694$). VOCs emitted by EC8 grown on agar medium increased the biomass of *Arabidopsis* shoots (Fig. 4E, t-Test, $P = 0.001$) and roots (t-Test, $P < 0.001$), and of lettuce shoots (t-Test, $P = 0.004$) and roots (Fig. 4F, t-Test, $P = 0.036$).

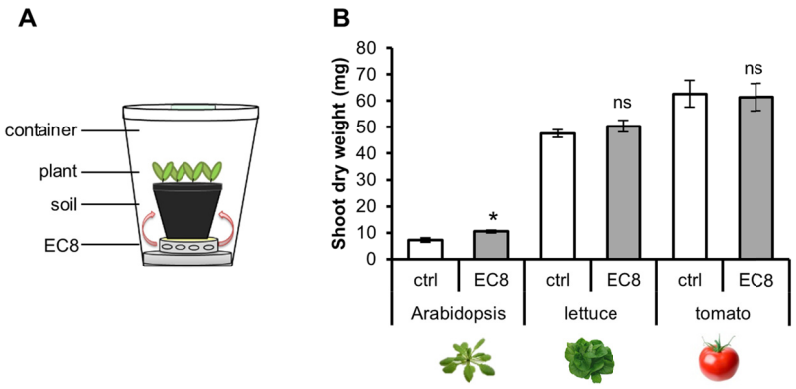


Fig. 3 | Plant shoot exposure to volatile organic compounds (VOCs) emitted by *Microbacterium* sp. EC8. **(A)** Experimental set-up used to expose plant shoots to bacterial VOCs: plants were grown in potting soil and EC8 on agar medium. **(B)** Shoot dry biomass (mean ± SE, n = 5-8) of *Arabidopsis*, lettuce and tomato plants. Control plants are referred as 'ctrl' and were exposed to agar medium only; plants exposed to VOCs emitted by EC8 are referred as 'EC8'; asterisks indicate a statistically significant difference between VOC-exposed and control seedlings; *ns* indicates no statistical differences (Student's t-Test, $P < 0.05$).

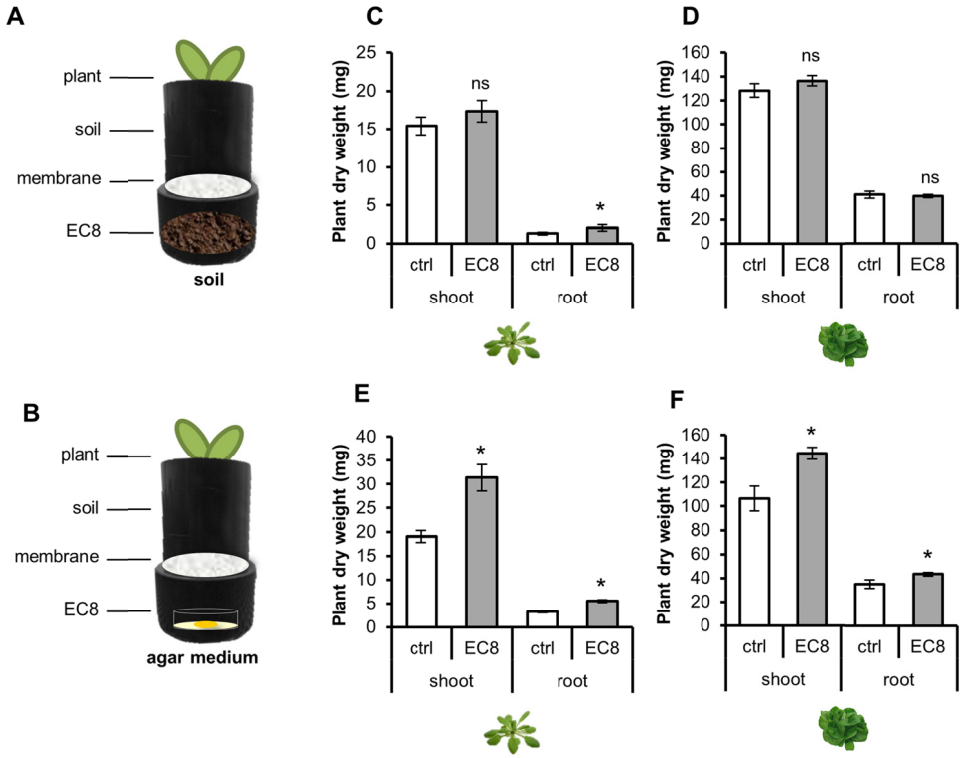


Fig. 4 | Plant root exposure to volatile organic compounds (VOCs) emitted by *Microbacterium* sp. EC8. Two-compartment pots were used where the bacterial cells was inoculated in soil **(A)** or on agar medium **(B)**. Dry biomass (mean ± SE, n = 6-9) of *Arabidopsis* shoots and roots **(C)** and lettuce shoots and roots **(D)** exposed to VOCs emitted by EC8 grown in soil. Dry biomass (mean ± SE, n = 8-9) of *Arabidopsis* shoots and roots **(E)** and lettuce shoots and roots **(F)** exposed to VOCs emitted by EC8 grown on agar medium. Control plants are referred as 'ctrl' and were exposed to agar medium or soil only; plants exposed to VOCs emitted by EC8 are referred as 'EC8'; asterisks indicate a statistically significant difference between VOC-exposed and control seedlings; *ns* indicates no statistical differences (Student's t-Test, $P < 0.05$).

VOC profiling of *Microbacterium* in soil

Previous studies in our lab showed that EC8 produced a variety of sulfur-containing VOCs when grown on TSA agar medium (Chapter 4). Here, we set out to investigate if these and possibly other VOCs could also be detected in soil inoculated with strain EC8. In the headspace of soil inoculated with EC8 and in the non-inoculated soil, the two sulfur-containing VOCs dimethyl disulfide and dimethyl trisulfide were detected at similar levels (FC > 2, $P < 0.05$) (Fig. 5). However, soil inoculated with EC8 showed an increase of five VOCs not detected when EC8 was grown on TSA medium. These compounds were tentatively

identified as 2,2,4,6,6-pentamethyl heptane (RI = 992), 3-carene (RI = 1011), 2,2,4,4-tetramethyloctane (RI = 1029), unknown (RI = 1036) and unknown (RI = 1049) (Fig. 5).

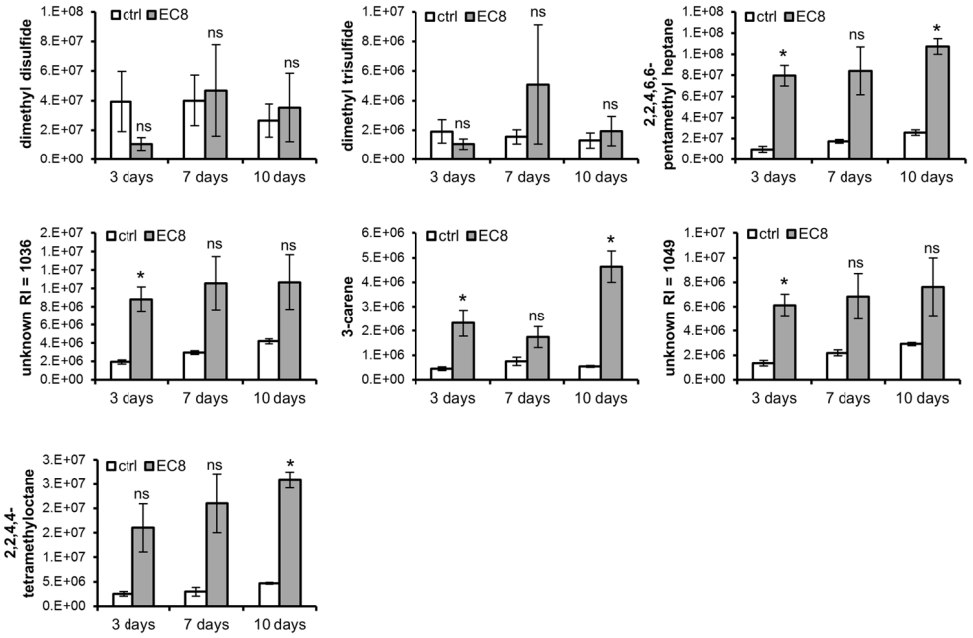


Fig. 5 | Volatile organic compounds (VOCs) detected in soil inoculated with *Microbacterium* sp. EC8. Peak area (mean ± SE, n = 4) of compounds detected by Gas Chromatography-Mass Spectrometry (GC-MS) after 3, 7 and 10 days of VOC collection. 'ctrl' refers to VOCs from soil only (control); 'EC8' refers to VOCs detected in soil inoculated with EC8; asterisks indicate a statistically significant difference between VOC-exposed and control seedlings; *ns* indicates no statistical differences (Student's t-Test, $P < 0.05$).

Effects of cell density and successive application of *Microbacterium* on plant growth promotion and biocontrol

To evaluate the practical application of EC8 as a biostimulant of crop plants in horticulture, a series of experiments were conducted to determine the plant growth-promoting efficacy of strain EC8 in commercial potting soil and to assess the required cell density and number of applications for plant growth promotion. The results of these preliminary soil bioassays indicated that the effects of *Microbacterium* on plant growth are dependent on bacterial cell density, the plant species and soil type. No significant differences were found in shoot biomass between *Arabidopsis* plants grown in soil inoculated with EC8 or in non-inoculated soil (Fig. S2). For lettuce, the results showed that EC8 significantly increased

shoot biomass of lettuce in a dose-dependent manner. No significant increase in shoot biomass of lettuce was observed for plants grown in soil inoculated with *Microbaterium* at cell densities of 10^6 cfu g^{-1} soil applied at sowing only (t-Test, $P = 0.296$). However, a second application resulted in 23% (t-Test, $P = 0.060$) and 25% (t-Test, $P = 0.029$) increases of lettuce shoot biomass at cell densities of 10^6 and 10^7 cfu g^{-1} soil, respectively (Fig. S2). These results indicate that successive applications of EC8 are required for growth promotion of lettuce. Inoculation of EC8 in soil did not show a significant effect on damping-off disease of lettuce plants caused by *Rhizoctonia solani* (Fig. S3). Also short *in vitro* exposure of the lettuce seedlings to the EC8 VOCs did not enhance plant resistance to infection by *R. solani* (Fig. S3). These results indicate that *Microbacterium* EC8 does not provide protection of lettuce plants against infection by *R. solani* but does enhance lettuce biomass.

Discussion

Volatile organic compounds (VOCs) emitted by plant-associated bacteria have great potential for stimulating plant growth and health. However, the current understanding of bacterial VOCs and their effects on plants results from experiments performed mostly under artificial lab conditions. Moreover, the bioactive VOCs responsible for the plant-growth promoting effects have been identified in only a few cases (Ryu *et al.*, 2003; Meldau *et al.*, 2013; Shao *et al.*, 2014; Park *et al.*, 2015). Here, we showed that VOCs emitted by EC8 stimulated the growth of two crop plants, lettuce and tomato, both *in vitro* and *in vivo*. We further showed that a short *in vitro* exposure of seedlings to VOCs emitted by EC8 stimulated the growth of plants transplanted to soil, thus eliminating the need for root colonization and long-term survival of the VOCs-producing bacterial strain. Our results further indicated that VOC perception is plant tissue-dependent: root exposure to the bacterial VOCs increased shoot and root of *Arabidopsis* and lettuce plants, whereas exposure of lettuce shoots to the VOCs did not trigger the growth-promoting effects.

Current knowledge on plant perception of VOCs is still limited. Most results on plant perception of VOCs originate from studies on aboveground plant-plant communication. For example, plants can sense VOCs emitted by neighboring plants under herbivore attack and enhance resistance (Engelberth *et al.*, 2004; Heil & Silva Bueno, 2007). Our results showed that roots play an important role in sensing and responding to the plant growth-promoting bacterial VOCs but the underlying mechanisms remain to be investigated. We further showed that the plant growth-promoting effects by VOCs emitted by EC8 were also dependent on the substrate available for bacterial growth. VOC

production by microorganisms in soil strongly depends on nutrient and oxygen availability, pH, temperature and the physiological state of the microorganisms (Insam & Seewald, 2010). Wheatley *et al.* (1996) highlighted that even small variations in nutrient composition may considerably change the type and the amount of VOCs produced.

From this study, it is not yet clear which VOCs produced by EC8 are responsible for plant growth promotion. Bacterial VOCs first described for their involvement in plant growth promotion include 2,3-butanediol and acetoin (Ryu *et al.*, 2003). Other VOCs, such as the plant hormones indole-3-acetic acid and ethylene are produced by several bacterial species (Costacurta & Vanderleyden, 1995; Lin & Xu, 2013; Kudoyarova *et al.*, 2015) and have been reported in literature for their plant growth promoting effects (Bailly *et al.*, 2014). So far, we have no evidence that these VOCs are produced by EC8. VOC profiling of *Microbacterium* EC8 grown on agar medium showed a great diversity of sulfur-containing compounds. Meldau *et al.* (2013) showed that dimethyl disulfide produced by *Bacillus* sp. promoted plant growth presumably by enhancing the availability of reduced sulfur for plants growing in sulfur-deficient soils. Two sulfur-containing compounds were detected in soil for EC8, however not in concentrations significantly different from the non-inoculated soil. However, we did detect five VOCs from soil inoculated with EC8 that were significantly different from the levels found in non-inoculated soil. These VOCs included two branched chain alkanes (tentatively identified as 2,2,4,6,6-pentamethyl heptane and 2,2,4,4-tetramethyloctane) and two unknown compounds. The VOC 2,2,4,4,6-pentamethyl heptane has been described to be produced by a *Mycobacterium* sp., but its function remains unknown (McNerney *et al.*, 2012). If and how these specific VOCs of EC8 impact plant growth remains to be investigated.

In conclusion, VOCs produced by *Microbacterium* represent a new source of natural compounds for stimulation of crop growth. Priming seedlings by a short exposure to *Microbacterium* VOCs provides an exciting new strategy for plant growth promotion, as it does not require environmental introduction of the bacterial strain. Exploration of these VOCs in lab and open field conditions and characterization of their ecological functions will contribute to reveal novel mechanisms for improving crop production for sustainable agriculture.

Supplementary Materials

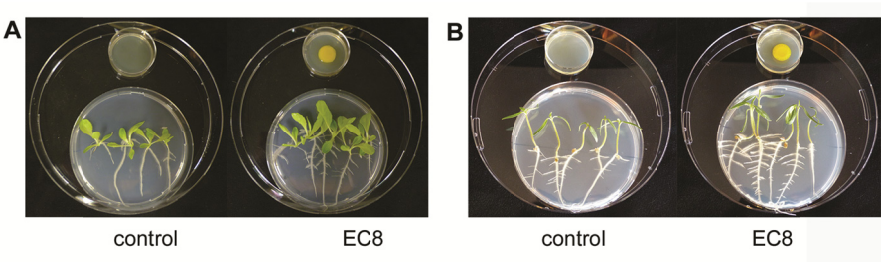


Fig. S1 | Plant growth-promoting effects of volatile organic compounds (VOCs) emitted by *Microbacterium* sp. EC8 *in vitro*. Lettuce (A) and tomato (B) seedlings were exposed *in vitro* to bacterial VOCs for 7 and 10 days, respectively.

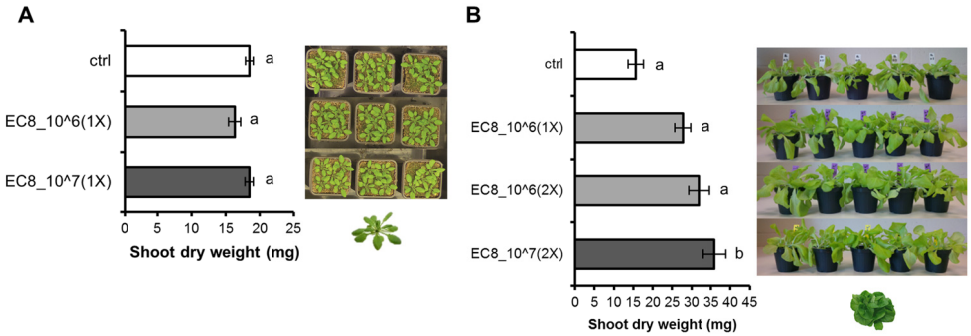


Fig. S2 | Plant growth-promoting effects of *Microbacterium* sp. EC8 in soil. Shoot dry biomass (mean ± SE, n = 10) of *Arabidopsis* (A) and lettuce (B) plants grown in soil inoculated with *Microbacterium* sp. EC8 at cell densities of 10^6 and 10^7 cfu g^{-1} soil inoculated once (1X: at the day of sowing only) and twice (2X: at the day of sowing and 7 days after sowing). Control plants ('ctrl') refer to plants in non-inoculated soil and 'EC8' refers to plants grown in soil inoculated with *Microbacterium* sp. EC8. Different letters indicate statistically significant differences between the treatments (One-way ANOVA, Tukey HSD post-hoc Test, $P < 0.05$). Pictures of *Arabidopsis* and lettuce plants were taken 21 and 15 days after sowing, respectively.

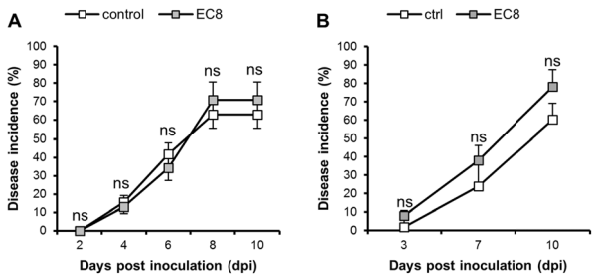


Fig. S3 | Biocontrol potential of *Microbacterium* sp. EC8. (A) *Microbacterium* sp. EC8 was inoculated in soil where lettuce plants were grown. (B) Lettuce seedlings were exposed *in vitro* to the VOCs emitted by *Microbacterium* sp. EC8 and then transplanted to soil. Control plants (ctrl) refer to plants grown in soil without bacterial inoculation (A) or plants not exposed to the bacterial VOCs prior to soil transplantation (B); plants exposed to the bacterial VOCs are referred as 'EC8'; *ns* indicates no statistical difference between VOC-exposed and control plants (Student's t-Test, $P < 0.05$).

Chapter 6

Volatiles from a soil-borne pathogenic fungus modulate the trade-off between plant growth and insect resistance

Viviane Cordovez, Liesje Mommer, Kay Moisan, Dani Lucas-Barbosa, Ronald Pierik, Roland Mumm, Victor J. Carrion, Jos M. Raaijmakers

Submitted for publication

Abstract

Beneficial soil microorganisms affect plant growth by the production of volatile organic compounds (VOCs). If and how VOCs from soil-borne plant pathogenic fungi affect plant growth, development and resistance is largely unknown. We studied VOC-mediated interactions between the fungal root pathogen *Rhizoctonia solani* and *Arabidopsis thaliana*. We investigated how fungal VOCs affect seed germination, plant biomass, root architecture, plant gene expression, plant VOC emission and resistance to the insect herbivore *Mamestra brassicae*. We show that *R. solani* produces an array of VOCs that promote growth, accelerate development, change VOC emission and reduce insect resistance of plants. Plant growth-promoting effects induced by the fungal VOCs were not transgenerational. Whole-genome transcriptome analysis of *Arabidopsis* seedlings revealed that genes involved in auxin signaling were up-regulated, whereas ethylene and jasmonic acid signaling were down-regulated upon exposure to fungal VOCs. In conclusion, *R. solani* modulates, *via* VOCs, plant growth, development and insect resistance. We hypothesize that soil-borne pathogens use VOCs to predispose plants for infection by stimulating lateral root formation and enhancing root biomass while suppressing defense mechanisms. Alternatively, upon perception of VOCs from soil-borne pathogens, plants invest in root biomass and accelerate development, while minimizing investments in defensive mechanisms, to survive infection.

Keywords

Fungal and plant volatiles, plant growth promotion, plant defense, plant transcriptome, auxin

Introduction

During their life cycle, plants interact with a multitude of (micro)organisms, including beneficial symbionts, fungal pathogens and insects. In order to cope with pests and pathogens, plants have evolved complex and inducible defensive strategies, which include the biosynthesis of secondary metabolites and volatile organic compounds (VOCs) (Dicke & Baldwin, 2010; Niinemets *et al.*, 2013). Due to their low molecular weight, high vapor pressure and low boiling point, VOCs can travel long distances, facilitating a multitude of interactions with other organisms both below- and aboveground (Schulz & Dickschat, 2007; Das *et al.*, 2012; Junker & Tholl, 2013). For example, during insect herbivore attack, plants trigger the emission of VOCs to attract natural enemies of the herbivores and to elicit resistance mechanisms in neighboring plants (Paré & Tumlinson, 1999; Dicke & Loreto, 2010; Mumm & Dicke, 2010). In addition, plants use VOCs during resource limitation to detect the presence of proximate competitors, such as neighboring plants, and to inhibit the competitors' growth and development (Kegge & Pierik, 2010). Thus, plants do not only emit VOCs in response to stress but also perceive VOCs emitted by other (micro)organisms present in the environment.

It is now well established that VOCs emitted by beneficial soil microorganisms can mediate, from a distance, interactions with plants and other microorganisms (Bailly & Weisskopf, 2012; Effmert *et al.*, 2012; Bitas *et al.*, 2013). For example, bacterial VOCs have been reported to promote plant growth, to induce plant systemic resistance, and to affect motility and antibiotic resistance in other bacteria (Ryu *et al.*, 2003; Ryu *et al.*, 2004; Lee *et al.*, 2012; D'Alessandro *et al.*, 2014; Park *et al.*, 2015; Schmidt *et al.*, 2015). Fungal VOCs are also widespread in nature, but less studied than bacterial VOCs. To date, approximately 250 fungal VOCs have been described, including acids, alcohols, aldehydes, esters, short-chain fatty acids, lipid oxides, terpenes and phenolics (Morath *et al.*, 2012; Roze *et al.*, 2012). The most well-known fungal VOC is 1-octen-3-ol, also referred to as the "mushroom smell", which functions as a developmental signal for several fungal species (Chitarra *et al.*, 2004; Herrero-Garcia *et al.*, 2011).

To date, information on the ecological functions of fungal VOCs is limited and fragmented, in particular for VOCs produced by soil-borne plant pathogenic fungi. Upon exposure to VOCs emitted by the plant beneficial fungus *Trichoderma viride*, biomass and chlorophyll content of *Arabidopsis thaliana* seedlings increased (Hung *et al.*, 2013). In contrast, Kottb *et al.* (2015) showed that VOCs emitted by *Trichoderma asperellum* inhibited plant growth and increased the levels of the plant hormones salicylic acid and abscisic acid, resulting in enhanced resistance against pathogenic fungi. Bitas *et al.* (2015) showed that VOCs emitted by pathogenic *Fusarium oxysporum* promoted the growth of

Arabidopsis and *Nicotiana tabacum*, and affected auxin transport and signaling. A more recent study showed that VOCs emitted by *Alternaria alternata* enhanced growth, early flowering and photosynthesis rates of *Arabidopsis*, maize and pepper by affecting the levels of plastidic cytokinin (Sanchez-Lopez *et al.*, 2016). Collectively, these few studies suggest that VOCs from plant pathogenic fungi may modulate the trade-off between plant growth, development and defense, but the underlying molecular mechanisms and ecological functions remain largely unknown.

Here, we profiled the VOCs emitted by the soil-borne plant pathogenic fungus *Rhizoctonia solani* and investigated if and how these VOCs modulate growth, development and insect resistance of *Arabidopsis*. For that, we exposed seeds and seedlings to *R. solani* VOCs and monitored their impact on seed germination, shoot and root biomass, root architecture, VOC emission and resistance of *Arabidopsis* to the generalist herbivore *Mamestra brassicae*. To further understand the underlying mechanisms, we performed genome-wide transcriptome analysis of *Arabidopsis* seedlings exposed to *R. solani* VOCs. Our results show that VOCs from the soil-borne pathogenic fungus *R. solani* promoted plant growth and development, suppressed insect resistance and induced multiple transcriptional changes in genes involved in photosynthetic-, metabolic- and hormone-related processes. The putative ecological functions of the VOCs in pathogen-plant interactions will be discussed.

Materials and Methods

Plant, fungus and insect materials

Arabidopsis thaliana wild-type Col-0 was obtained from the collection of the Department of Phytopathology at Wageningen University, the Netherlands. The ethylene-insensitive mutants, *etr1-4* (Chang *et al.*, 1993) and *ein3eil1* (Alonso et al., 2003), were provided by G.E. Schaller and J.R. Ecker, respectively. Seeds were surface sterilized as previously described (van de Mortel *et al.*, 2012) and kept in the dark at 4 °C for 3-4 days before sowing. Plates and pots containing plants were grown in climate cabinets (21 °C; 180 µmol light m^{-2} s^{-1} at plant level; 16 h : 8 h, light : dark; 60-70% R.H.).

The fungus *Rhizoctonia solani* AG2-2 IIIB was obtained from the collection of the Sugar Beet Research Institute, Bergen op Zoom, the Netherlands. Fungal cultures were started with a mycelial plug (∅ 5 mm) on 1/5th strength potato dextrose agar (PDA, Oxoid) at 25 °C. Sclerotia were obtained from a fungal culture incubated on 1/5th PDA at 25 °C for 3 weeks.

The generalist insect herbivore *Mamestra brassicae* L. (Lepidoptera: Noctuidae; cabbage moth) was reared on *Brassica oleracea* L. var. gemmifera cv. Cyrus in a controlled

growth chamber (22 ± 2 °C; 16 h : 8 h, light : dark; 40-50% R.H.). Newly-emerged larvae were used in the experiments.

Effects of fungal VOCs on plant growth and development

To investigate the effects of VOCs emitted by the fungal pathogen *R. solani* on the growth and development of *Arabidopsis,* 7-day-old seedlings were exposed to the fungal VOCs. To physically separate the plants from the fungus, a 3-compartment set-up was used (Fig. 1a). Sterile *Arabidopsis* seeds were sown on Petri dish (∅ 90 mm) containing 25 ml of half-strength Murashige and Skoog medium (Murashige & Skoog, 1962) supplemented with 5 % sucrose (0.5xMS). These Petri dishes (without lids) were kept inside a larger Petri dish (∅ 145 mm) which were sealed and kept in climate cabinets. After 7 days, seedlings were exposed to the fungal VOCs or to agar medium by introducing a small Petri dish (∅ 35 mm) containing a 7-day-old fungal culture (referred to as VOC-exposed) or containing the agar medium or a soil-sand mixture only (control). Petri dishes (∅ 145 mm) were re-sealed and kept in the climate cabinets for 2 weeks. For testing the effect of different substrates on VOC production by the fungus, 5 different media were used. The media tested include water-agar (HA), water-agar supplemented with sucrose (HS), water-agar supplemented with sucrose and yeast extract (HSY), malt-agar (Oxoid) (Ezra & Strobel, 2003) and 1/5th strength potato dextrose agar (PDA). Additionally, *R. solani* was grown in a soil-sand mixture. For that, a mycelium plug was added into 2.5 g of sterile soil-sand mixture (12:5 v/v), previously sterilized by autoclaving for 20 min twice with 24 h interval. Fresh and dry weight (overnight incubation at 65 °C) of shoots and roots of VOCs-exposed plants were determined and compared to control plants. For each treatment, five to 6 plants per plate were pooled and treated as a single biological replicate, and a total of 5-8 biological replicates were used.

To test the effects of fungal VOCs on seed germination and emergence of the radicle and cotyledons, 2-compartment Petri dishes (∅ 90 mm) were used. Six seeds were placed in one compartment containing 0.5xMS medium and the Petri dish (∅ 35 mm) with a 7-day-old fungal culture or 1/5th PDA medium only were added to the other compartment. Seed germination and emergence of cotyledons and radicles were recorded daily. Length of primary root was measured after 7 days of exposure.

To study the effects of a short exposure to fungal VOCs on plant growth and development, 7-day-old *Arabidopsis* seedlings grown *in vitro* were exposed to *R. solani* VOCs (VOC-exposed) or to the agar medium (control) for one week as described above and then transferred to pots containing a soil-sand mixture (1:1 v/v) sterilized by autoclaving for 20 min twice with a 24 h interval (Fig. 2A). Shoot fresh weight, length of floral stem, and

number of flowers were determined 2 weeks after soil transplantation. To test if the observed effects of fungal VOCs on plant development could be transferred via the seeds to the next generation (transgenerational effect), 10 VOC-exposed and 10 control plants were kept for seed collection. Seeds were harvested, surface-sterilized, sown in Petri dishes (∅ 90 mm) containing 0.5xMS and grown for 2 weeks under controlled conditions. Fresh and dry weight (overnight incubation at 65 °C) of shoots and roots were determined and compared to control plants.

Statistical differences were determined by pairwise comparison of VOC-exposed and control plants using Student t-Test and One-way ANOVA.

Effects of CO_2 on plant growth

To investigate potential effects of increased levels of fungal-produced CO_2 on plant growth, 7-day-old *Arabidopsis* seedlings grown on square Petri dishes (∅ 100 mm) were exposed to ambient (400 ppm) and elevated (1330 ppm) CO_2 levels in 22.4 l desiccators. A total of 4-7 replicates were used per treatment. CO_2 levels were manipulated through mass flow controllers mixing air and CO_2 to the desired concentrations and monitored with infrared gas analyzers. Desiccators were kept at 20 °C; 180 µmol light m^{-2} s^{-1} at plant level; 16 h : 8 h, light : dark. Plant shoot and root biomass was determined after 7 days of exposure.

Collection and analysis of fungal VOCs

Since also ethylene, a gaseous hormone, is known to be produced by soil microorganisms and plays a role in plant growth and development, we determined the ethylene concentration emitted by *R. solani.* For that, a mycelial plug (∅ 5 mm) was grown in sterile 10 ml glass vials containing 2.5 ml of 1/5th PDA or 0.5xMS media. Vials containing the media only were used as controls. All vials were closed with silicone/PTFE lids and incubated at 25 °C for 10 days. Ethylene concentration was measured with a gas chromatograph (Syntech GC 955-100) equipped with a HayeSep 80/100 column and flame-ionization detector as previously described (Pierik *et al.*, 2009). Measurements were performed using 5 replicates. Statistical differences were determined with Student t-Test.

To identify other VOCs produced by *R. solani* and potentially involved in the modulation of plant growth and development, fungal cultures were prepared as described for the ethylene measurements. Fungal VOCs were collected by a dynamic headspace system on stainless steel cartridges filled with 200 mg Tenax TA as adsorbent. For this, a custom-made 'needle inlet' connected to a Tenax cartridge was penetrated through the septum in the lids. VOCs were collected on cartridges by sucking the air out of the vials with a flow of 40 ml min^{-1} for 5 hours. A second clean Tenax cartridge was placed similarly to

clean the incoming air and to prevent an under pressure. Before thermodesorption, cartridges with the headspace samples were flushed with helium at 50 ml min^{-1} for 5 min to remove moisture and oxygen and after that analyzed by Thermodesorption Gas Chromatography-Mass Spectrometry (TDGC-MS). VOCs were thermally desorbed at 220 °C for 7 min (Ultra, Markes Llantrisant, UK) with a helium flow of 30 ml min^{-1}. Analytes were focused at 4 °C on a cooled trap (Unity, Markes, Llantrisant, UK) and were then transferred to the analytical column (ZB-5Msi, 30 m, 0.25 mm i.d., 1.0 µm film thickness, Phenomenex, Torrance, CA, USA) by rapid heating of the cold trap to 260 °C for 4 min using a split flow of 5 ml min^{-1}. The temperature gradient of the GC oven was as follows: 45 °C hold for 3 min, 10 °C min^{-1} gradient to 280 °C, with a 2 min hold, at a constant gas flow of 1 ml min^{-1}. Mass spectra were acquired by electron impact ionization (70 eV) with a scanning from m/z 35-400 with a scan rate of 5 scans s^{-1}.

GC-MS raw data were processed by an untargeted metabolomics approach as previously described by Cordovez *et al.* (2015). VOCs were tentatively identified by comparison of the mass spectra with those of NIST (National Institute of Standards and Technology, USA), Wiley libraries and the Wageningen Mass Spectral Database of Natural Products and by comparing the experimentally calculated LRI with the literature values. Three replicates per treatment were used and vials containing 1/5th PDA medium only were used as controls.

Plant exposure to synthetic VOCs

The synthetic compounds 2-methyl-1-propanol (analytical standard), 2-pentanone (99.5%), 2-methyl-1-butanol (≥ 98.0%), 1-octen-3-ol (98%) and 3-octanone (≥ 98%) were purchased from Sigma-Aldrich. Experiments were performed in Petri dishes (∅ 90 mm) with 2-compartments. Four 7-day-old *Arabidopsis* seedlings were placed on one compartment containing 0.5xMS medium and different dilutions of the 5 synthetic compounds and the mixture were applied to a sterile paper filter (1.5 x 1.5 cm) placed in the other compartment. Dilutions of the synthetic compounds were made as previously described by Blom *et al.* (2011). Briefly, synthetic compounds were diluted with dichloromethane (DCM) to concentrations of 1 ng, 10 ng, 100 ng, 10 µg, 100 µg and 1 mg per 10 µl. Each compound was mixed in a 1:1 ratio with the lanolin solution (1.6 g of lanolin in 10 ml DCM) and 20 µl of this mixture was added to the paper filter. For negative controls, the second compartment was left empty or a mixture of DCM and lanolin was added. For the positive control, seedlings were exposed to the one-week-old *R. solani* culture grown on a Petri dish (∅ 35 mm) containing 1/5th PDA medium. Petri dishes were immediately sealed and incubated in a growth cabinet. Shoot and root biomass was determined after 2

weeks. Experiments were performed using 5 biological replicates and statistical differences were determined by pairwise comparison between VOC-exposed and control (exposed to solvent) plants using Student t-Test.

Plant RNA extraction, sequencing and transcriptome analyses

To investigate how VOCs from a soil-borne pathogenic fungus modulate plant growth and development at the transcriptomic level, we sequenced and analyzed the genome-wide transcriptome of *Arabidopsis* seedlings exposed to *R. solani* VOCs for 7 days *in vitro*. Seedlings exposed to 1/5th PDA medium only were used as control. For the sequencing of plant RNA, total RNA was extracted from roots and shoots. A total of 4 replicates were used, and each replicate consisted of 4 plates with 6 seedlings each in order to obtain enough biomass. RNA was obtained from frozen tissues with Trizol reagent (Invitrogen). The RNA samples were further purified using the NucleoSpin RNA II kit (Macherey-Nagel). Samples were processed using the NEBNext Ultra Directional RNA Library Prep Kit for Illumina at ServiceXS (GenomeScan B.V., Leiden, the Netherlands). Briefly, mRNA was isolated from the total RNA using the oligo-dT magnetic beads. After fragmentation of the mRNA, cDNA was synthesized, ligated with sequencing adapters and amplified by PCR in order to obtain cDNA libraries. Each cDNA library was individually analyzed for quality and yield using a Fragment Analyzer. cDNA was then clustered and a concentration of 1.6 pM was sequenced with an Illumina NextSeq 500 sequencer.

For the transcriptome analysis, Illumina sequences were trimmed and filtered with FASTQC with a threshold of 25 (Q > 25). Quality-trimmed reads were counted using RSEM software package (Li & Dewey, 2011) transformed into RPKM (Reads Per Kilobases per Million reads). Reads were mapped to the *Arabidopsis* reference genes using the software Bowtie2 v.2.1.0 (Langmead & Salzberg, 2012). The Bioconductor package DESeq2 for R Statistical Analysis (Love *et al.*, 2014) was used for normalization and differential expression analyses. The *P*-value was obtained from the differential gene expression test. FDR (False Discovery Rate) correction was used to determine the *P*-value threshold in multiple tests and analyses. Significant differentially expressed genes (DEGs) were selected using FDR < 0.05 and the absolute value of the log2Ratio ≥ 0.585 (at least 1.5x higher than the expression level in control) as thresholds.

Biological interpretation of the DEGs was carried out with a GO-term enrichment analysis performed using AgriGO (http://bioinfo.cau.edu.cn/agriGO/index.php). TAIR AGI IDs of significantly up- and down-regulated genes were subjected to a singular enrichment analysis using Fisher test with FDR (Hochberg) at 0.05. *Arabidopsis* TAIR 9 database was

used as a background. Identification of transcription factors was carried out using the *Arabidopsis* transcription factor database (http://*Arabidopsis*.med.ohio-state.edu/).

Collection and analysis of plant VOCs

To investigate if a previous exposure to VOCs from a pathogenic fungus changes the VOC profile of plants, 7-day-old *Arabidopsis* seedlings were exposed to *R. solani* VOCs for one week and transplanted to soil-sand mixture without the fungus (Fig. 5A). After 2 weeks, headspace VOCs from the aerial parts of *Arabidopsis* plants exposed to the *R. solani* VOCs and plants exposed to the 1/5th PDA medium (control) were collected (n = 8 pots containing 4 plants each). Prior to collection, pots and soil surface were covered with tin foil and plant aerial parts were enclosed in an oven bag (Toppits® Brat-Schlauch, polyester, 32 × 32 × 70 cm, Minden, Germany) (Fig. 5A). Synthetic air was flushed through the bag at a flow rate of 300 ml min^{-1} (224-PCMTX8, air-sampling pump Deluxe, Dorset, UK) by inserting Teflon tubing through an opening in the upper part of the bag. Air was sucked out, and headspace VOCs were collected in the Tenax TA for 4 hours at a flow rate of 250 ml min^{-1}.

Plant VOCs were analyzed by Gas Chromatography-Quadrupole Time of Flight-Mass Spectrometry (GC-QTOF-MS) (Agilent 7890B GC and Agilent 7200A QTOF, Santa Clara, USA). VOCs were desorbed from the Tenax tubes in a thermal desorption unit (Unity TD-100, Markes International Ltd., Llantrisant, UK) at 210 °C for 12 min (Helium flow 50 ml min−1) and focused at -10°C in a cold trap. VOCs were introduced into the GC (column:RXI-5MS, 30 m, 0.25 mm i.d., 0.25 µm film thickness, Restek 13424-6850, Bellefonte, PA, USA) by heating the cold trap for 12 min to 250 °C with 1:10 split ratio. Temperature gradient of the GC oven was: 39 °C for 2 min, from 39 °C to 95 °C at 3.5 °C min^{-1}, from 95 °C to 165 °C at 4 °C min^{-1}, from 165 °C to 280 °C at 15 °C min^{-1} and from 280 °C to 320 °C at 30 °C min^{-1}, 7 min hold at a constant gas flow of 1.2 ml min^{-1}. Mass spectra were acquired by electron impact ionization (70 eV) with a scanning from m/z 30-400 with a scan rate of 5 scans s^{-1}.

VOCs were tentatively identified by comparison of the mass spectra with those of NIST (National Institute of Standards and Technology, USA), Wiley libraries and the Wageningen Mass Spectral Database of Natural Products and by comparing the experimentally calculated LRI with the literature values. Chromatograms were integrated using MassHunter Qualitative Analysis Software V B.06.00 Build 6.0.633.0 (Agilent Technologies, Santa Clara, USA). Emission rates were quantified using the relative peak area and normalized by plant fresh weight. Normalized emission rates were used to perform a Projection to Latent Structures Discriminant Analysis (PLS-DA) to determine qualitative and quantitative differences in terms of VOC profile of VOC-exposed and control plants. Multivariate analysis was performed with SIMCA P+ 12.0 software, Umetrics AB

(Umeå, Sweden). To determine significant differences between the total VOCs emitted by the VOC-exposed and the control plants, related samples Wilcoxon signed rank test was performed ($P < 0.05$).

Effects of fungal VOCs on plant resistance to insect herbivory

To test the effect of a short exposure to fungal VOCs on plant resistance to a generalist insect herbivore, one-week-old *Arabidopsis* seedlings were exposed to *R. solani* VOCs for 7 days *in vitro* and transplanted to the soil-sand mixture. After 2 weeks, plants were infested with 30 neonates of *Mamestra brassicae* using a fine brush (n = 8 pots containing 3 plants each) (Fig. 6A). Plant pots were kept inside a plastic container (Duchefa, Haarlem, the Netherlands; height: 140 mm, upper ∅: 115 mm, lower ∅: 90 mm), covered with insect-proof mesh cloth and sealed with elastic bands and kept in growth cabinet. Caterpillar density was reduced to 15 and 10 larvae at 3 and 7 days post-infestation (dpi), respectively. Larval performance was measured by weighing the larvae on a microbalance (accuracy: ± 1 µg; Mettler-Toledo MT5 Electrobalance) at 3, 7 and 10 dpi. A Generalized Linear Model (GLM) was performed to statistically assess the effects of VOC-exposure, time, replicate and their interactions.

Results

VOCs emitted by fungal pathogen promote plant growth and prime plant development

To study the effects of VOCs from a fungal pathogen on plant growth, *Arabidopsis* seedlings were co-cultivated with *R. solani* but physically separated, allowing only VOC-mediated interaction (Fig. 1A). After 2 weeks, seedlings exposed to VOCs from *R. solani* (hereafter referred to as VOC-exposed) grown on agar medium showed an increase in shoot and root dry weight of 150% (t-Test, $P < 0.001$) and 265% (t-Test, $P < 0.001$), respectively, compared to seedlings exposed to the agar medium only (hereafter referred to as control) (Fig. 1B). Also seedlings exposed to VOCs from fungal mycelium inoculated into a soil-sand mixture showed a 62% increase in root dry weight (Fig. 1B, t-Test, $P = 0.002$). Because *R. solani* survives in soil as mycelium or as sclerotia, we also tested if VOCs emitted from sclerotia affect plant growth. Sclerotial VOCs increased shoot and root dry weight by 60% (t-Test, $P < 0.001$) and 44% (t-Test, $P = 0.012$), respectively (Fig. 1B). Since the production of VOCs by microorganisms is typically influenced by the substrate composition, we tested, in addition to the 1/5th PDA medium, 4 other agar media with a composition ranging from nutrient-poor to nutrient-rich. Dry weight of plant shoots and roots were significantly higher in the

VOC-exposed seedlings than in the control for all tested media, except for water agar (Fig. S1).

To study if *R. solani* VOCs can modulate *Arabidopsis* development, we investigated their effects on seed germination as well as radicle and cotyledon emergence. *R. solani* VOCs had no effect on seed germination nor cotyledon emergence, however a delay in radicle emergence was observed for VOC-exposed seeds (Fig. 1C, t-Test, $P = 0.015$). Seven-day-old seedlings resulting from seeds exposed to fungal VOCs displayed shorter primary roots compared with control (Fig. 1D; t-Test, $P < 0.001$). However, plant growth-promoting effects were still observed for these seedlings when growth was continued for 18 days, but to a lower extent than observed when seedlings instead of seeds were exposed to fungal VOCs (Fig. 1E,F; t-Test, $P < 0.001$).

To investigate whether *R. solani* VOCs can prime development of plants grown in soil, *Arabidopsis* seedlings were exposed *in vitro* to fungal VOCs for 7 days, transplanted to a soil-sand mixture and further grown in absence of the fungus (Fig. 2A). After 2 weeks, VOC-exposed plants showed significant increases in shoot weight, length of the flower stem and number of flowers by 96%, 40% and 45%, respectively (Fig. 2B, C,D). For 10 VOC-exposed and 10 control plants, seeds were collected and subsequently grown on 0,5xMS agar medium to determine if the VOC-mediated effects on growth and root architecture were transgenerational. Seedlings originated from seeds of VOCs-exposed plants showed no difference in root weight (t-Test, $P = 0.216$) and even a slight reduction in shoot weight (t-Test, $P = 0.034$) compared to seedlings originated from seeds of control plants (Fig. 2A,E).

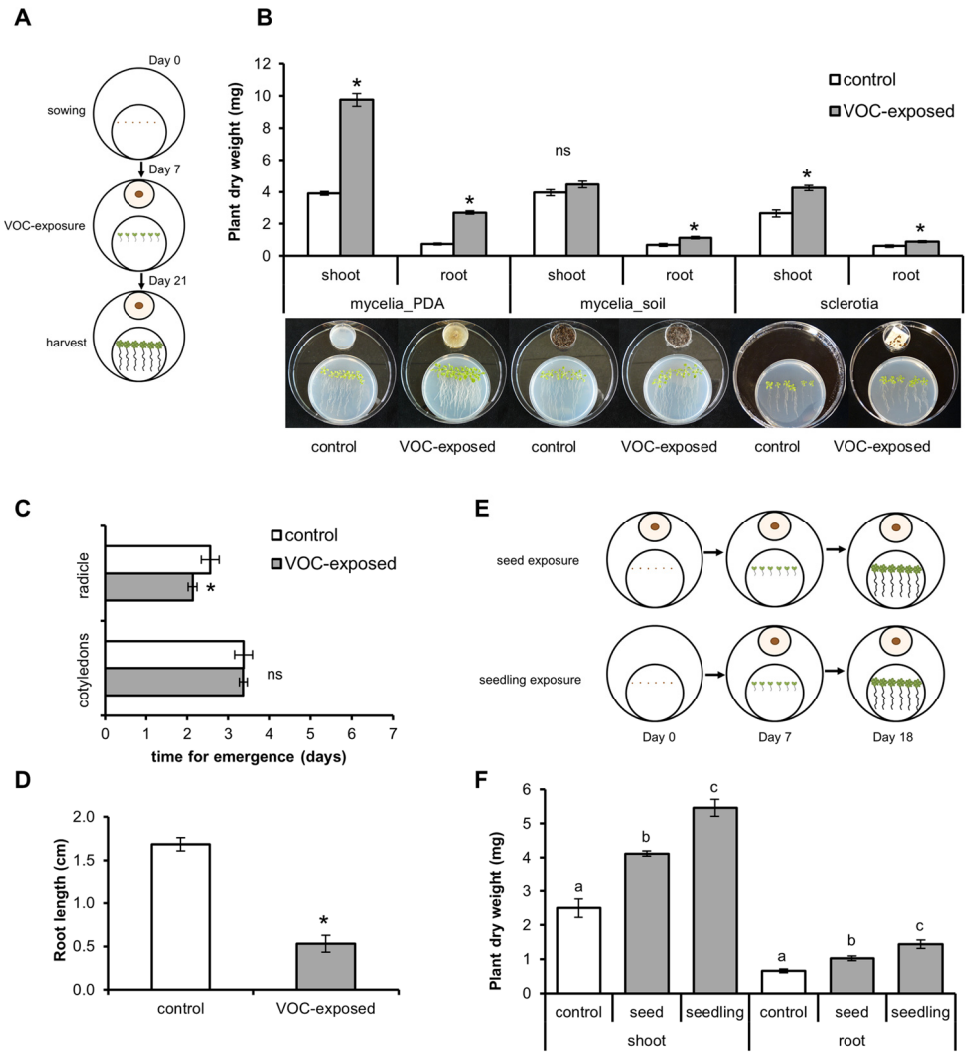


Fig. 1 | Effects of volatile organic compounds (VOCs) produced by *Rhizoctonia solani* on the growth and development of *Arabidopsis thaliana* seedlings *in vitro*. Plants exposed to fungal VOCs are referred as VOC-exposed whereas plants exposed to agar medium or soil-sand mixture are referred as control. Asterisks indicate statistically significant differences resulted from pairwise comparisons between VOC-exposed and control plants (Student's t-Test, $P < 0.05$). Non-significant differences are displayed as 'ns'. **(A)** Schematic representation of the experimental set-up to exposure seedlings to fungal VOCs. **(B)** Biomass (mean ± SE, n = 5-8) of shoots and roots exposed to VOCs produced by fungal mycelium grown on 1/5th Potato Dextroxe Agar medium (mycelia_PDA) and on soil-sand mixture (soil_mycelia), and by the VOCs produced by fungal sclerotia for 14 days. **(C)** Time of emergence (mean ± SE, n = 8) of radicle and cotyledons VOC-exposed and control plants. **(D)** Primary root length of VOC-exposed and control plants after 7 days. **(E)** Schematic representation of the experimental set-up to exposure seeds and seedlings to fungal VOCs. **(F)** Biomass (mean ± SE, n = 5) of shoots and roots of VOC-exposed and control plants at the seed and 7-day-old seedling stages. Different letters indicate statistically significant differences between treatments (One-way ANOVA, Tukey post-hoc Test, $P < 0.05$).

Identification of fungal VOCs involved in plant growth promotion

Previous studies have reported increased CO_2 concentrations in closed systems due to microbial catabolism. Higher CO_2 concentrations lead to enhanced photosynthetic efficiency, chlorophyll content and sugar accumulation, leading to an increase in plant biomass (Poorter & Navas, 2003; Kai & Piechulla, 2009; Jauregui et al., 2015). To evaluate a role of CO_2 in the VOC-mediated plant growth-promoting effects, the first approach used was to trap CO_2 with different amounts of the CO_2 absorber, sodalime. The results showed that even small amounts of sodalime were harmful to the *Arabidopsis* seedlings, suggesting a deleterious depletion of CO_2 (Fig. S2A). A second approach involved exposing the *Arabidopsis* seedlings to an elevated CO_2 concentration of 1330 ppm which is approximately 3 times higher than the ambient CO_2 concentration of 400 ppm. After 7 days of plant growth, no differences in plant shoot (t-Test, $P = 0.219$) and root (t-Test, $P = 0.277$) weight were observed between CO_2-exposed and control seedlings (Fig. S2B,C).

Table 1 | Volatile organic compounds (VOCs) emitted by the fungal pathogen *Rhizoctonia solani*. VOCs displayed are significantly different (Student's t-Test, $P < 0.05$, n = 3), detected at peak intensities at least twice as high as in the control (medium only) and with a match factor higher than 800. Compounds were putatively annotated by comparing their mass spectra with those of NIST and in-house mass spectral libraries.

Compound	RI[a]	Annotation[b]	VOC emission[c]	
			control	*R. solani*
2-methyl-1-propanol*	665	1	258225 ± 59955	739090 ± 106560
unknown	706	4	3664 ± 1222	318349 ± 121863
2-pentanone*	709	1	145709 ± 18534	329667 ± 47446
methyl thiocyanate	732	2	17812 ± 8687	583890 ± 36242
2-methylbutanenitrile	740	1	24682 ± 18783	414931 ± 118132
3-methyl-butanenitrile	745	1	72109 ± 11955	1169000 ± 273882
2-methyl-1-butanol*	751	1	33574 ± 4041	470300 ± 181198
unknown	956	4	11654 ± 10118	30769 ± 5295
1-octen-3-ol*	978	1	4620 ± 2684	2255670 ± 1373443
3-octanone*	986	1	12785 ± 10514	3549774 ± 1893459
2-ethyl-1-hexanol	1027	2	264835 ± 59710	507060 ± 88147
2-nonen-1-ol	1172	1	1956 ± 281	4839 ± 1900
unknown	1343	4	3468 ± 869	5971 ± 1257
alpha-copaene	1403	2	5092 ± 3553	45013 ± 21268

[a]Calculated retention indices (RI)

[b]Annotation level according to the Metabolomics Standards Initiative (MSI)

[c]Emission values refer to peak intensity (mean ± SD)

*Compounds tested for *in vitro* plant growth-promoting effects

We next determined if *R. solani* produces ethylene, a volatile hormone produced by several microorganisms (Ilag & Curtis, 1968; Considine *et al.*, 1977). Among the VOCs emitted by a 10-day-old fungal culture no differences in ethylene concentration were found relative to the control (Fig. 3A; t-Test, $P = 0.965$). However, we cannot not exclude that ethylene production occurs at an earlier or later developmental stages of fungal growth, during the interaction with plants, or when grown in soil. To exclude a major role of ethylene in our experimental system, we tested two independent ethylene-insensitive *Arabidopsis* mutants: *etr1-4* (ethylene receptor mutant) and *ein3 eil1* (transcription factor double mutant) for their response to the fungal VOCs. After 2 weeks, VOC-mediated plant growth promotion was observed for both mutants and to the same extent as observed in wild-type *Arabidopsis* Col-0 (Fig. 3B,C). In these assays, we also included *Verticillium dahliae*, a soil-borne pathogenic fungus that produces ethylene when grown on $1/5^{th}$ PDA. *V. dahliae* still promoted plant growth in the two ethylene-insensitive *Arabidopsis* mutants as observed for *R. solani* (data not shown). Collectively, these results rule out a major role of ethylene in the plant growth promotion observed in our study system.

Further GC-MS analysis detected a total of 14 VOCs in the headspace of *R. solani* cultures that were significantly different from the control (medium only) (t-Test, $P < 0.05$) and with peak intensities at least twice as high as those in the control (Table 1). Among these, typical fungal VOCs such as 1-octen-3-ol and 3-octanone were detected. A total of 5 VOCs, which could be reliably annotated, based on RI and mass spectral similarity, and which were commercially available as authentic reference standards, were selected to test their effects on plant growth. When *Arabidopsis* seedlings were exposed to 2-methyl-1-propanol, 2-pentanone, 2-methyl-1-butanol, 1-octen-3-ol or 3-octanone at concentrations ranging from 1 ng to 1 mg per 10 µl or a mix of all 5 synthetic compounds, no growth promotion was observed compared to seedlings exposed to the solvent only (Fig. S3A). Higher concentrations (10 µg, 100 µg and 1 mg) of 1-octen-3-ol, 3-octanone and the mix even inhibited plant growth (Fig. S3B). These results indicate the involvement of other yet unknown VOCs, not detected by the method used.

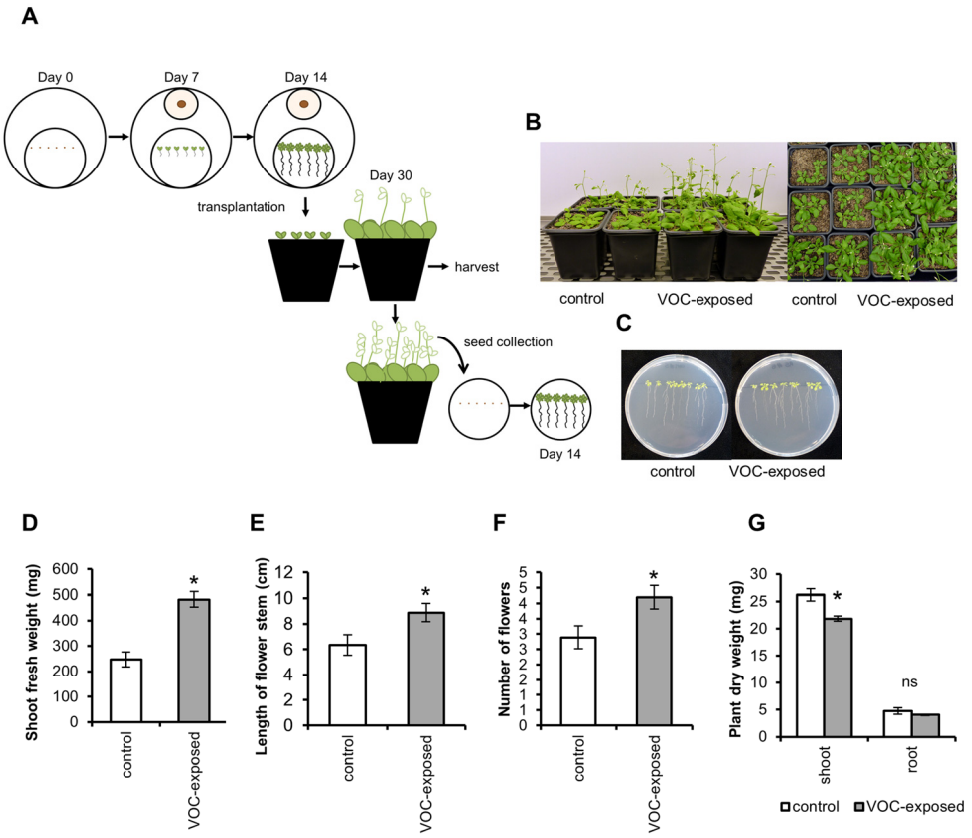


Fig. 2 | Effects of short exposure to volatile organic compounds (VOCs) produced by *Rhizoctonia solani* on the growth and development of *Arabidopsis thaliana* plants *in situ*. Plants exposed to fungal VOCs are referred as VOC-exposed whereas plants exposed to agar medium or soil-sand mixture are referred as control. Asterisks indicate statistically signifcant differences resulted from pairwise comparisons between VOC-exposed and control plants (Student's t-Test, $P < 0.05$). Non-significant differences are displayed as 'ns'. **(A)** Schematic representation of the set-up experiment. **(B)** Pictures of VOC-exposed and control plants after 1-week-exposure to fungal VOCs and agar medium, respectively, and subsequent 2 weeks after transplantation to soil-sand mixture. **(C)** Pictures of the first generation seedlings harvested from VOC-exposed plants taken 14 days after sowing. Shoot biomass **(D)**, length of flower stem **(E)**, and number of flowers **(F)** (mean ± SE, n = 12) of VOC-exposed and control plants. **(G)** Shoot and root biomass (mean ± SE, n = 4) of the first generation seedlings harvested from VOC-exposed plants.

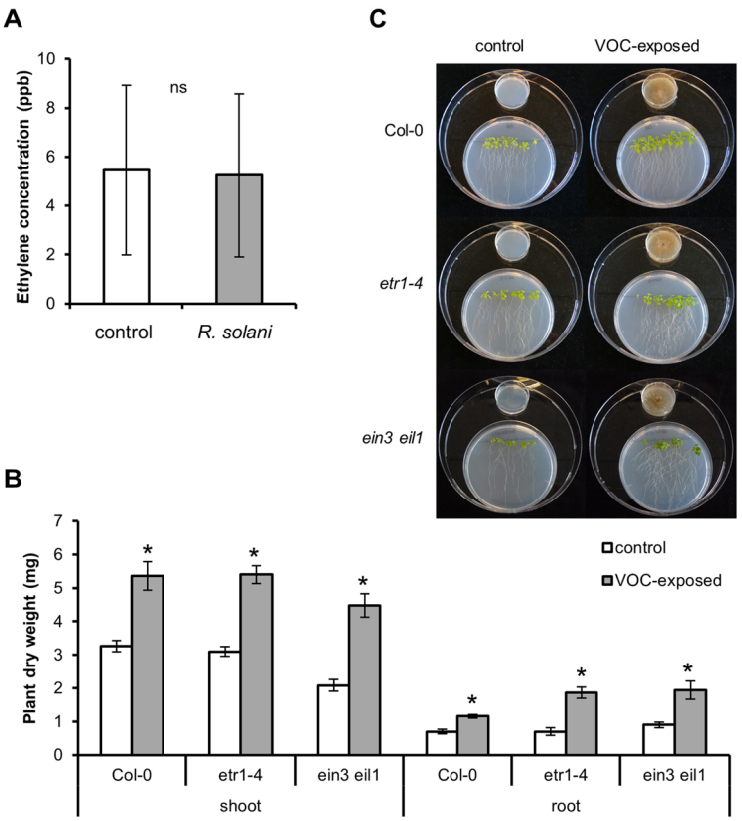


Fig. 3 | Production of ethylene by *Rhizoctonia solani* and its effects on plant growth. **(A)** Gas-chromatographic measurement of ethylene (mean ± SE, n = 4-5) emitted by 7-day-old culture of *Rhizoctonia solani* grown on 1/5th Potato Dextroxe Agar medium. Control refers to medium only and non-significant difference is displayed as 'ns' (Student's t-Test, $P < 0.05$). **(B)** Shoot and root biomass (mean ± SE, n = 4-5) of *Arabidopsis* wild-type Col-0 and the mutants *etr1-4* and *ein3xeil* exposed to volatile organic compounds (VOCs) emitted by *R. solani*. Asterisks indicate a statistically significant difference between VOC-exposed and control (exposed to agar medium only) plants (Student's t-Test, $P < 0.05$). **(C)** Phenotype of *Arabidopsis* wild-type and mutants after 14 days of exposure to fungal VOCs.

Fungal VOCs induce plant genome-wide transcriptional changes

To study plant genes transcriptionally responding to VOCs from the fungal pathogen, significant DEGs were selected with a log2 fold change ≥ + 0.058 (1.5 X higher) or ≤ - 0.585 (1.5 X lower) and FDR < 0.05. Following these criteria, a total of 477 (267 up-regulated and 210 down-regulated) and 12 (6 up-regulated and 6 down-regulated) genes were identified in *Arabidopsis* shoot and root tissues exposed to *R. solani* VOCs, respectively. To identify

enriched Gene Ontology (GO) terms, a singular enrichment analysis (SEA) of GO categories was conducted using AgriGO (Table S1,S2). In shoot tissue, up-regulated genes included genes regulating processes related to response to auxin stimulus, response to abscisic acid stimulus, and photosynthesis, whereas down-regulated genes included genes involved in ethylene- and jasmonic acid (JA) signaling pathways. Genes involved in response to gibberellin (GA) and brassinosteroid (BR) stimuli were both up- and down-regulated in shoot tissue. (Table S3,S4). It was not possible to perform an enrichment analysis for root DEGs since the total number of DEGs was lower than the required minimum number of genes in the query.

Several genes involved in growth-related processes were up-regulated whereas genes involved in defense were down-regulated in seedlings exposed to fungal VOCs (Fig. 4). Up-regulated shoot genes involved in growth-related processes included *IAA-2* (AT3G23030.1), *IAA-19* (AT3G15540.1), *IAA-29* (AT4G32280.1), *WES1* (At4G27260.1), *HAT2* (AT5G47370.1), and *DFL1* (At5g54510.1), all involved in the homeostasis of auxin (Fig. S4A). In addition, *CYP707A3* (AT5G45340.1), involved in abscisic acid catabolic process was 6.3-fold up-regulated in root genes of VOC-exposed seedlings. Down-regulated shoot genes involved in defense-related processes included *RCD1* (AT1G32230.1), *ARR4* (AT1G10470.1) and *EBF2* (AT5G25350.1), involved in ET/JA-, ET- and cytokinin-mediated signaling pathways, respectively.

To shed light on the regulatory genes of *Arabidopsis* modulated by *R. solani* VOCs, we identified transcription factors encoded by the DEGs using a web-based tool (http://*Arabidopsis*.med.ohio-state.edu/AtTFDB). We found a total of 20 and 22 transcription factors (TFs) that were up- and down-regulated, respectively, when seedlings were exposed to the fungal VOCs (Table S5). Among the up-regulated TFs, C2C2-CO-like family was the most abundant whereas among the down-regulated, C2H2, C2C2-CO-like, AP2-EREBP and WRKY families were the most abundant (Fig. S4B). Previous studies with *Arabidopsis* have demonstrated that members of the WRKY and the MYB transcription factor families control various processes involved in the responses to biotic and abiotic stresses, development, metabolism, and defense. Our analyses identified several members of the MYB and the WRKY families: *WRKY53* (AT4G23810) was up-regulated, whereas *WRKY26* (AT5G07100), *WRKY39* (ATt3G04670), *WRKY45* (AT3G01970), and *MYB3R-3* (AT3G09370) were down-regulated in plants exposed to fungal VOCs. These down-regulated transcription factors have been described to be involved in plant resistance to pathogens. In addition, we also found *PIF5* (AT3G59060) and *HB-2* (AT4G16780.1) among the up-regulated genes of *Arabidopsis* shoots exposed to fungal VOCs. The first acts negatively in the phytochrome B signaling pathway promoting the shade-avoidance

response and the latter regulates root development. Collectively, the transcriptome data indicates an impact of *R. solani* VOCs on the trade-off between growth/development and defense to biotic stress of *Arabidopsis* seedlings.

To determine the effects of fungal VOCs on plant resistance to herbivores, the performance of the larvae *M. brassicae* feeding on *Arabidopsis* plants previously exposed to *R. solani* VOCs was investigated (Fig. 6A). Biomass of larvae feeding on leaves of VOC-exposed plants increased in fresh weight by 49% and 70% at 7 and 10 dpi, respectively, compared to non-exposed control plants (Fig. 6B,C). Statistically significant effects of the time point (GLM, $P \leq 0.001$), VOC exposure (GLM, $P \leq 0.001$), replicate (GLM, $P = 0.005$) as well as the interactions between VOC exposure and time point and VOC exposure (GLM, $P \leq 0.001$) and replicate (GLM, $P = 0.001$) were also observed. These analyses show that exposure to *R. solani* VOCs suppressed *Arabidopsis* resistance to larval feeding.

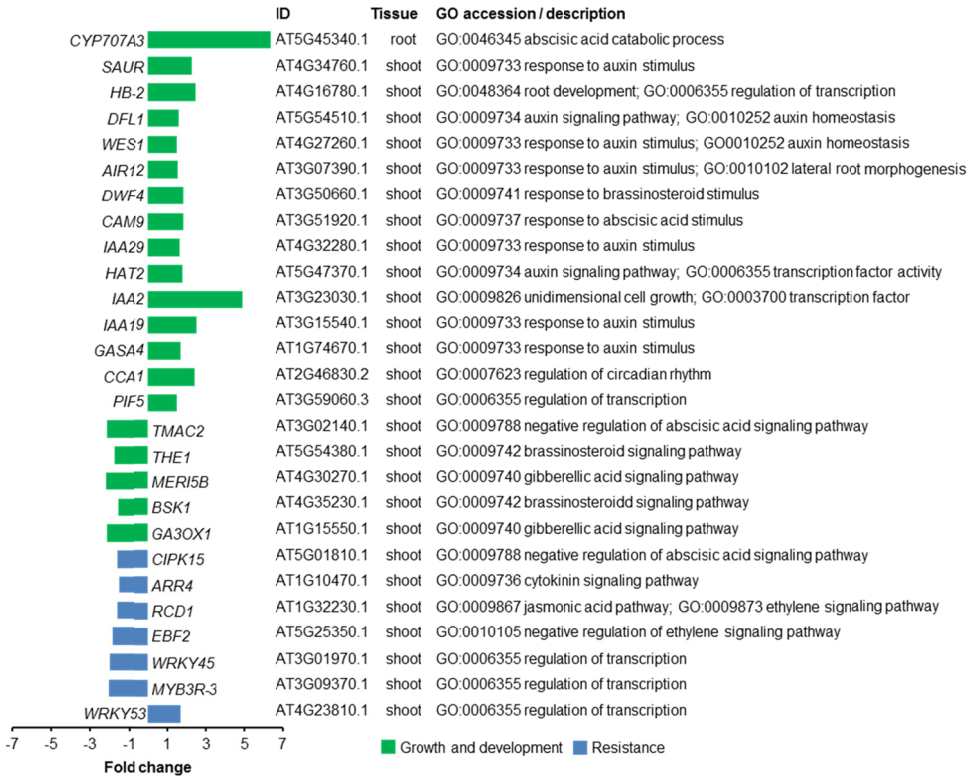


Fig. 4 | Effects of volatile organic compounds (VOCs) emitted by *Rhizoctonia solani* on the trade-off between growth/development and resistance of *Arabidopsis thaliana* seedlings at the transcriptional level. Differentially expressed genes (DEGs) for seedlings exposed to fungal VOCs. Genes previously described to be involved in plant growth and development are displayed in green and genes involved in resistance to biotic stress in blue. Gene Ontology (GO) description was performed using AgriGO.

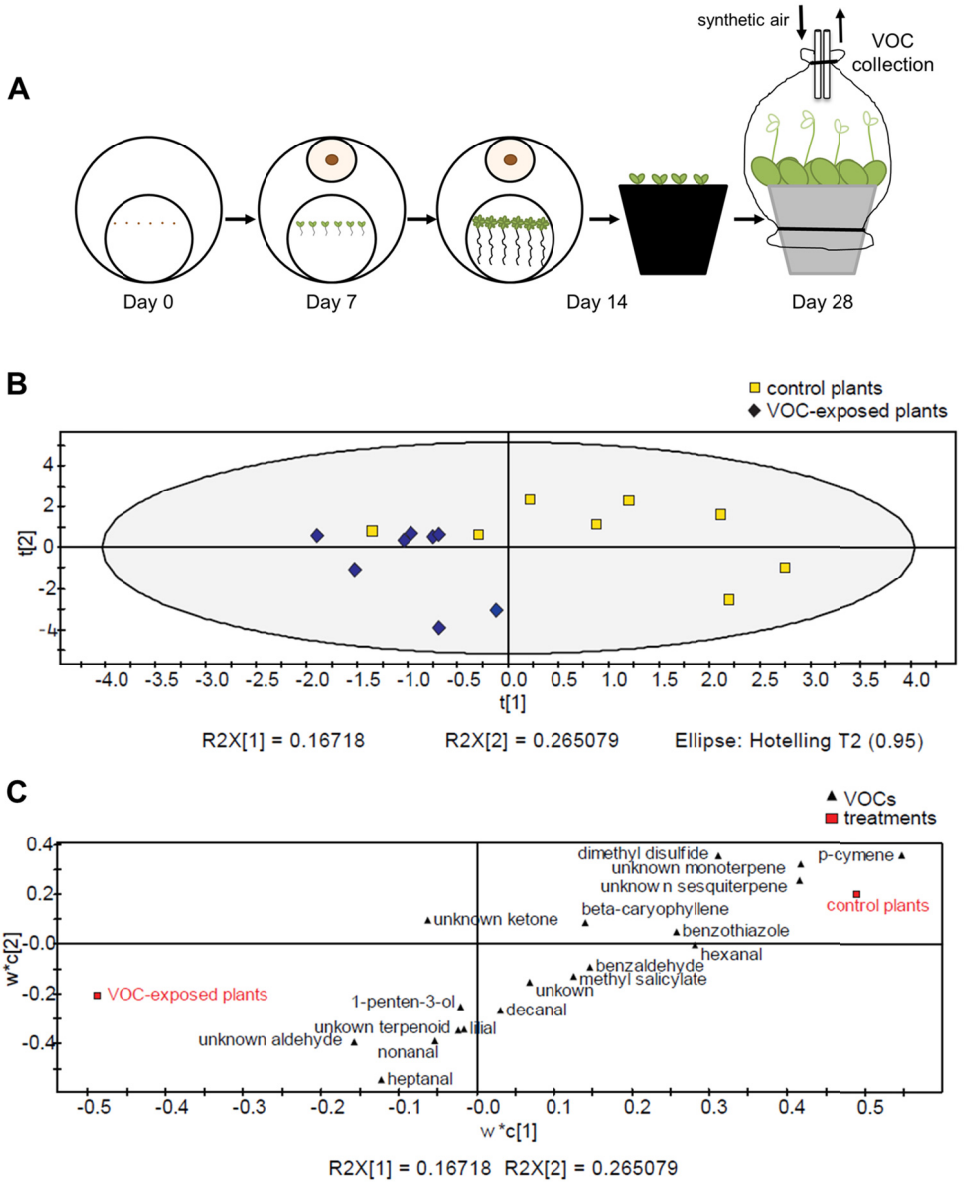


Fig. 5 | Profiling of *Arabidopsis thaliana* volatile organic compounds (VOCs) after exposure to VOCs emitted by *Rhizoctonia solani*. (A) Schematic representation of the experimental set-up. (B) Projection to Latent Structures Discriminant Analysis (PLS-DA) of volatile organic compounds (VOCs) collected from the headspace of aerial parts of *Arabidopsis* plants exposed to VOCs emitted by *Rhizoctonia solani* and of control (exposed to agar medium only) plants. Grouping pattern of samples according to the first two principal components, and the Hotelling's T2 ellipse confining the confidence region (95%) of the score plot. (C) Contribution of individual VOCs to the first two principal components is shown in the loading plot of the PLS-DA components.

Fungal VOCs change plant VOC emission and insect resistance

Analysis of the VOCs in the headspace of *Arabidopsis* aerial parts previously exposed to R. solani VOCs, showed a total of 18 VOCs, including alcohols, aldehydes, ketones, terpenoids and sulfur-containing compounds (Table S6). A Projection to Latent Structures Discriminant Analysis (PLS-DA) analysis resulted in two principal components explaining 17 and 27 % of the total variance between the VOC-exposed plants and the control plants, respectively (Fig. 5A,B). The first principal component showed a separation of samples exposed to fungal VOCs from the control and can be explained by quantitative differences in p-cymene (RI = 1028, VIP = 1.94), unknown monoterpene (RI = 1012, VIP = 1.58), unknown sesquiterpene (RI = 1447, VIP = 1.47), hexanal (RI = 801, VIP = 1.09), dimethyl disulfide (RI = 749, VIP = 1.09), an unknown ketone (RI = 699, VIP = 1.00) and benzothiazole (RI = 1220, VIP = 1.00) (Fig 5C, Table S6). After VOC collection, plant weight, stem length, and number of flowers were measured (Fig. S5A). VOC-exposed plants showed an increase in fresh weight of aerial parts compared to control (t-Test, P = 0.029). Flower stem length and the number of flowers were not significantly affected by VOC exposure (Fig. S5B; t-Test, P = 0.160 and 0.198, respectively).

Discussion

Over the past decade, microbial VOCs have been increasingly recognized as an important mechanism in microbe-microbe and microbe-plant interactions. Here we show that VOCs emitted by the soil-borne fungal pathogen *R. solani* promote plant growth, prime and accelerate plant development, alter plant chemistry and reduce resistance to a generalist insect herbivore. These findings suggest a trade-off between growth and defense in VOC-mediated interactions between *R. solani* and *Arabidopsis*. In nature, plants must be able to grow fast enough to compete and yet defend against herbivores and pathogens, also referred to as the 'dilemma of plants'. Hence, fine-tuned regulation is crucial to balance these energetically costly processes, as activation of defense mechanisms generally comes at a cost of plant growth and *vice-versa* (Huot *et al.*, 2014).

Genome-wide transcriptome analysis further supported the modulation of plant growth, development and defense by the fungal VOCs at the molecular level. Our data show that *R. solani* modulated hormone signaling in *Arabidopsis* seedlings *via* the production of VOCs. Genes involved in response to auxin and ABA stimuli were up-regulated in seedlings exposed to fungal VOCs, whereas genes involved in ET- and JA-mediated signaling pathways were down-regulated. In a recent study, Garnica-Vergara *et al.* (2016) showed that auxin signaling in *Arabidopsis* is affected by the volatile 6-pentyl-2H-

pyran-2-one emitted by the beneficial fungus *Trichoderma atroviride*. Also Bitas *et al.* (2015) showed that the pathogenic fungus *Fusarium oxysporum* can manipulate auxin transport and signaling by production of VOCs. Our results confirm and extend these observations and indicate that modulation of the auxin-mediated signaling pathway appears to be a common effect of fungal VOCs, either from plant beneficial or plant pathogenic fungi.

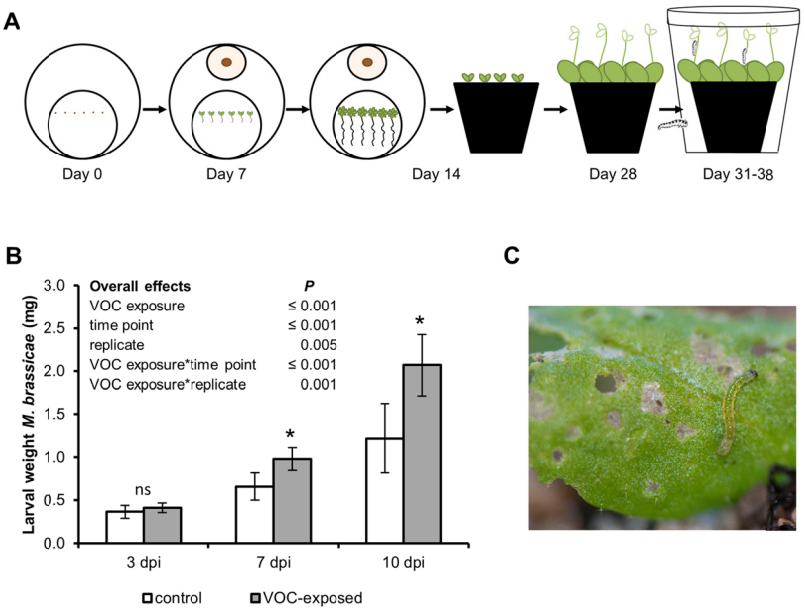


Fig. 6 | Effects of volatile organic compounds (VOCs) emitted by *Rhizoctonia solani* on *Arabidopsis thaliana* resistance to a generalist insect herbivore. (A) Schematic representation of the experimental set-up. (B) Biomass of *Mamestra brassicae* larvae (mean ± SE, n=8) feeding on plants previously exposed to fungal VOCs or to the medium only (control). Each pot contained 3 plants which were infested with 30 neonates. Larval weight was measured at 3, 7 and 10 days post-infestation (dpi). Generalized linear model (GLM) was performed to test the main effects of the VOC exposure, time point of larval performance, replicate, as well as the interaction between VOC exposure and time point and VOC exposure and replicate. (C) Pictures of *M. brassicae* feeding on *Arabidopsis* plants 7 dpi.

More specifically, the up-regulated genes included several genes involved in auxin homeostasis such as *IAA2, IAA19, IAA29*, *HAT2*, *DFL1*, *WES1*. Several of these genes, in particular *IAA2*, *IAA19*, *IAA29* and *HAT2,* have been reported to be associated with shade-avoidance responses (de Wit *et al.*, 2013). When competing for light, *Arabidopsis* plants invest in shade-avoidance responses such as increased leaf angles, stem elongation, apical dominance and early flowering, phenotypes that allow competition with neighboring plants. Our results further show the up-regulation in shoot tissues of *PIF5,* a phytochrome interacting-factor also involved in shade-avoidance responses. *PIF5* regulates elongation growth by controlling the expression of genes that code for auxin biosynthesis and auxin signaling components (Lorrain *et al.*, 2008; Hornitschek *et al.*, 2012). Interestingly, plant investment in growth during shade-avoidance responses compromises pathogen resistance by repression of JA-dependent defense mechanisms (de Wit *et al.*, 2013). Our genome-wide transcriptome analysis also points to a suppression of resistance mechanisms. In contrast to the genes involved in growth and developmental processes, genes involved in plant defense responses, such as JA and ET, were down-regulated in shoot tissue of *Arabidopsis* seedlings exposed to *R. solani* VOCs. These hormones have been associated with resistance against pathogens and with priming of plants against insect herbivores (Engelberth *et al.*, 2004; Pieterse & Dicke, 2007). Beneficial as well as pathogenic fungi are known to change plant defense signaling for their own benefit by targeting JAZ repressor proteins which regulate JA signaling in plants (Thines *et al.*, 2007; Plett *et al.*, 2014). Our results show that plants previously exposed to the fungal VOCs are indeed more susceptible to herbivory. However, JAZ regulators were not differentially expressed in *Arabidopsis* shoot tissue exposed to *R. solani* VOCs. Instead, we found up-regulation of *WRKY53,* a negative regulator of plant resistance, and down-regulation of *WRKY45*, a regulator involved in rice resistance to fungal and bacterial pathogens (Shimono *et al.*, 2012; Cheng *et al.*, 2015). Whether modulation of plant resistance by *R. solani* VOCs involves the JAZ and WRKY regulators will be subject of future studies involving multiple *Arabidopsis* mutant lines.

We further show, for the first time, that a soil-borne fungal pathogen can change, *via* VOCs, the plant chemistry. *Arabidopsis* exposed to *R. solani* VOCs produced significantly lower levels of a group of volatile mono- and sesquiterpenes. It is known that plants change their VOC profile during herbivore attack and pathogen infection (Fiers *et al.*, 2013), but that they also do this upon perception of VOCs emitted by a soil-borne fungal pathogen has, to our knowledge, not been described before. Modulation of plant secondary metabolites *via* VOCs may represent a strategy employed by fungal pathogens to predispose plants to infection. In turn, plants will anticipate and prepare for the

upcoming attack. For example, the emission of terpenoids is a well-known defensive mechanism used by plants to attract natural enemies of insect herbivores, also known as the plant indirect defense (Kessler & Baldwin, 2001; War *et al.*, 2012). Our results suggest that the plant direct resistance to an insect herbivore may be compromised after exposure to fungal VOCs, but whether the reduced VOC emission by the plant also adversely impacts on its ability to recruit the natural enemies remains to be investigated.

VOC profiling of *R. solani* showed the presence of typical fungal VOCs such as 1-octen-3-ol and 3-octanone, which belong to the family of oxylipins. Oxylipins are a large and diverse group of secondary metabolites that have been proposed as developmental and communication signals between plants and fungi (Tsitsigiannis & Keller, 2007). In plants, oxylipins are involved in the regulation of plant growth and development as well as defense to biotic and abiotic stresses, whereas in fungi they are used as a developmental signal for fungal spore germination and growth (Brodhun & Feussner, 2011). Several lines of evidence suggest that plant oxylipins can be partly substituted for fungal oxylipins and that fungal oxylipins can influence plant development by mimicking the plant endogenous signal communication (Tsitsigiannis & Keller, 2007; Brodhagen *et al.*, 2008). Here, we tested the plant growth-promoting effects of 5 synthetic fungal VOCs, including two belonging to the oxylipins. However, none of these VOCs showed a significant effect on plant growth when tested individually or in a mixture; several were even toxic at higher concentrations. Future studies will focus on the extraction and identification of other yet unknown VOCs for their involvement in the plant phenotypic and molecular responses to *R. solani*.

In conclusion, our study indicates that fungal VOCs can influence the trade-off between plant growth/development and resistance. From the fungal point of view, this is likely a successful strategy. The increased root biomass and the stimulation of lateral root formation provide a greater root surface area for fungal colonization and infection. Alternatively, plants may use growth promotion upon perception of a potential pathogen as a defensive strategy, sacrificing part of the root biomass and reallocating resources into reproduction rather than in defense processes.

Acknowledgments

We thank Prof. Dr Teris van Beek and Hans Zweers for the assistance with the analysis of plant volatiles by GC-MS and Victor de Jager for the quality check of the transcriptome data. We also thank Prof. Dr Marcel Dicke for the fruitful discussions about this research project.

Supplementary Materials

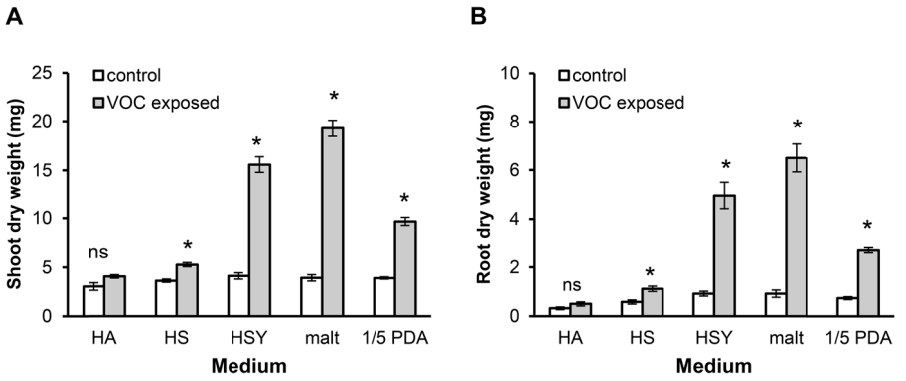


Fig. S1 | Biomass of *Arabidopsis thaliana* shoots (A) and roots (B) exposed to volatile organic compounds (VOCs) emitted by *Rhizoctonia solani* grown on different media (HA: water-agar, HS: water-agar with sucrose, HSY: water-agar with sucrose, HSY: yeast extract, malt-agar and 1/5th PDA: 1/5th potato dextrose agar). Data represent the mean ± SE (n = 4). Asterisks indicate statistically significant differences between VOC-exposed and control (exposed to agar media only) plants (Student's t-Test, $P < 0.05$).

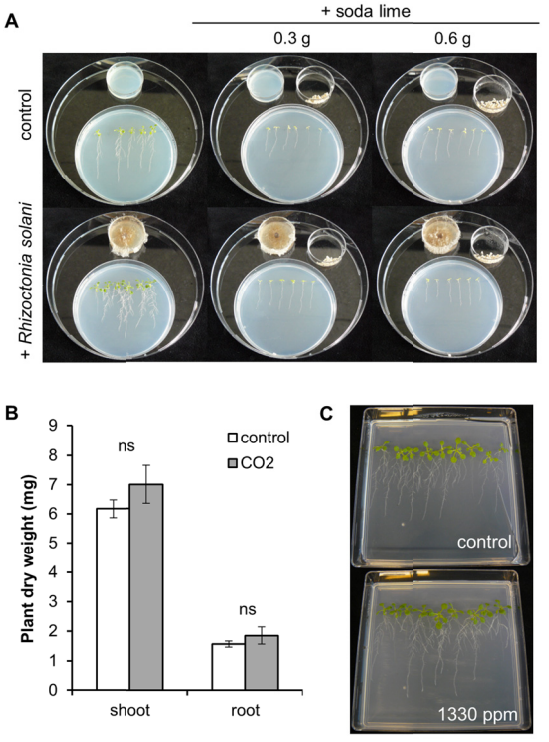


Fig. S2 | (A) Effects of different concentrations of sodalime on *Arabidopsis thaliana* growth in the presence and absence of *Rhizoctonia solani*. (B) *Arabidopsis* shoot and root biomass (mean ± SE, n = 4) after exposure of 1330 ppm CO_2. ns indicates no statistically significant difference between CO_2-exposed and non-exposed seedlings (Student's t-Test, $P < 0.05$). (C) Pictures were taken after 14 days CO_2 exposure.

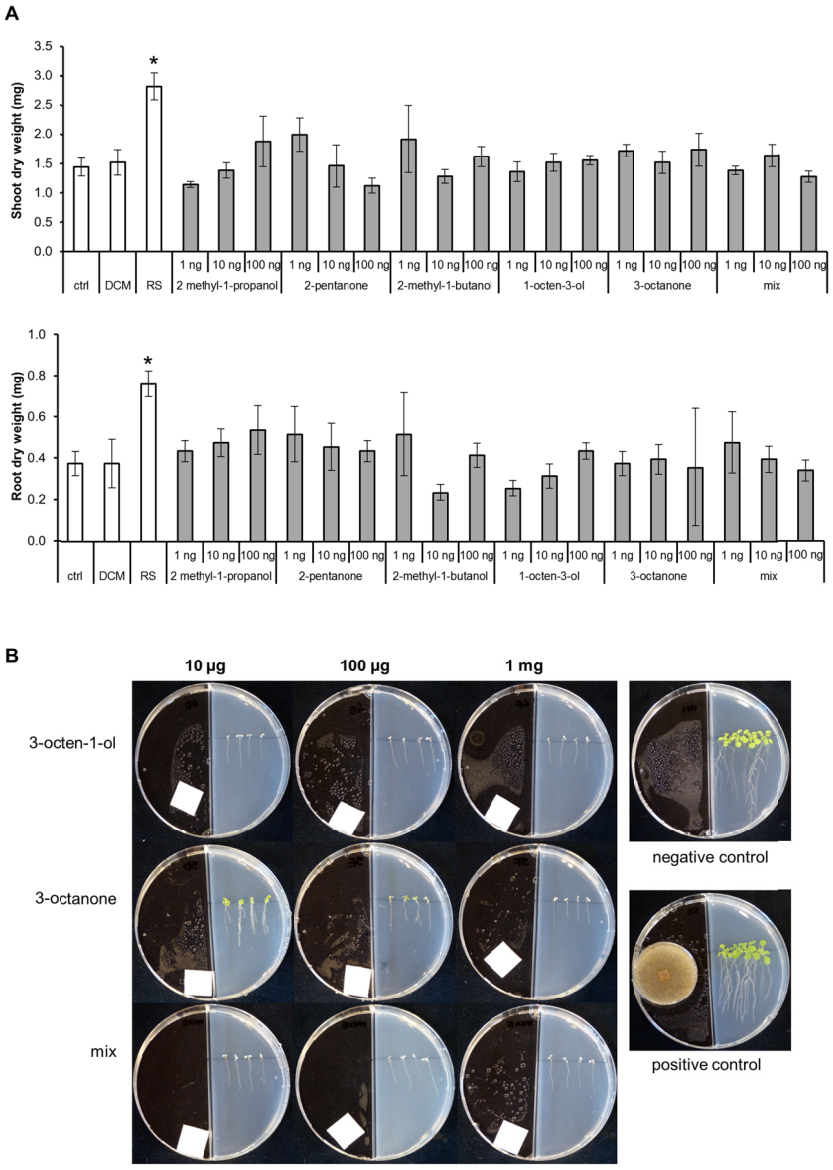


Fig. S3 | Effects of exposure of synthetic volatile compounds (VOCs) on plant growth. **(A)** Shoot (top) and root (bottom) biomass (mean ± SE, n = 5) of *Arabidopsis thaliana* exposed to concentrations of 1, 10 and 100 ng of 2-methyl-1-propanol, 2-pentanone, 2-methyl-1-butanol, 1-octen-3-ol, 3-octanone and the mixture of all 5 VOCs. A mixture of dichloromethane and lanolin (DCM) was used as solvent. Plants exposed to *Rhizoctonia solani* mycelia were used a positive control (RS). Asterisks indicate statistical differences as compared to control (exposure to DCM) (Student's t-Test, $P < 0.05$). **(B)** Phytotoxic effects of 3-octen-1-ol, 3-octanone and the mix at high concentrations (10 µg, 100 µg and 1 mg) after 14 days of exposure.

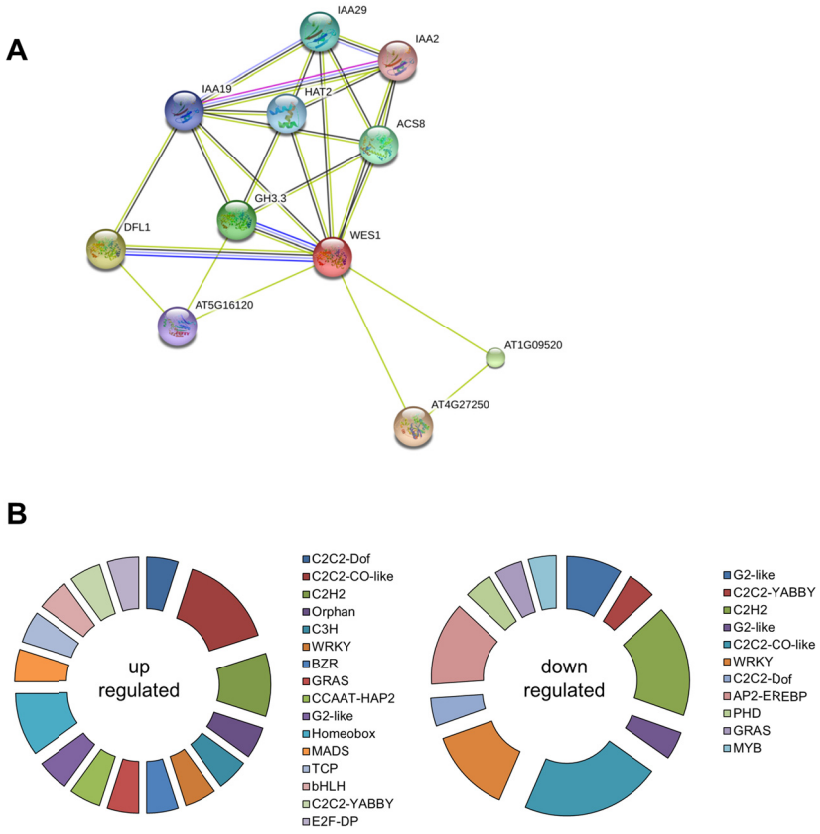


Fig. S4 | (A) Network of predicted functional partners of the WES1 gene (indole-3-acetic acid-amido synthetase GH3.5) generated with STRING10 (Search Tool for the Retrieval of Interacting Genes/Proteins). (B) Graphs show the transcription factor families identified in the differentially expressed genes *Arabidopsis thaliana* upon exposure to volatile organic compounds emitted by *Rhizoctonia solani*.

Table S1 | Output of Single Enrichment Analysis (SEA) performed for the up-regulated genes of *Arabidopsis thaliana* shoots exposed to VOCs emitted by *Rhizoctonia solani*. SEA was performed using AgriGO (http://bioinfo.cau.edu.cn/agriGO/index.php) using Fisher test with FDR (Hochberg) at 0.05.

GO term	Ontology	Description	Number in input list	Number in reference	*P*-value	FDR
GO:0009628	P	response to abiotic stimulus	44	1471	1.30E-16	1.80E-14
GO:0015979	P	photosynthesis	18	162	3.00E-16	2.10E-14
GO:0009416	P	response to light stimulus	28	596	2.00E-15	9.40E-14
GO:0009314	P	response to radiation	28	613	4.00E-15	1.40E-13
GO:0010218	P	response to far red light	10	57	2.30E-11	6.50E-10
GO:0009637	P	response to blue light	10	69	1.30E-10	2.90E-09
GO:0009639	P	response to red or far red light	14	210	3.70E-10	7.30E-09
GO:0050896	P	response to stimulus	61	4057	1.00E-09	1.70E-08
GO:0010114	P	response to red light	9	77	6.10E-09	9.40E-08
GO:0046148	P	pigment biosynthetic process	8	112	1.40E-06	2.00E-05
GO:0042440	P	pigment metabolic process	8	134	5.10E-06	6.30E-05
GO:0015995	P	chlorophyll biosynthetic process	5	47	2.40E-05	2.80E-04
GO:0009725	P	response to hormone stimulus	19	982	4.30E-05	4.50E-04
GO:0006779	P	porphyrin biosynthetic process	5	65	1.00E-04	1.00E-03
GO:0015994	P	chlorophyll metabolic process	5	66	1.10E-04	1.00E-03
GO:0009719	P	response to endogenous stimulus	19	1068	1.30E-04	1.10E-03
GO:0033014	P	tetrapyrrole biosynthetic process	5	72	1.60E-04	1.30E-03
GO:0042221	P	response to chemical stimulus	29	2085	1.70E-04	1.30E-03
GO:0009733	P	response to auxin stimulus	10	360	1.90E-04	1.40E-03
GO:0010033	P	response to organic substance	21	1342	3.10E-04	2.10E-03
GO:0006778	P	porphyrin metabolic process	5	89	4.10E-04	2.70E-03
GO:0033013	P	tetrapyrrole metabolic process	5	93	5.00E-04	3.10E-03
GO:0006091	P	generation of precursor metabolites and energy	8	285	7.90E-04	4.50E-03
GO:0019684	P	photosynthesis, light reaction	5	103	7.70E-04	4.50E-03
GO:0009414	P	response to water deprivation	7	229	1.00E-03	5.70E-03
GO:0009415	P	response to water	7	240	1.30E-03	7.10E-03
GO:0018130	P	heterocycle biosynthetic process	5	123	1.60E-03	8.40E-03

GO term	Ontology	Description	Number in input list	Number in reference	*P*-value	FDR
GO:0009605	P	response to external stimulus	9	429	2.70E-03	1.30E-02
GO:0009266	P	response to temperature stimulus	9	485	5.90E-03	2.80E-02
GO:0009409	P	response to cold	7	328	7.20E-03	3.30E-02
GO:0034641	P	cellular nitrogen compound metabolic process	9	506	7.60E-03	3.40E-02
GO:0051188	P	cofactor biosynthetic process	5	191	1.00E-02	4.30E-02
GO:0048519	P	negative regulation of biological process	8	442	1.10E-02	4.40E-02
GO:0016168	F	chlorophyll binding	10	38	6.90E-13	3.20E-11
GO:0046906	F	tetrapyrrole binding	11	136	4.50E-09	1.00E-07
GO:0022803	F	passive transmembrane transporter activity	5	152	4.00E-03	3.70E-02
GO:0022838	F	substrate-specific channel activity	5	151	3.90E-03	3.70E-02
GO:0015267	F	channel activity	5	152	4.00E-03	3.70E-02
GO:0044435	C	plastid part	31	867	7.10E-14	1.90E-12
GO:0044434	C	chloroplast part	29	746	6.40E-14	1.90E-12
GO:0009579	C	thylakoid	21	376	3.40E-13	5.90E-12
GO:0034357	C	photosynthetic membrane	18	273	1.30E-12	1.70E-11
GO:0009521	C	photosystem	11	66	3.80E-12	4.00E-11
GO:0030076	C	light-harvesting complex	9	31	4.70E-12	4.20E-11
GO:0010287	C	plastoglobule	10	81	5.30E-10	4.00E-09
GO:0005622	C	intracellular	107	9671	3.90E-09	2.60E-08
GO:0044422	C	organelle part	44	2562	8.60E-09	5.00E-08
GO:0044424	C	intracellular part	103	9302	9.40E-09	5.00E-08
GO:0009507	C	chloroplast	45	2740	2.10E-08	1.00E-07
GO:0044446	C	intracellular organelle part	43	2561	2.50E-08	1.10E-07
GO:0009536	C	plastid	47	2965	2.80E-08	1.10E-07
GO:0005623	C	cell	144	15217	4.00E-08	1.40E-07
GO:0044464	C	cell part	144	15217	4.00E-08	1.40E-07
GO:0043227	C	membrane-bounded organelle	87	7622	7.30E-08	2.40E-07
GO:0031976	C	plastid thylakoid	13	293	1.50E-07	4.20E-07
GO:0031984	C	organelle subcompartment	13	295	1.70E-07	4.20E-07
GO:0043231	C	intracellular membrane-bounded organelle	86	7615	1.50E-07	4.20E-07

GO term	Ontology	Description	Number in input list	Number in reference	*P*-value	FDR
GO:0042651	C	thylakoid membrane	12	244	1.60E-07	4.20E-07
GO:0009534	C	chloroplast thylakoid	13	290	1.40E-07	4.20E-07
GO:0043234	C	protein complex	29	1443	1.80E-07	4.20E-07
GO:0043226	C	organelle	90	8155	1.80E-07	4.20E-07
GO:0044436	C	thylakoid part	13	307	2.60E-07	5.70E-07
GO:0043229	C	intracellular organelle	89	8149	3.60E-07	7.40E-07
GO:0044444	C	cytoplasmic part	74	6289	3.50E-07	7.40E-07
GO:0005737	C	cytoplasm	78	6822	4.70E-07	9.20E-07
GO:0055035	C	plastid thylakoid membrane	11	231	7.20E-07	1.30E-06
GO:0009535	C	chloroplast thylakoid membrane	11	231	7.20E-07	1.30E-06
GO:0009522	C	photosystem I	5	22	8.60E-07	1.50E-06
GO:0009523	C	photosystem II	6	45	1.10E-06	1.90E-06
GO:0031090	C	organelle membrane	20	842	1.40E-06	2.40E-06
GO:0032991	C	macromolecular complex	31	2180	6.60E-05	1.10E-04
GO:0005773	C	vacuole	11	383	7.00E-05	1.10E-04
GO:0016020	C	membrane	46	4068	2.60E-04	3.90E-04
GO:0009570	C	chloroplast stroma	7	249	1.60E-03	2.40E-03
GO:0044425	C	membrane part	19	1360	2.20E-03	3.10E-03
GO:0009532	C	plastid stroma	7	322	6.50E-03	9.10E-03
GO:0031975	C	envelope	9	595	2.00E-02	2.60E-02
GO:0031967	C	organelle envelope	9	595	2.00E-02	2.60E-02
GO:0009526	C	plastid envelope	6	331	2.50E-02	3.20E-02
GO:0005886	C	plasma membrane	16	1456	3.60E-02	4.60E-02

Table S2 | Output of Single Enrichment Analysis (SEA) performed for the down-regulated genes of *Arabidopsis thaliana* shoots exposed to VOCs emitted by *Rhizoctonia solani*. SEA was performed using AgriGO (http://bioinfo.cau.edu.cn/agriGO/index.php) using Fisher test with FDR (Hochberg) at 0.05.

GO term	Ontology	Description	Number in input list	Number in reference	*P*-value	FDR
GO:0048511	P	rhythmic process	9	75	6.00E-10	3.60E-08
GO:0007623	P	circadian rhythm	9	75	6.00E-10	3.60E-08
GO:0006807	P	nitrogen compound metabolic process	45	3826	1.80E-07	7.00E-06
GO:0009791	P	post-embryonic development	17	705	2.70E-07	8.00E-06
GO:0050896	P	response to stimulus	44	4057	2.20E-06	5.30E-05
GO:0034641	P	cellular nitrogen compound metabolic process	13	506	3.70E-06	7.30E-05
GO:0044271	P	cellular nitrogen compound biosynthetic process	11	394	9.90E-06	1.70E-04
GO:0010228	P	vegetative to reproductive phase transition of meristem	6	112	3.70E-05	5.50E-04
GO:0009755	P	hormone-mediated signaling pathway	9	321	6.20E-05	7.40E-04
GO:0032870	P	cellular response to hormone stimulus	9	321	6.20E-05	7.40E-04
GO:0010114	P	response to red light	5	77	7.10E-05	7.60E-04
GO:0009639	P	response to red or far red light	7	210	1.50E-04	1.50E-03
GO:0046483	P	heterocycle metabolic process	10	460	1.90E-04	1.70E-03
GO:0003006	P	reproductive developmental process	15	978	2.30E-04	1.80E-03
GO:0048608	P	reproductive structure development	15	978	2.30E-04	1.80E-03
GO:0065007	P	biological regulation	39	4188	2.60E-04	1.80E-03
GO:0042221	P	response to chemical stimulus	24	2085	2.60E-04	1.80E-03
GO:0006139	P	nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	32	3198	3.00E-04	2.00E-03
GO:0008152	P	metabolic process	78	10614	3.50E-04	2.20E-03
GO:0050789	P	regulation of biological process	35	3697	4.20E-04	2.50E-03
GO:0048583	P	regulation of response to stimulus	6	188	5.50E-04	3.10E-03
GO:0044237	P	cellular metabolic process	66	8722	5.90E-04	3.20E-03
GO:0044248	P	cellular catabolic process	12	746	6.50E-04	3.40E-03
GO:0050794	P	regulation of cellular process	32	3375	7.50E-04	3.40E-03
GO:0010556	P	regulation of macromolecule biosynthetic process	21	1843	7.40E-04	3.40E-03
GO:0070887	P	cellular response to chemical stimulus	9	452	7.30E-04	3.40E-03

GO term	Ontology	Description	Number in input list	Number in reference	*P*-value	FDR
GO:0007242	P	intracellular signaling cascade	11	659	8.10E-04	3.60E-03
GO:0009889	P	regulation of biosynthetic process	21	1881	9.50E-04	3.90E-03
GO:0031326	P	regulation of cellular biosynthetic process	21	1881	9.50E-04	3.90E-03
GO:0009987	P	cellular process	82	11684	1.00E-03	4.00E-03
GO:0006350	P	transcription	21	1923	1.20E-03	4.70E-03
GO:0009416	P	response to light stimulus	10	596	1.30E-03	4.70E-03
GO:0045449	P	regulation of transcription	20	1802	1.30E-03	4.70E-03
GO:0022414	P	reproductive process	15	1161	1.30E-03	4.70E-03
GO:0080090	P	regulation of primary metabolic process	21	1952	1.50E-03	5.10E-03
GO:0000003	P	reproduction	15	1186	1.60E-03	5.30E-03
GO:0009314	P	response to radiation	10	613	1.60E-03	5.30E-03
GO:0019219	P	regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	20	1847	1.80E-03	5.60E-03
GO:0009743	P	response to carbohydrate stimulus	6	240	1.90E-03	5.70E-03
GO:0048856	P	anatomical structure development	19	1726	1.90E-03	5.80E-03
GO:0010033	P	response to organic substance	16	1342	2.00E-03	5.80E-03
GO:0009628	P	response to abiotic stimulus	17	1471	2.00E-03	5.80E-03
GO:0009607	P	response to biotic stimulus	10	638	2.20E-03	6.10E-03
GO:0051171	P	regulation of nitrogen compound metabolic process	20	1888	2.30E-03	6.20E-03
GO:0031323	P	regulation of cellular metabolic process	21	2036	2.50E-03	6.50E-03
GO:0060255	P	regulation of macromolecule metabolic process	21	2060	2.80E-03	7.30E-03
GO:0032501	P	multicellular organismal process	21	2094	3.40E-03	8.60E-03
GO:0044238	P	primary metabolic process	64	8995	3.60E-03	9.00E-03
GO:0010035	P	response to inorganic substance	6	279	3.90E-03	9.40E-03
GO:0006810	P	transport	19	1846	4.00E-03	9.60E-03
GO:0051234	P	establishment of localization	19	1851	4.10E-03	9.70E-03
GO:0048580	P	regulation of post-embryonic development	5	200	4.50E-03	1.00E-02
GO:0010468	P	regulation of gene expression	20	2001	4.40E-03	1.00E-02
GO:0051707	P	response to other organism	9	599	4.70E-03	1.00E-02
GO:0032502	P	developmental process	22	2304	4.80E-03	1.00E-02

GO term	Ontology	Description	Number in input list	Number in reference	*P*-value	FDR
GO:0051716	P	cellular response to stimulus	11	840	5.10E-03	1.10E-02
GO:0044249	P	cellular biosynthetic process	39	4925	5.10E-03	1.10E-02
GO:0009058	P	biosynthetic process	40	5118	5.70E-03	1.20E-02
GO:0019222	P	regulation of metabolic process	21	2210	6.20E-03	1.20E-02
GO:0051179	P	localization	19	1922	6.10E-03	1.20E-02
GO:0044262	P	cellular carbohydrate metabolic process	7	417	6.90E-03	1.40E-02
GO:0043170	P	macromolecule metabolic process	51	7127	9.60E-03	1.80E-02
GO:0007275	P	multicellular organismal development	19	2020	1.00E-02	1.90E-02
GO:0044265	P	cellular macromolecule catabolic process	7	465	1.20E-02	2.20E-02
GO:0006812	P	cation transport	6	357	1.20E-02	2.20E-02
GO:0016070	P	RNA metabolic process	16	1657	1.40E-02	2.50E-02
GO:0044260	P	cellular macromolecule metabolic process	46	6447	1.50E-02	2.60E-02
GO:0050793	P	regulation of developmental process	6	377	1.50E-02	2.60E-02
GO:0009725	P	response to hormone stimulus	11	982	1.50E-02	2.60E-02
GO:0009908	P	flower development	6	377	1.50E-02	2.60E-02
GO:0051239	P	regulation of multicellular organismal process	5	277	1.60E-02	2.70E-02
GO:0006950	P	response to stress	20	2320	2.00E-02	3.30E-02
GO:0051704	P	multi-organism process	9	776	2.20E-02	3.60E-02
GO:0006811	P	ion transport	6	427	2.60E-02	4.10E-02
GO:0009719	P	response to endogenous stimulus	11	1068	2.60E-02	4.10E-02
GO:0016310	P	phosphorylation	11	1079	2.80E-02	4.30E-02
GO:0010154	P	fruit development	7	557	2.90E-02	4.40E-02
GO:0007165	P	signal transduction	12	1228	2.90E-02	4.40E-02
GO:0006979	P	response to oxidative stress	5	332	3.20E-02	4.80E-02
GO:0003824	F	catalytic activity	81	9638	1.40E-06	6.10E-05
GO:0016491	F	oxidoreductase activity	18	1463	7.60E-04	1.70E-02
GO:0016740	F	transferase activity	31	3321	1.20E-03	1.80E-02
GO:0005488	F	binding	78	11258	2.20E-03	2.50E-02

GO term	Ontology	Description	Number in input list	Number in reference	*P*-value	FDR
GO:0016301	F	kinase activity	17	1641	6.00E-03	4.50E-02
GO:0016772	F	transferase activity, transferring phosphorus-containing groups	19	1887	5.10E-03	4.50E-02
GO:0043231	C	intracellular membrane-bounded organelle	62	7615	1.30E-04	1.60E-03
GO:0043227	C	membrane-bounded organelle	62	7622	1.30E-04	1.60E-03
GO:0043229	C	intracellular organelle	62	8149	8.20E-04	3.50E-03
GO:0005622	C	intracellular	71	9671	7.90E-04	3.50E-03
GO:0005886	C	plasma membrane	18	1456	7.20E-04	3.50E-03
GO:0043226	C	organelle	62	8155	8.40E-04	3.50E-03
GO:0005773	C	vacuole	8	383	1.10E-03	3.80E-03
GO:0005634	C	nucleus	26	2621	1.30E-03	4.10E-03
GO:0044424	C	intracellular part	67	9302	2.00E-03	5.60E-03
GO:0005737	C	cytoplasm	50	6822	6.60E-03	1.70E-02
GO:0044464	C	cell part	95	15217	1.50E-02	3.10E-02
GO:0005623	C	cell	95	15217	1.50E-02	3.10E-02

Table S3 | Gene Ontology (GO) terms associated with hormone-signaling pathway identified for up-regulated genes of *Arabidopsis thaliana* shoots exposed to VOCs emitted by *Rhizoctonia solani*.

Gene name	GO terms	Description
AT4G34760.1	GO:0009733 response to auxin stimulus	protein_coding auxin-responsive family protein auxin-responsive family protein
AT4G16780.1	GO:0009733 response to auxin stimulus GO:0043565 sequence-specific DNA binding GO:0009735 response to cytokinin stimulus GO:0008283 cell proliferation GO:0009826 unidimensional cell growth GO:0005634 nucleus GO:0010218 response to far red light GO:0006355 regulation of transcription, DNA-dependent GO:0048364 root development GO:0003700 transcription factor activity GO:0010016 shoot morphogenesis GO:0010017 red or far-red light signaling pathway GO:0016481 negative regulation of transcription GO:0009641 shade avoidance GO:0042803 protein homodimerization activity	protein_coding ATHB-2 (*ARABIDOPSIS* THALIANA HOMEOBOX PROTEIN 2)
AT5G54510.1	GO:0005737 cytoplasm GO:0009826 unidimensional cell growth GO:0010279 indole-3-acetic acid amido synthetase activity GO:0010252 auxin homeostasis GO:0009734 auxin mediated signaling pathway	protein_coding DFL1 (DWARF IN LIGHT 1)
AT2G36830.1	GO:0015250 water channel activity GO:0006810 transport GO:0009740 gibberellic acid mediated signaling pathway GO:0009705 plant-type vacuole membrane	protein_coding GAMMA-TIP (GAMMA TONOPLAST INTRINSIC PROTEIN)

Gene name	GO terms	Description
AT4G27260.1	GO:0010252 auxin homeostasis GO:0009733 response to auxin stimulus GO:0010279 indole-3-acetic acid amido synthetase activity	protein_coding WES1
AT3G07390.1	GO:0009733 response to auxin stimulus GO:0005201 extracellular matrix structural constituent GO:0030198 extracellular matrix organization GO:0005576 extracellular region GO:0005886 plasma membrane GO:0010102 lateral root morphogenesis GO:0031225 anchored to membrane	protein_coding AIR12
AT3G22060.1	GO:0005618 cell wall GO:0009737 response to abscisic acid stimulus GO:0005773 vacuole	protein_coding receptor protein kinase-related
AT3G50660.1	GO:0009826 unidimensional cell growth GO:0005783 endoplasmic reticulum GO:0009741 response to brassinosteroid stimulus GO:0016132 brassinosteroid biosynthetic process GO:0010012 steroid 22-alpha hydroxylase activity GO:0010358 leaf shaping	protein_coding DWF4 (DWARF 4)
AT3G51920.1	GO:0005513 detection of calcium ion GO:0009737 response to abscisic acid stimulus GO:0019722 calcium-mediated signaling GO:0005509 calcium ion binding GO:0009414 response to water deprivation GO:0009651 response to salt stress	protein_coding CAM9 (CALMODULIN 9)

Gene name	GO terms	Description
AT4G32280.1	GO:0003700 transcription factor activity GO:0009733 response to auxin stimulus GO:0010218 response to far red light GO:0010114 response to red light	protein_coding IAA29 (INDOLE-3-ACETIC ACID INDUCIBLE 29)
AT3G62030.1	GO:0006457 protein folding GO:0009570 chloroplast stroma GO:0009737 response to abscisic acid stimulus GO:0006979 response to oxidative stress GO:0010555 response to mannitol stimulus GO:0019344 cysteine biosynthetic process GO:0007165 signal transduction GO:0003755 peptidyl-prolyl cis-trans isomerase activity GO:0009651 response to salt stress GO:0009642 response to light intensity GO:0045036 protein targeting to chloroplast	protein_coding ROC4 (chloroplast / cyclophilin / rotamase / cyclosporin A-binding protein cyclophilin CYP20-3)
AT5G47370.1	GO:0009826 unidimensional cell growth GO:0009734 auxin mediated signaling pathway GO:0003700 transcription factor activity GO:0005634 nucleus GO:0006355 regulation of transcription, DNA-dependent GO:0016564 transcription repressor activity GO:0009641 shade avoidance	protein_coding HAT2
AT3G23030.1	GO:0009733 response to auxin stimulus GO:0003700 transcription factor activity GO:0005634 nucleus	protein_coding IAA2 (INDOLE-3-ACETIC ACID INDUCIBLE 2)

Gene name	GO terms	Description
AT1G20450.1	GO:0005737 cytoplasm GO:0009631 cold acclimation GO:0009737 response to abscisic acid stimulus GO:0005634 nucleus GO:0016020 membrane GO:0003779 actin binding GO:0009414 response to water deprivation	protein_coding ERD10 (EARLY RESPONSIVE TO DEHYDRATION 10)
AT1G29395.1	GO:0009535 chloroplast thylakoid membrane GO:0009737 response to abscisic acid stimulus GO:0042631 cellular response to water deprivation GO:0009631 cold acclimation	protein_coding COR414-TM1
AT3G17510.2	GO:0009651 response to salt stress GO:0016301 kinase activity GO:0009737 response to abscisic acid stimulus GO:0005515 protein binding GO:0005886 plasma membrane	protein_coding CIPK1 (CBL-INTERACTING PROTEIN KINASE 1)
AT3G15540.1	GO:0009733 response to auxin stimulus GO:0003700 transcription factor activity GO:0009638 phototropism GO:0009630 gravitropism	protein_coding IAA19 (INDOLE-3-ACETIC ACID INDUCIBLE 19)
AT1G74670.1	GO:0009744 response to sucrose stimulus GO:0009750 response to fructose stimulus GO:0009740 gibberellic acid mediated signaling pathway GO:0009749 response to glucose stimulus GO:0012505 endomembrane system	protein_coding gibberellin-responsive protein

Gene name	GO terms	Description
AT2G46830.2	GO:0009733 response to auxin stimulus GO:0009737 response to abscisic acid stimulus GO:0048574 long-day photoperiodism, flowering GO:0009753 response to jasmonic acid stimulus GO:0003700 transcription factor activity GO:0005634 nucleus GO:0009723 response to ethylene stimulus GO:0016564 transcription repressor activity GO:0009739 response to gibberellin stimulus GO:0007623 circadian rhythm GO:0009651 response to salt stress GO:0046686 response to cadmium ion GO:0009751 response to salicylic acid stimulus GO:0010243 response to organic nitrogen GO:0016563 transcription activator activity	protein_coding CCA1 (CIRCADIAN CLOCK ASSOCIATED 1)

Table S4 | Gene Ontology (GO) terms associated with hormone-signaling pathway identified for down-regulated genes of *Arabidopsis thaliana* shoots exposed to VOCs emitted by *Rhizoctonia solani*.

Gene name	GO terms	Description
AT3G02140.1	GO:0009651 response to salt stress GO:0005634 nucleus GO:0010581 regulation of starch biosynthetic process GO:0009788 negative regulation of abscisic acid mediated signaling pathway	protein_coding TMAC2 (TWO OR MORE ABRES-CONTAINING GENE 2)
AT5G54380.1	GO:0009742 brassinosteroid mediated signaling pathway GO:0051510 regulation of unidimensional cell growth GO:0004672 protein kinase activity GO:0009791 post-embryonic development GO:0005886 plasma membrane GO:0046777 protein amino acid autophosphorylation	protein_coding THE1 (THESEUS1)
AT1G32230.1	GO:0005737 cytoplasm GO:0005515 protein binding GO:0042542 response to hydrogen peroxide GO:0009816 defense response to bacterium, incompatible interaction GO:0010102 lateral root morphogenesis GO:0005634 nucleus GO:0009867 jasmonic acid mediated signaling pathway GO:0000303 response to superoxide GO:0012501 programmed cell death GO:0010193 response to ozone GO:0009414 response to water deprivation GO:0009873 ethylene mediated signaling pathway GO:0009651 response to salt stress GO:0009790 embryonic development GO:0006809 nitric oxide biosynthetic process	protein_coding RCD1 (RADICAL-INDUCED CELL DEATH1)

Gene name	GO terms	Description
AT4G30270.1	GO:0005737 cytoplasm GO:0007568 aging GO:0009740 gibberellic acid mediated signaling pathway GO:0016798 hydrolase activity, acting on glycosyl bonds GO:0016762 xyloglucan:xyloglucosyl transferase activity GO:0009505 plant-type cell wall	protein_coding MERI5B (meristem-5)
AT4G35230.1	GO:0016301 kinase activity GO:0009742 brassinosteroid mediated signaling pathway GO:0005886 plasma membrane	protein_coding BSK1 (BR-SIGNALING KINASE 1)
AT1G15550.1	GO:0005737 cytoplasm GO:0009740 gibberellic acid mediated signaling pathway GO:0016707 gibberellin 3-beta-dioxygenase activity GO:0008134 transcription factor binding GO:0009686 gibberellin biosynthetic process GO:0010114 response to red light	protein_coding GA3OX1 (GIBBERELLIN 3-OXIDASE 1)
AT5G01810.1	GO:0009788 negative regulation of abscisic acid mediated signaling pathway GO:0004672 protein kinase activity	protein_coding CIPK15 (CBL-INTERACTING PROTEIN KINASE 15)
AT5G25350.1	GO:0005634 nucleus GO:0010105 negative regulation of ethylene mediated signaling pathway GO:0019005 SCF ubiquitin ligase complex GO:0005515 protein binding GO:0006511 ubiquitin-dependent protein catabolic process	protein_coding EBF2 (EIN3-BINDING F BOX PROTEIN 2)

Gene name	GO terms	Description
AT1G10470.1	GO:0005737 cytoplasm GO:0042752 regulation of circadian rhythm GO:0009736 cytokinin mediated signaling pathway GO:0005634 nucleus GO:0009793 embryonic development ending in seed dormancy GO:0006950 response to stress GO:0005515 protein binding GO:0010017 red or far-red light signaling pathway GO:0010114 response to red light GO:0000156 two-component response regulator activity	protein_coding ARR4 (RESPONSE REGULATOR 4)

Table S5 | List of transcription factors among the differentially expressed genes of *Arabidopsis thaliana* exposed to VOCs emitted by *Rhizoctonia solani*. Identification of transcription factors was carried out using the *Arabidopsis* transcription factor database (http://*Arabidopsis*.med.ohio-state.edu/).

TF Family Name	TF Locus ID	Protein Name	Sub Family	Gene Name , Synonym
Shoot up-regulated genes				
C2C2-Dof	AT5G62430		NA	CDF1
C2C2-CO-like	AT4G27310		NA	
C2C2-CO-like	AT5G57660		NA	COL5
C2H2	AT3G02830		NA	ZFN1
Orphan	AT1G06040		NA	STO
C3H	AT3G60080		NA	
WRKY	AT4G23810	AtWRKY53	NA	ATWRKY53, WRKY53
BZR	AT3G50750		NA	
GRAS	AT3G54220		NA	SCR, SGR1
C2C2-CO-like	AT1G73870		NA	COL7
CCAAT-HAP2	AT5G12840		NA	ATHAP2A, EMB2220, HAP2A, NF-YA1
G2-like	AT5G44190		NA	ATGLK2, GLK2, GPRI2
Homeobox	AT4G16780	AtHB2	HD-Zip II	ATHB-2, ATHB2, HAT4
MADS	AT5G65060		TypeII	AGL70, FCL3, MAF3
TCP	AT1G69690		NA	
bHLH	AT3G59060	AtbHLH65	NA	PIF5, PIL6
C2C2-YABBY	AT4G00180		NA	YAB3
C2H2	AT1G14580		NA	
E2F-DP	AT3G48160		NA	DEL1, E2FE, E2FF, E2L3
Homeobox	AT5G47370		HD-Zip II	HAT2
Shoot down-regulated genes				
G2-like	AT3G46640		NA	LUX, PCL1
G2-like	AT5G59570		NA	
C2C2-YABBY	AT4G00180		NA	YAB3
C2H2	AT2G23740		NA	
G2-like	AT3G46640		NA	LUX, PCL1
C2C2-CO-like	AT4G39070		NA	
C2H2	AT5G09740		NA	HAM2
C2C2-CO-like	AT5G48250		NA	COL10
WRKY	AT3G01970	AtWRKY45	NA	AtWRKY45, WRKY45
C2C2-Dof	AT1G69570		NA	
C2H2	AT3G05160		NA	
AP2-EREBP	AT1G21910		NA	
C2C2-CO-like	AT3G07650		NA	COL9
C2C2-CO-like	AT1G28050		NA	COL15
AP2-EREBP	AT3G61630		NA	CRF6
WRKY	AT5G07100	AtWRKY26	NA	WRKY26
AP2-EREBP	AT2G40340		NA	
PHD	AT1G05380		NA	
C2H2	AT4G06634		NA	
GRAS	AT1G07520		NA	
MYB	AT3G09370	AtMYB3R3	MYB3Rs	AtMYB3R3, MYB3R-3
Root down-regulated genes				
WRKY	AT3G04670	AtWRKY39	NA	AtWRKY39, WRKY39

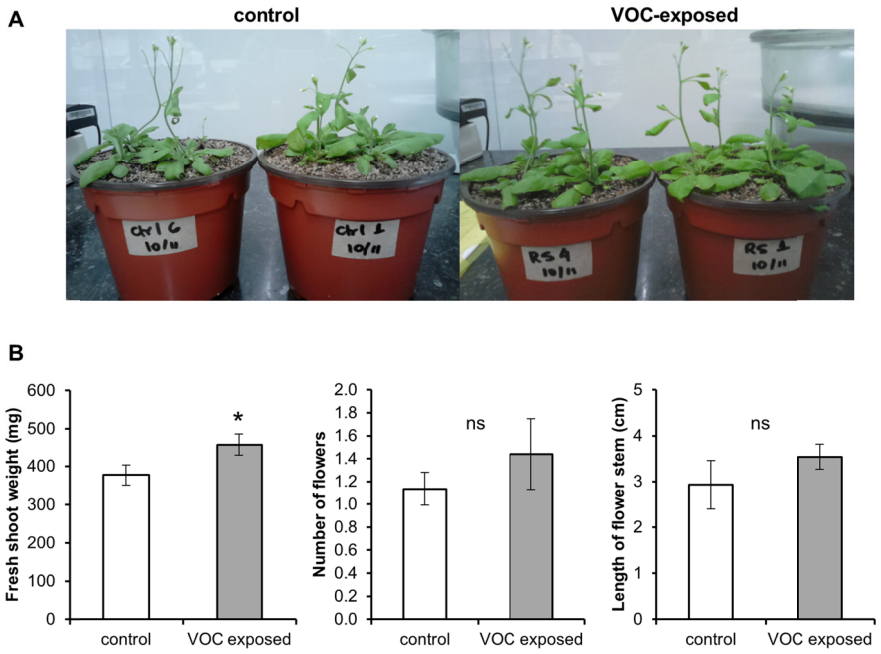


Fig. S5 | (A) Three-week-old *Arabidopsis thaliana* plants used for collection of volatile organic compounds (VOCs). **(B)** Fresh weight, number of flowers and length of flower stem (mean ± SE, n = 8) of control *Arabidopsis* plants previously exposed to volatiles emitted by *Rhizoctonia solani*. Asterisks indicate statistical differences between plants exposed to fungal VOCs (VOC-exposed) and to the agar medium only (control), 'ns' indicates no statistically significant differences (Student's t-Test, $P < 0.05$).

Table S6 | List of volatile organic compounds (VOCs) detected for aerial parts of *Arabidopsis thaliana* leaves exposed (VOC-exposed) and non-exposed (control) to VOCs emitted by *Rhizoctonia solani*.

Compound	RI[a]	VOC emission[b]	
		control	VOC-exposed
1-penten-3-ol	685	291 ± 259	300 ± 171
unknown ketone	699	60 ± 56	65 ± 21
dimethyl disulfide	**749**	**981 ± 1298**	**394 ± 407**
hexanal	**801**	**161 ± 126**	**110 ± 35**
unknown	**880**	**40 ± 19**	**38 ± 21**
heptanal	899	55 ± 20	60 ± 22
benzaldehyde	963	644 ± 385	551 ± 283
unknown monoterpene	**1012**	**81 ± 70**	**36 ± 27**
p-cymene	**1028**	**74 ± 39**	**36 ± 21**
unknown aldehyde	1032	821 ± 476	963 ± 481
nonanal	1103	562 ± 300	589 ± 233
methyl salicylate	1186	52 ± 67	40 ± 26
decanal	1201	955 ± 608	924 ± 493
benzothiazole	**1220**	**354 ± 484**	**181 ± 86**
unkown terpenoid	1381	2254 ± 3519	2419 ± 4153
beta-caryophyllene	1424	251 ± 62	225 ± 126
unknown sesquiterpene	**1447**	**383 ± 170**	**255 ± 130**
lilial	1519	131 ± 147	135 ± 90
Total VOC emission		8152 ± 8107	7320 ± 6828

[a]Calculated retention indices (RI)
[b]Values for peak area (mean ± SD) were divided by 10^4 and expressed per gram of shoot fresh weight
VIP value > 1 are shown in bold

Chapter 7

General discussion

Volatile affairs in the rhizosphere

Volatile organic compounds (VOCs) are a diverse group of carbon-based compounds that are products of primary and secondary metabolism of (micro)organisms. VOCs represent a relatively small proportion of the full repertoire of metabolites produced by these organisms (Pichersky *et al.*, 2006; Mendes *et al.*, 2013). Nevertheless, current advances in chemical ecology have demonstrated an array of natural functions of VOCs in long-distance communication between organisms. They not only play a role in plant-plant and plant-insect interactions, but also in microbe-microbe and microbe-plant interactions. The overall aims of my thesis were to i) characterize VOCs emitted by plant beneficial and plant pathogenic rhizosphere microorganisms, ii) investigate their effects on plant growth, development and resistance, and iii) understand the molecular mechanisms underlying VOC-mediated plant growth promotion and induced resistance. Here I discuss the major findings of my thesis in the context of other published studies. I will proceed with raising new hypotheses on the ecological roles of VOCs in soil and rhizosphere environments, both from the perspective of the plant and of the VOC-producing microorganisms.

Volatile repertoire of soil microorganisms

Currently, more than 1.000 bacterial and fungal VOCs have been described in literature (Effmert *et al.*, 2012; Lemfack *et al.*, 2014). A recent meta-survey showed that terpenes, alcohols, ketones, aromatic compounds, and organic acids composed the majority of soil microbial VOCs, whereas alcohols, aldehydes, and ketones composed the majority of plant root VOCs (Schenkel *et al.*, 2015). Ketones, esters, sulfur-containing compounds and furans were found in similar frequencies for plant roots and soil microorganisms (Fig. 1).

VOC profiling of different *Streptomyces* species and strains showed that most of the 381 VOCs were specific for some strains, whereas a small number of VOCs were produced by all (Chapter 3). For example, the terpenoid trans-1,10-dimethyl-trans-9-decalol (geosmin) was found for all 12 *Streptomyces* strains, whereas 2-methylisoborneol (MIB) was detected for 8 out of 12 strains. No terpenes were detected for *Microbacterium* strains, but instead a large number of sulfur-containing compounds was detected. These included sulfur-containing VOCs commonly found for other bacterial genera such as dimethyl disulfide and dimethyl trisulfide, but also more rare sulfur-containing VOCs were detected, including S-methyl-2-methylpropanethioate and S-methyl-pentanethioate (Chapter 4). I further characterized VOCs produced by the soil-borne fungal pathogen

Rhizoctonia solani. A total of 14 VOCs were detected, including the two oxylipins 1-octen-3-ol and 3-octanone, and the tentatively identified terpene alpha-copaene (Chapter 6).

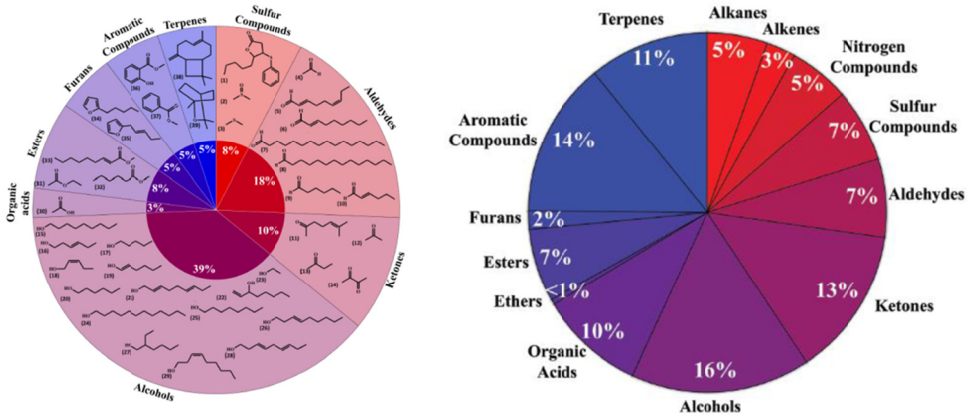


Fig. 1 | Diversity of volatile organic compounds (VOCs) emitted by plant roots and soil microorganisms. Meta-analysis of the diversity and specificity of belowground VOCs, which included 39 root VOCs (A) emitted by *Arabidopsis*, barley, maize and bean plants as well as 1093 microbial VOCs (B) emitted by 135 fungi and 356 bacteria (reproduced from Schenkel *et al*. 2015).

Underground chemical warfare: VOCs as microbial weapons

Microorganisms are part of a dynamic soil food web occupying the same or physically distinct ecological niches. To compete or cooperate with one another for nutrients or space, soil and rhizosphere microorganisms have developed intercellular signaling mechanisms to sense other organisms and to adapt to host defenses. In the rhizosphere, VOCs provide an advantage in signaling over other compounds (in liquid phase), since they can diffuse through soil pores over longer distances and are not dependent on water availability. Intercellular signaling is in part mediated by VOCs, acting as developmental signals, aiding in reproduction, and attracting or repulsing other (micro)organisms (Hung *et al.*, 2015). For example, several bacterial VOCs can inhibit the germination and growth of fungi including plant pathogens (Moore-Landecker & Stotzky, 1973; Vespermann *et al.*, 2007; Garbeva *et al.*, 2014). Conversely, fungal VOCs can inhibit bacterial growth and also alter swarming motility of bacteria (Strobel *et al.*, 2001; Schmidt *et al.*, 2015).

VOC-mediated underground chemical warfare has been proposed as a key mechanism of natural disease-suppressive soils (Lockwood, 1977). In this thesis, I studied the potential role of VOCs in soil suppressiveness to *R. solani*, the fungal root pathogen

responsible for damping-off disease of sugar beet. Actinobacteria were prominently more represented in the suppressive soil than in the non-suppressive (conducive) soil and were subjected to isolation, VOC profiling and antifungal activity. I specifically focused on *Streptomyces* and *Microbacterium* species as the two representatives of the most abundant Actinobacterial families detected by PhyloChip-based analysis of the rhizosphere microbiome of sugar beet seedlings grown in the suppressive soil (Mendes *et al.*, 2011). Several of these isolates inhibited hyphal growth and showed fungicidal effects via the production of specific VOCs (Chapters 3 and 4). The antifungal VOCs identified for two *Streptomyces* strains include methyl 2-methylpentanoate and 1,3,5-trichloro-2-methoxy benzene (Chapter 3). Whether these VOCs also play a role in soil suppressiveness to *R. solani* requires additional analyses to demonstrate that the fungicidal VOCs are actually produced *in situ* at concentrations sufficient to suppress plant infection by the pathogenic fungus. For the *Microbacterium* species, the specific antifungal VOCs have not yet been identified. In other fungistatic soils, different VOCs have been detected *in situ*. These include trimethylamine, 3-methyl-2-pentanone, dimethyl disulfide, methyl pyrazine, 2,5-dimethyl-pyrazine, benzaldehyde, N,N-dimethyloctylamine and nonadecane, some of which exhibit antifungal activity (Chuankun *et al.*, 2004).

Long distance relationships: VOCs as a common language

Microbial VOCs play an important role in microbe-microbe communication and can induce transcriptional and phenotypic changes in competitors (Kim *et al.*, 2013; Garbeva *et al.*, 2014; Chapter 2). Plants also use VOCs for communication and, in this way, they compensate for limitations resulting from their immobility. For instance, VOC emission contributes to communication between distal plant parts and is used to attract pollinators and enemies of plant herbivores (Paré & Tumlinson, 1999; Dicke & Loreto, 2010; Mumm & Dicke, 2010). The use of VOCs as a 'language' by plants to pursue communication has been proposed in many studies. Holopainen and Blande (2012) proposed that the complexity and species-specificity of VOCs might act as a plant vocabulary, where individual VOCs are words and the different VOC blends are sentences. In this context, Kikuta *et al.* (2011) showed that, upon mechanical wounding, the VOC blend and not the individual VOCs were required to elicit the production of pyrethrins, natural insecticides produced by plants.

It is now well established that plants use VOCs aboveground to 'cry for help' (Dicke & Sabelis, 1987; Dicke, 2009). Also underground, VOCs from plants under herbivore attack are involved in recruiting enemies of the insect herbivores. Rasmann *et al.* (2005) showed that maize roots damaged by insects emit (E)-β-caryophyllene that acts as an attractant for

entomopathogenic nematodes. Whether plant VOCs released underground also 'cry for help' to call upon beneficial microorganisms to support plant growth and to defend against pathogens is, to our knowledge, not yet known.

Although these and other ecological functions of microbial VOCs are not well understood yet, a role in long-distance communication is evident. For example, oxylipins, a large and diverse group of eight-carbon secondary metabolites, have been suggested as developmental and communication signals between plants and fungi (Tsitsigiannis & Keller, 2007). In plants, oxylipins are involved in the regulation of plant growth and development as well as defense to (a)biotic stresses, whereas in fungi they act as a developmental signal for fungal spore germination and growth (Brodhun & Feussner, 2011). Several lines of evidence have suggested that plant oxylipins can be partly substituted by fungal oxylipins and that fungal oxylipins can influence plant development by mimicking these plant endogenous communication signals (Tsitsigiannis & Keller, 2007; Brodhagen *et al.*, 2008). Interestingly, a relatively high proportion of VOCs from soil seems to be specific to the (micro)organisms in defined habitats (Schenkel *et al.*, 2015). For example, the oxylipins 1-octen-3-ol and octan-3-one, predominantly produced by rhizospheric fungi living in symbiotic association with plant roots, likely serve as symbiotic signals to a potential host plant. Thus I can speculate that a higher resemblance between microbial and plant VOCs may translate into more intimate associations in the rhizosphere between the VOC-producing soil microorganisms and the plant species.

In addition to oxylipins, also terpenoids have been proposed as a common 'language' between plants and other (micro)organisms. Approximately 50.000 terpenoids have been isolated from plants and fungi, but also from prokaryotes (Ebel, 2010; Cane & Ikeda, 2012; Piccoli & Bottini, 2013; Song *et al.*, 2015; Yamada *et al.*, 2015). Both the number and structural diversity of plant terpenoids provide enormous potential for mediating significant ecological interactions. Several of these terpenoids, as for example mono- and sesquiterpenes, are volatile and have been described to act as infochemicals in plant-plant, plant-insect and plant-fungus interactions (Dicke & Sabelis, 1987; Keeling & Bohlmann, 2006; Ditengou *et al.*, 2015).

To grow or not to grow: the role of VOCs in the plant's dilemma

In nature, plants must grow fast enough to compete with other plant species and yet defend against herbivores and pathogens, also referred to as the 'dilemma of plants' (Herms & Mattson, 1992). Plant growth and defense occur with an expenditure of metabolic energy. Hence, a fine-tuned regulation is crucial to balance these energetically costly

processes. Generally, the activation of defense mechanisms comes at a cost of plant growth and vice-versa (Huot *et al.*, 2014). This is referred to as the trade-off between growth and defense (Simms, 1992; Heil & Bostock, 2002; Messina *et al.*, 2002; Huot *et al.*, 2014).

Ryu *et al.* (2003) were the first to demonstrate that VOCs emitted by *Bacillus* promoted plant growth. Since then, numerous studies have shown that VOCs emitted by other beneficial bacteria, such as *Pseudomonas*, *Burkholderia* and *Serratia,* and beneficial fungi, such as *Trichoderma,* can enhance plant growth (Blom *et al.*, 2011; Hung *et al.*, 2013; Lee *et al.*, 2015; Park *et al.*, 2015). Remarkably, VOCs from *Bacillus* not only enhanced the growth but also induced systemic resistance of *Arabidopsis* plants. In this thesis, I demonstrated that rhizospheric *Streptomyces* and *Microbacterium* species enhance, via VOCs, shoot and root biomass (Chapters 3 and 4). I further showed that plant growth promotion was accompanied by several transcriptional changes, which included up-regulation of genes involved in sulfur and nitrogen metabolism in shoot and root tissues, respectively. These VOC-mediated transcriptional changes presumably favor the plant's assimilation of sulfate and nitrogen, essential nutrients for plant growth, development and also resistance. VOC profiling and genomic analyses of *Microbacterium* sp. EC8 further identified several sulfur-related genes and compounds. These bacterial genes and compounds will be subject of future studies to resolve their role in VOC-mediated plant growth promotion and induced resistance. I also found that VOCs emitted by the fungal pathogen *R. solani* enhanced the growth but decreased resistance of *Arabidopsis* plants to an insect herbivore (Chapter 6). Genome-wide transcriptome analysis of the *Arabidopsis* seedlings revealed that genes involved in auxin signaling were up-regulated, whereas ethylene and jasmonic acid signaling were down-regulated upon exposure to fungal VOCs.

While interactions between plants and various soil-borne microorganisms are beneficial for plant growth and development, some interactions can be harmful and cause disease. To maximize interactions with beneficial microorganisms and minimize interactions with pathogens, plants not only need to recognize but also differentiate between these microorganisms (Pel & Pieterse, 2012; Dicke, 2016). Plant perception of microorganisms is commonly prompted by pattern recognition receptors (PRRs) that detect microbe- or pathogen-associated molecular patterns (MAMPs/PAMPs), which in turn lead to the induction of plant defense responses (MAMP-triggered immunity). Many of the identified microbial patterns are conserved among diverse pathogens, such as chitin present in fungal cell walls or bacterial flagellin (Gomez-Gomez & Boller, 2000; Nurnberger & Brunner, 2002; Parker, 2003). It is not yet clear what the relative importance of VOCs is as MAMPs/PAMPs in plant perception of beneficial and pathogenic microbes. Runyon *et al.* (2006) have shown that parasitic plant seedlings can determine the host location by

eavesdropping on their host plant VOCs. Consequently, it is well possible that plants not only sense other (micro)organisms, but also distinguish between them. In this context, it is an intriguing finding from my thesis and other studies that VOCs from both beneficial and pathogenic microorganisms can promote plant growth. One hypothesis is that plants use increased growth and development upon perception of VOCs from a pathogen as a defensive strategy, sacrificing part of the biomass and reallocating resources into reproduction rather than in defense processes. Thus, perception of VOCs from harmful microorganisms could allow plants to adjust their phenotype and physiology prior to colonization and infection. An alternative hypothesis is that stimulation of root biomass and lateral root formation by the pathogen provide a greater root surface area for colonization and infection. Taken into account the life cycle of *R. solani*, a fungus that survives in soil mostly in the form of immobile sclerotia, the fact that sclerotial VOCs also promote root growth may be an effective way to favor and safe-guard its proliferation (Chapter 6).

Plant pathogenic microorganisms have evolved several strategies to modulate growth and defense of their host. For instance, compounds secreted by pathogens may suppress or interfere with plant defense responses and in turn facilitate host colonization and infection (Jones & Dangl, 2006; Ottmann *et al.*, 2009; Demkura *et al.*, 2010). These compounds include phytohormones which control and regulate various functions related to plant growth, metabolism and reproduction. Hence, modulation of endogenous hormone levels in plants by microbial production of these hormones has been proposed as a mechanism for plant-growth promotion by soil-microorganisms (Costacurta & Vanderleyden, 1995; Lin & Xu, 2013; Kudoyarova *et al.*, 2015). I showed that significant levels of ethylene are produced by the soil-borne fungal pathogen *Verticillium dahliae* but not by *R. solani* (Chapter 6). In the assays with *Arabidopsis*, I have ruled out the involvement of fungal ethylene in the promotion of *Arabidopsis*, but whether that is also the case for other pathogen-plant interactions remains to be investigated.

To date, the mechanisms underlying plant perception of microbial VOCs are poorly understood. Holopainen and Blande (2012) suggested that the receiver plant employs different mechanisms or sensors for detecting VOCs and that receiver cells are located in another organ than those of the emitting plant. I have shown that shoot exposure to *Microbacterium* VOCs stimulated the growth of *Arabidopsis*, but not of lettuce, whereas VOC exposure of root tissue led to a biomass increase in both *Arabidopsis* and lettuce (Chapter 5). These new findings suggest that plant perception of microbial VOCs is dependent on the plant tissue as well as on the plant species.

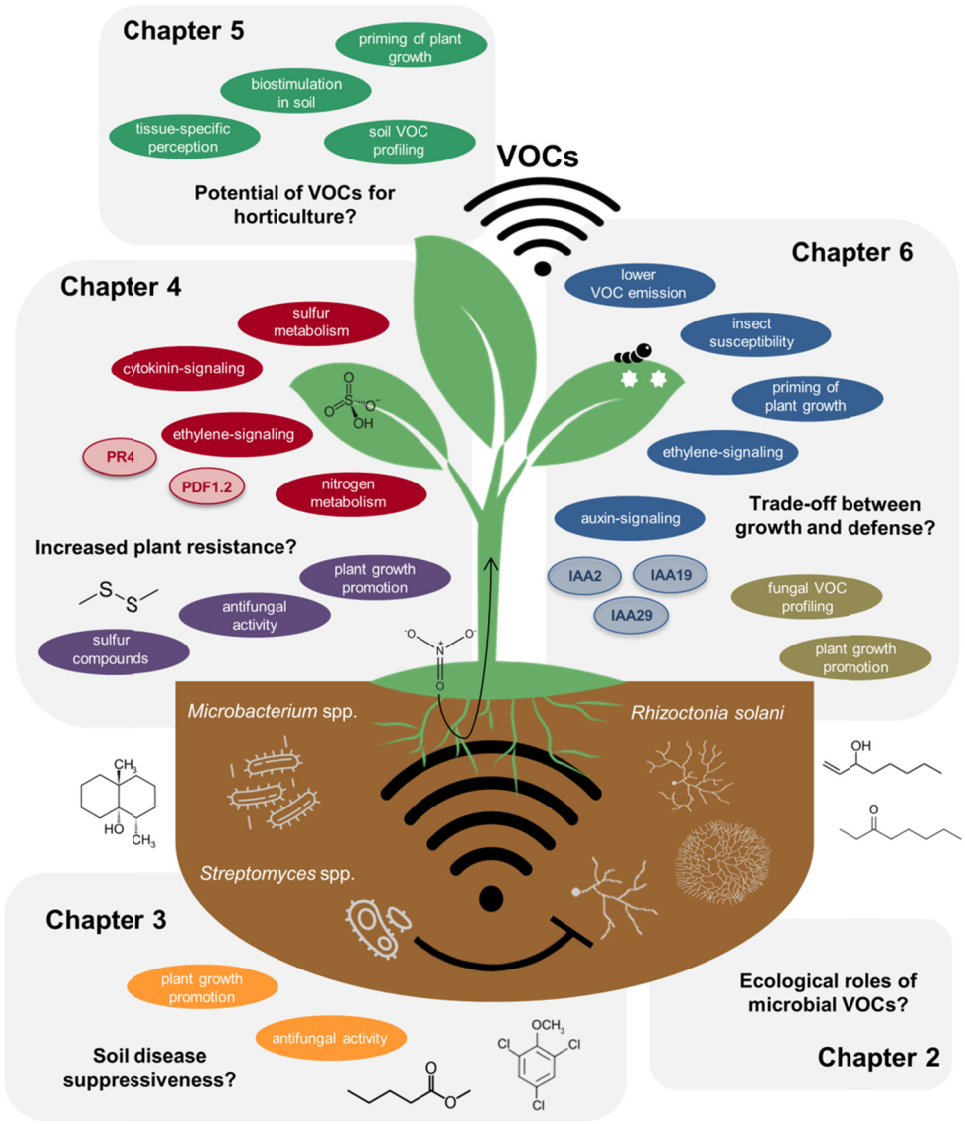


Fig. 2 | Volatile-mediated interactions in the rhizosphere among plants, the beneficial Actinobacteria and the fungal root pathogen *Rhizoctonia solani*. Schematic overview of the main topics, research questions and most important findings described in this thesis.

From the lab to the field: challenges and perspectives of microbial VOCs

Microbial VOCs are of interest to various applications in agriculture, aquaculture, and pharma (Morath *et al.*, 2012). Fungal and bacterial VOCs have been extensively used for food flavoring and aroma and also as biomarkers. For example, fungal VOCs are used for detection of mycotoxin-producing fungi in food products, while bacterial VOCs are used to detect a broad range of diseases, including cancer and infections (Phillips *et al.*, 2003; Boots *et al.*, 2012; Polizzi *et al.*, 2012; Sethi *et al.*, 2013). Some of the microbial VOCs have been found to be common to several phylogenetic groups, while other VOCs seem to be unique for some species (Larsen & Frisvad, 1995; Schnürer *et al.*, 1999; Muller *et al.*, 2013). In fact, recent studies have shown that chemotyping allows not only to identify species- and strain-specific VOCs, but also to study soil microbial activity and shifts in microbial community compositions (McNeal & Herbert, 2009; Muller *et al.*, 2013; Trefz *et al.*, 2013). I have demonstrated that VOC profiling allows to discriminate between *Streptomyces* isolates that are phylogenetically close but phenotypically different (Chapter 3).

In agriculture, microbial VOCs have potential for biological control of plant pathogens and insect pests as well as for stimulation of plant growth. Thus, VOCs and VOC-producing microorganisms provide an alternative to the use of pesticides to protect plants and to improve crop production. In the past decades, several *in vitro* studies have described the effects of microbial VOCs on other (micro)organisms (Blom *et al.*, 2011; Hernández-León *et al.*, 2015; Chapters 3 and 4). However, so far, little is known on the potential of VOCs in large-scale agriculture and horticulture. An example of successful application of VOCs in the field has been described by Song and Ryu (2013), who demonstrated that 3-pentanol and 2-butanone can effectively induce plant resistance against the bacterial leaf pathogen *Pseudononas syringae* pv. *lachrymans* and the sucking insect *Myzus persicae*.

To investigate if and how the growth-promoting effects by VOCs from *Microbacterium* on *Arabidopsis* plants grown *in vitro* (Chapter 4) could be translated to crop plants grown in soil, studies were conducted with lettuce and tomato plants (Chapter 5). *Microbacterium* VOCs promoted the growth of lettuce and tomato plants, but did not suppress disease caused by the fungal pathogen *R. solani.* Furthermore, VOC profiling of soil inoculated with *Microbacterium* identified the two sulfur-containing compounds dimethyl disulfide and dimethyl trisulfide, previously detected for *Microbacterium* grown on agar medium. Also two branched chain alkanes (tentatively identified as 2,2,4,6,6-pentamethyl heptane and 2,2,4,4-tetramethyloctane) and two unknown compounds were detected in soil but not on agar medium. The production of VOCs in soils by microorganisms is influenced by the soil microbiome composition, but also by abiotic

factors such as temperature, water, oxygen and nutrient availability (Asensio *et al.*, 2007; Insam & Seewald, 2010). Furthermore, the detection of VOC production *in situ* and determination of their concentrations by GC-MS is not trivial, as soil physico-chemical characteristics can lead to a rapid and uneven evaporation of VOCs resulting in inconsistent outcomes (Ryu, 2015). Thus, investigating VOC composition and relevant biological concentration *in situ* is essential to evaluate their biological relevance and ecological roles. For that, a close collaboration between biologists and analytical chemists is imperative.

Concluding remarks

The research presented in this thesis indicated that both plants and microorganisms engage *via* VOCs in long-distance interactions. VOCs produced by the beneficial *Streptomyces* and *Microbacterium* strains as well as VOCs from the fungal root pathogen *R. solani* affected plant growth, development and resistance. The phenotypic and transcriptional changes induced in plants by the beneficial and the pathogenic microorganisms indicated that they modulate plant physiology in different ways. How plants sense and differentiate among VOCs from beneficial and pathogenic soil microorganisms will be an intriguing subject for future studies. More specifically, a larger collection of beneficial and pathogenic microorganisms is currently being investigated for their temporal effects on plant phenotype, transcriptome and metabolome. The transcriptome analysis of VOC-exposed plants presented in this thesis provided a first step in identifying candidate plant genes involved in VOC perception and signal transduction. Similarly, the combined genome analysis and VOC profiling of the plant growth-promoting *Microbacterium* sp. EC8 revealed candidate genes involved in the regulation of biologically active VOCs. Mutagenesis combined with proteomic and metabolomic analyses will be required to obtain a more complete understanding of the underlying molecular mechanisms of VOC-mediated microbe-microbe and microbe-plant interactions.

References

A

Adrian TG, Tan PW, Chen JW, Yin WF, Chan KG. 2016. Draft genome sequence of *Kocuria rhizophila* strain TPW45, an Actinobacterium isolated from freshwater. *J Genomics* 4: 16-18.

Ågren J, Sundström A, Håfström T, Segerman B. 2012. Gegenees: fragmented alignment of multiple genomes for determining phylogenomic distances and genetic signatures unique for specified target groups. *PLoS One* 7(6): e39107.

Ahmad A, Viljoen AM, Chenia HY. 2014. The impact of plant volatiles on bacterial quorum sensing. *Lett Appl Microbiol* 60: 8-19.

Alonso JM, Stepanova AN, Solano R, Wisman E, Ferrari S, Ausubel FM, Ecker JR. 2003. Five components of the ethylene-response pathway identified in a screen for weak ethylene-insensitive mutants in *Arabidopsis*. *Proc Natl Acad Sci U S A* 100(5): 2992-2997.

Anand S, Bala K, Saxena A, Schumann P, Lal R. 2012. *Microbacterium amylolyticum* sp. nov., isolated from soil from an industrial waste site. *Int J Syst Evol Microbiol* 62(Pt 9): 2114-2120.

Anderson AS, Wellington EMH. 2001. The taxonomy of *Streptomyces* and related genera. *Int J Syst Evol Microbiol* 51: 797–814.

Asensio D, Peñuelas J, Filella I, Llusià J. 2007. On-line screening of soil VOCs exchange responses to moisture, temperature and root presence. *Plant and Soil* 291(1-2): 249-261.

Atmosukarto I, Castillo U, Hess WH, Sears J, Strobel G. 2005. Isolation and characterization of *Muscodor albus* I-41.3s, a volatile antibiotic producing fungus. *Plant Sci* 169: 854-861.

Audrain B, Létoffé S, Ghigo J-M. 2015. Airborne bacterial interactions: functions out of thin air? *Front Microbiol* 6: 1476.

Aziz M, Nadipalli RK, Xie X, Sun Y, Surowiec K, Zhang J-L, Paré PW. 2016. Augmenting sulfur metabolism and herbivore defense in *Arabidopsis* by bacterial volatile signaling. *Front Plant Sci* 7: 458.

B

Bailly A, Groenhagen U, Schulz S, Geisler M, Eberl L, Weisskopf L. 2014. The inter-kingdom volatile signal indole promotes root development by interfering with auxin signalling. *Plant J* 80(5): 758-771.

Bailly A, Weisskopf L. 2012. The modulating effect of bacterial volatiles on plant growth: current knowledge and future challenges. *Plant Signal Behav* 7(1): 79-85.

Barnett SJ, Roget DK, Ryder MH. 2006. Suppression of *Rhizoctonia solani* AG-8 induced disease on wheat by the interaction between *Pantoea*, *Exiguobacterium*, and Microbacteria. *Aust J Soil Res* 44(4): 331.

Barth C, Jander G. 2006. *Arabidopsis* myrosinases TGG1 and TGG2 have redundant function in glucosinolate breakdown and insect defense. *Plant J* 46(4): 549-562.

Bernier SP, Letoffe S, Delepierre M, Ghigo JM. 2011. Biogenic ammonia modifies antibiotic resistance at a distance in physically separated bacteria. *Mol Microbiol* 81(3): 705-716.

Berthelot M, Andre G. 1891. Sur l'odeur propre de la terre. *Compt Rend* 112: 598–599.

Bindea G, Mlecnik B, Hackl H, Charoentong P, Tosolini M, Kirilovsky A, Fridman W-H, Pagès F, Trajanoski Z, Galon J. 2009. ClueGO: a Cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks. *Bioinformatics* 25(8): 1091-1093.

Bitas V, Kim HS, Bennett JW, Kang S. 2013. Sniffing on microbes: diverse roles of microbial volatile organic compounds in plant health. *Mol Plant Microbe Interact* 26(8): 835-843.

Bitas V, McCartney N, Li N, Demers J, Kim JE, Kim HS, Brown KM, Kang S. 2015. *Fusarium oxysporum* volatiles enhance plant growth via affecting auxin transport and signaling. *Front Microbiol* 6: 1248.

Blom D, Fabbri C, Connor EC, Schiestl FP, Klauser DR, Boller T, Eberl L, Weisskopf L. 2011a. Production of plant growth modulating volatiles is widespread among rhizosphere bacteria and strongly depends on culture conditions. *Environ Microbiol* 13(11): 3047-3058.

Blom D, Fabbri C, Eberl L, Weisskopf L. 2011b. Volatile-mediated killing of *Arabidopsis thaliana* by bacteria is mainly due to hydrogen cyanide. *Appl Environ Microbiol* 77(3): 1000-1008.

Boots AW, van Berkel JJ, Dallinga JW, Smolinska A, Wouters EF, van Schooten FJ. 2012. The versatile use of exhaled volatile organic compounds in human health and disease. *J Breath Res* 6(2): 027108.

Boukaew S, Plubrukam A, Prasertsan P. 2013. Effect of volatile substances from *Streptomyces philanthi* RM-1-138 on growth of *Rhizoctonia solani* on rice leaf. *BioControl* 58(4): 471-482.

Brana AF, Rodriguez M, Pahari P, Rohr J, Garcia LA, Blanco G. 2014. Activation and silencing of secondary metabolites in *Streptomyces albus* and *Streptomyces lividans* after transformation with cosmids containing the thienamycin gene cluster from *Streptomyces cattleya*. *Arch Microbiol* 196(5): 345-355.

Brodhagen M, Tsitsigiannis DI, Hornung E, Goebel C, Feussner I, Keller NP. 2008. Reciprocal oxylipin-mediated cross-talk in the *Aspergillus*-seed pathosystem. *Mol Microbiol* 67(2): 378-391.

Brodhun F, Feussner I. 2011. Oxylipins in fungi. *FEBS J* 278(7): 1047-1063.

Brown SD, Palumbo AV, Panikov N, Ariyawansa T, Klingeman DM, Johnson CM, Land ML, Utturkar SM, Epstein SS. 2012. Draft genome sequence for *Microbacterium laevaniformans* strain OR221, a bacterium tolerant to metals, nitrate, and low pH. *J Bacteriol* 194(12): 3279-3280.

Bustos I, Martinez-Bartolome MA, Achemchem F, Pelaez C, Requena T, Martinez-Cuesta MC. 2011. Volatile sulphur compounds-forming abilities of lactic acid bacteria: C-S lyase activities. *Int J Food Microbiol* 148(2): 121-127.

C

Camehl I, Sherameti I, Venus Y, Bethke G, Varma A, Lee J, Oelmuller R. 2010. Ethylene signalling and ethylene-targeted transcription factors are required to balance beneficial and nonbeneficial traits in the symbiosis between the endophytic fungus *Piriformospora indica* and *Arabidopsis thaliana*. *New Phytol* 185(4): 1062-1073.

Cane DE, Ikeda H. 2012. Exploration and mining of the bacterial terpenome. *Acc Chem Res* 45(3): 463-472.

Castillo MC, Martínez C, Buchala A, Métraux J-P, León J. 2004. Gene-specific involvement of β-oxidation in wound-activated responses in *Arabidopsis*. *Plant Physiology* 135(1): 85-94.

Cepl J, Blahuskova A, Cvrckova F, Markos A. 2014. Ammonia produced by bacterial colonies promotes growth of ampicillin-sensitive *Serratia* sp. by means of antibiotic inactivation. *FEMS Microbiol Lett* 354: 126-132.

Chan XY, Hong KW, Yin WF, Chan KG. 2016. Microbiome and biocatalytic bacteria in monkey up (*Nepenthes Pitcher*) digestive fluid. *Sci Rep* 6: 20016.

Chang C, Kwok SF, Bleecker AB, Meyerowitz EM. 1993. *Arabidopsis* ethylene-response gene ETR1: similarity of product to two-component regulators. *Science* 262(5133): 539-544.

Chapelle E, Mendes R, Bakker PAHM, Raaijmakers JM. 2016. Fungal invasion of the rhizosphere microbiome. *ISME J* 10(1): 265-268.

Chauhan A, Green S, Pathak A, Thomas J, Venkatramanan R. 2013. Whole-genome sequences of five oyster-associated bacteria show potential for crude oil hydrocarbon degradation. *Genome Announc* 1(5).

Cheng H, Liu H, Deng Y, Xiao J, Li X, Wang S. 2015. The WRKY45-2 WRKY13 WRKY42 transcriptional regulatory cascade is required for rice resistance to fungal pathogen. *Plant Physiol* 167(3): 1087-1099.

Chernin L, Toklikishvili N, Ovadis M, Kim S, Ben-Ari J, Khmel I. 2011. Quorum-sensing quenching by rhizobacterial volatiles. *Environ Microbiol Rep* 3: 698-704.

Chin C-S, Alexander DH, Marks P, Klammer AA, Drake J, Heiner C, Clum A, Copeland A, Huddleston J, Eichler EE, et al. 2013. Nonhybrid, finished microbial genome assemblies from long-read SMRT sequencing data. *Nat Meth* 10(6): 563-569.

Chitarra GS, Abee T, Rombouts FM, Posthumus MA, Dijksterhuis J. 2004. Germination of *Penicillium paneum* conidia is regulated by 1-octen-3-ol, a volatile self-inhibitor. *Appl Environ Microbiol* 70(5): 2823-2829.

Chou WKW, Fanizza I, Uchiyama T, Komatsu M, Ikeda H, Cane DE. 2010. Genome mining in *Streptomyces avermitilis:* cloning and characterization of SAV_76, the synthase for a new sesquiterpene, avermitilol. *J Am Chem Soc* 132(26): 8850-8851.

Chuankun X, Minghe M, Leming Z, Keqin Z. 2004. Soil volatile fungistasis and volatile fungistatic compounds. *Soil Biol Biochem* 36(12): 1997-2004.

Claeson AS, Sandstrom M, Sunesson AL. 2007. Volatile organic compounds (VOCs) emitted from materials collected from buildings affected by microorganisms. *J Environ Monit* 9: 240-245.

Claessen D, Rozen DE, Kuipers OP, Sogaard-Andersen L, van Wezel GP. 2014. Bacterial solutions to multicellularity: a tale of biofilms, filaments and fruiting bodies. *Nat Rev Micro* 12(2): 115-124.

Cogan TM, Goerges S, Gelsomino R, Larpin S, Hohenegger M, Bora N, Jamet E, Rea MC, Mounier J, Vancanneyt M, et al. 2014. Biodiversity of the surface microbial consortia from Limburger, Reblochon, Livarot, Tilsit, and Gubbeen cheeses. *Microbiol Spectr* 2(1): Cm-0010-2012.

Combet E, Henderson J, Eastwood CD, Burton SK. 2006. Eight-carbon volatiles in mushrooms and fungi: properties, analysis, and biosynthesis. *Mycoscience* 47(6): 317-326.

Conolly JD, Hill RA. 1991. *Dictionary of terpenoids*. London: Chapman & Hall.

Considine PJ, Flynn N, Patching JW. 1977. Ethylene production by microorganisms. *Appl Environ Microbiol* 33(4): 977-979.

Cordero P, Principe A, Jofre E, Mori G, Fischer S. 2014. Inhibition of the phytopathogenic fungus *Fusarium proliferatum* by volatile compounds produced by *Pseudomonas*. *Arch Microbiol* 196(11): 803-809.

Cordovez V, Carrion VJ, Etalo DW, Mumm R, Zhu H, van Wezel GP, Raaijmakers JM. 2015. Diversity and functions of volatile organic compounds produced by *Streptomyces* from a disease-suppressive soil. *Frontiers in Microbiology* 6(1081).

Corretto E, Antonielli L, Sessitsch A, Kidd P, Weyens N, Brader G. 2015. Draft genome sequences of 10 *Microbacterium* spp., with emphasis on heavy metal-contaminated environments. *Genome Announc* 3(3).

Costacurta A, Vanderleyden J. 1995. Synthesis of phytohormones by plant-associated bacteria. *Crit Rev Microbiol* 21(1): 1-18.

Cruz-Morales P, Vijgenboom E, Iruegas-Bocardo F, Girard G, Yáñez-Guerra LA, Ramos-Aboites HE, Pernodet J-L, Anné J, van Wezel GP, Barona-Gómez F. 2013. The genome sequence of *Streptomyces lividans* 66 reveals a novel tRNA-dependent peptide biosynthetic system within a metal-related genomic island. *Genome Biol and Evol* 5(6): 1165-1175.

Curl EA, Truelove B 1986. The structure and function of roots. *The rhizophere*. New York: Springer Berlin Heidelberg 9–54.

Curson AR, Rogers R, Todd JD, Brearley CA, Johnston AW. 2008. Molecular genetic analysis of a dimethylsulfoniopropionate lyase that liberates the climate-changing gas dimethylsulfide in several marine alpha-proteobacteria and *Rhodobacter sphaeroides*. *Environ Microbiol* 10(3): 757-767.

Curson AR, Sullivan MJ, Todd JD, Johnston AW. 2011. DddY, a periplasmic dimethylsulfoniopropionate lyase found in taxonomically diverse species of Proteobacteria. *ISME J* 5(7): 1191-1200.

D

D'Alessandro M, Erb M, Ton J, Brandenburg A, Karlen D, Zopfi J, Turlings TC. 2014. Volatiles produced by soil-borne endophytic bacteria increase plant pathogen resistance and affect tritrophic interactions. *Plant Cell Environ* 37(4): 813-826.

Dandurishvili N, Toklikishvili N, Ovadis M, Eliashvili P, Giorgobiani N, Keshelava R. 2011. Broad-range antagonistic rhizobacteria *Pseudomonas fluorescens* and *Serratia plymuthica* suppress *Agrobacterium* crown gall tumours on tomato plants. *J Appl Microbiol* 110: 341-352.

Das A, Lee S-H, Hyun TK, Kim S-W, Kim J-Y. 2012. Plant volatiles as method of communication. *Plant Biotechnol Rep* 7(1): 9-26.

de Wit M, Spoel SH, Sanchez-Perez GF, Gommers CM, Pieterse CM, Voesenek LA, Pierik R. 2013. Perception of low red:far-red ratio compromises both salicylic acid- and jasmonic acid-dependent pathogen defences in *Arabidopsis*. *Plant J* 75(1): 90-103.

DeAngelis KM, Brodie EL, DeSantis TZ, Andersen GL, Lindow SE, Firestone MK. 2009. Selective progressive response of soil microbial community to wild oat roots. *ISME J* 3(2): 168-178.

Demkura PV, Abdala G, Baldwin IT, Ballare CL. 2010. Jasmonate-dependent and -independent pathways mediate specific effects of solar ultraviolet B radiation on leaf phenolics and antiherbivore defense. *Plant Physiol* 152(2): 1084-1095.

Dharmasiri N, Dharmasiri S, Estelle M. 2005. The F-box protein TIR1 is an auxin receptor. *Nature* 435(7041): 441-445.

Dicke M. 2009. Behavioural and community ecology of plants that cry for help. *Plant Cell Environ* 32(6): 654-665.

Dicke M. 2016. Induced plant volatiles: plant body odours structuring ecological networks. *New Phytol* 210(1): 10-12.

Dicke M, Baldwin IT. 2010. The evolutionary context for herbivore-induced plant volatiles: beyond the 'cry for help'. *Trends in Plant Sci* 15(3): 167-175.

Dicke M, Loreto F. 2010. Induced plant volatiles: from genes to climate change. *Trends Plant Sci* 15(3): 115-117.

Dicke M, Sabelis MW. 1987. How Plants Obtain Predatory Mites as Bodyguards. *Netherlands Journal of Zoology* 38(2): 148-165.

Dickschat JS, Bruns H, Riclea R. 2011. Novel fatty acid methyl esters from the actinomycete *Micromonospora aurantiaca*. *Beilstein J Org Chem* 7: 1697-1712.

Dickschat JS, Pahirulzaman KA, Rabe P, Klapschinski TA. 2014. An improved technique for the rapid chemical characterisation of bacterial terpene cyclases. *Chembiochem* 15(6): 810-814.

Ditengou FA, Müller A, Rosenkranz M, Felten J, Lasok H, van Doorn MM, Legué V, Palme K, Schnitzler J-P, Polle A. 2015. Volatile signalling by sesquiterpenes from ectomycorrhizal fungi reprogrammes root architecture. *Nat Commun* 6.

Dobbs CG, Hinson WH. 1953. A widespread fungistasis in soils. *Nature* 172: 197 - 199.

Dudareva N, Pichersky E, Gershenzon J. 2004. Biochemistry of plant volatiles. *Plant Physiol* 135(4): 1893-1902.

E

Ebel R. 2010. Terpenes from marine-derived fungi. *Mar Drugs* 8(8): 2340-2368.

Eckert C, Xu W, Xiong W, Lynch S, Ungerer J, Tao L, Gill R, Maness PC, Yu J. 2014. Ethylene-forming enzyme and bioethylene production. *Biotechnol Biofuels* 7(1): 33.

Edgar RC. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* 32(5): 1792-1797.

Effmert U, Kalderas J, Warnke R, Piechulla B. 2012. Volatile mediated interactions between bacteria and fungi in the soil. *J Chem Ecol* 38(6): 665-703.

Engelberth J, Alborn HT, Schmelz EA, Tumlinson JH. 2004. Airborne signals prime plants against insect herbivore attack. *Proc Natl Acad Sci U S A* 101(6): 1781-1785.

Epstein L, Lockwood JL. 1984. Effect of soil microbiota on germination of *Bipolaris victoriae* conidia. *Tras. Br. Mycol. Soc.* 82: 63-69.

Ezra D, Strobel GA. 2003. Effect of substrate on the bioactivity of volatile antimicrobials produced by *Muscodor albus*. *Plant Science* 165(6): 1229-1238.

F

Farag MA, Zhang H, Ryu CM. 2013. Dynamic chemical communication between plants and bacteria through airborne signals: induced resistance by bacterial volatiles. *J Chem Ecol* 39: 1007-1018.

Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap *Evolution* 39(4): 783-791

Fernando WGD, Ramarathnam R, Krishnamoorthy AS, Savchuk SC. 2005. Identification and use of potential bacterial organic antifungal volatiles in biocontrol. *Soil Biol Biochem* 37(5): 955-964.

Fiers M, Lognay G, Fauconnier ML, Jijakli MH. 2013. Volatile compound-mediated interactions between barley and pathogenic fungi in the soil. *PLoS One* 8(6): e66805.

Fujimoto SY, Ohta M, Usui A, Shinshi H, Ohme-Takagi M. 2000. *Arabidopsis* ethylene-responsive element binding factors act as transcriptional activators or repressors of GCC box-mediated gene expression. *Plant Cell* 12(3): 393-404.

G

Gallucci MN, Oliva M, Casero C, Dambolena J, Luna A, Zygadlo J. 2009. Antimicrobial combined action of terpenes against the food-borne microorganisms *Escherichia coli, Staphylococcus aureus* and *Bacillus cereus*. *Flavour Fragr J* 24: 348-354.

Gan HY, Gan HM, Savka MA, Triassi AJ, Wheatley MS, Smart LB, Fabio ES, Hudson AO. 2014. Whole-genome sequences of 13 endophytic bacteria isolated from shrub willow (salix) grown in geneva, new york. *Genome Announc* 2(3).

Garbeva P, Hol WHG, Termorshuizen AJ, Kowalchuk GA, de Boer W. 2011. Fungistasis and general soil biostasis – a new synthesis. *Soil Biology and Biochemistry* 43(3): 469-477.

Garbeva P, Hordijk C, Gerards S, De Boer W. 2014a. Volatile-mediated interactions between phylogenetically different soil bacteria. *Front Microbiol* 5: 285-290.

Garbeva P, Hordijk C, Gerards S, de Boer W. 2014b. Volatiles produced by the mycophagous soil bacterium *Collimonas*. *FEMS Microbiol Ecol* 87(3): 639-649.

Garnica-Vergara A, Barrera-Ortiz S, Munoz-Parra E, Raya-Gonzalez J, Mendez-Bravo A, Macias-Rodriguez L, Ruiz-Herrera LF, Lopez-Bucio J. 2016. The volatile 6-pentyl-2H-pyran-2-one from *Trichoderma atroviride* regulates *Arabidopsis thaliana* root morphogenesis via auxin signaling and ETHYLENE INSENSITIVE 2 functioning. *New Phytol* 209(4): 1496-1512.

Gerber NN. 1968. Geosmin, from microorganisms, is -1, 10-dimethyl-9-decalol. *Tetrahedron Lett* 25: 2971-2974.

Gershenzon J, Dudareva N. 2007. The function of terpene natural products in the natural world. *Nat Chem Biol* 3(7): 408-414.

Girard G, Traag BA, Sangal V, Mascini N, Hoskisson PA, Goodfellow M, van Wezel GP. 2013. A novel taxonomic marker that discriminates between morphologically complex actinomycetes. *Open Biol* 3(10): 130073.

Gomez-Gomez L, Boller T. 2000. FLS2: An LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in *Arabidopsis*. *Mol Cell* 5(6): 1003-1011.

Goodfellow M 2012. Phylum XXVI. Actinobacteria phyl. nov. In: Goodfellow M, Kämpfer P, Busse H-J, Trujillo ME, Suzuki K-I, Ludwig W, Whitman WB eds. *Bergey's Manual of Systematic Bacteriology*. New York: Springer, 1-2083.Grayston SJ, Wang S, Campbell CD, Edwards AD. 1998. Selective influence of plant species on microbial diversity in the rhizosphere. *Soil Biol Biochem* 30(3): 369-378.

Groenhagen U, Baumgartner R, Bailly A, Gardiner A, Eberl L, Schulz S. 2013a. Production of bioactive volatiles by different *Burkholderia ambifaria* strains. *J Chem Ecol* 39: 892-906.

Groenhagen U, Baumgartner R, Bailly A, Gardiner A, Eberl L, Schulz S, Weisskopf L. 2013b. Production of bioactive volatiles by different *Burkholderia ambifaria* strains. *J Chem Ecol* 39(7): 892-906.

Gu Y-Q, Wildermuth MC, Chakravarthy S, Loh Y-T, Yang C, He X, Han Y, Martin GB. 2002. Tomato transcription factors Pti4, Pti5, and Pti6 activate defense responses when expressed in *Arabidopsis*. *The Plant Cell* 14(4): 817-831.

Guo Y, Zheng W, Rong X, Huang Y. 2008. A multilocus phylogeny of the *Streptomyces griseus* 16S *rRNA* gene clade: use of multilocus sequence analysis for streptomycete systematics. *Int J Syst Evol Microbiol* 58(Pt 1): 149-159.

Gurtler H, Pedersen R, Anthoni U, Christophersen C, Nielsen PH, Wellington EMH. 1994. Albaflavenone, a sesquiterpene ketone with a zizaene skeleton produced by a streptomycete with a new rope morphology. *J Antibiot* 47: 434-439.

H

Hagai E, Dvora R, Havkin-Blank T, Zelinger E, Porat Z, Schulz S. 2014a. Surface-motility induction, attraction and hitchhiking between bacterial species promote dispersal on solid surfaces. *ISME J* 8: 1147-1151.

Hagai E, Dvora R, Havkin-Blank T, Zelinger E, Porat Z, Schulz S, Helman Y. 2014b. Surface-motility induction, attraction and hitchhiking between bacterial species promote dispersal on solid surfaces. *ISME J* 8(5): 1147-1151.

Han SR, Kim KH, Ahn DH, Park H, Oh TJ. 2016. Complete genome sequence of carotenoid-producing *Microbacterium* sp. strain PAMC28756 isolated from an Antarctic lichen. *J Biotechnol* 226: 18-19.

Hazelwood LA, Daran JM, van Maris AJ, Pronk JT, Dickinson JR. 2008. The Ehrlich pathway for fusel alcohol production: a century of research on *Saccharomyces cerevisiae* metabolism. *Appl Environ Microbiol* 74(8): 2259-2266.

Heil M, Bostock RM. 2002. Induced systemic resistance (ISR) against pathogens in the context of induced plant defences. *Ann Bot* 89(5): 503-512.

Heil M, Silva Bueno JC. 2007. Within-plant signaling by volatiles leads to induction and priming of an indirect plant defense in nature. *Proc Natl Acad Sci U S A* 104(13): 5467-5472.

Hemaiswarya S, Doble M. 2010. Synergistic interaction of phenylpropanoids with antibiotics against bacteria. *J Med Microbiol* 59: 1469-1476.

Herms DA, Mattson WJ. 1992. The dilemma of plants: to grow or defend. *The Quarterly Review of Biology* 67(3): 283-335.

Hernández-León R, Rojas-Solís D, Contreras-Pérez M, Orozco-Mosqueda MdC, Macías-Rodríguez LI, Reyes-de la Cruz H, Valencia-Cantero E, Santoyo G. 2015. Characterization of the antifungal and plant growth-promoting effects of diffusible and volatile organic compounds produced by *Pseudomonas fluorescen*s strains. *Biol Control* 81: 83-92.

Herrero-Garcia E, Garzia A, Cordobes S, Espeso EA, Ugalde U. 2011. 8-Carbon oxylipins inhibit germination and growth, and stimulate aerial conidiation in *Aspergillus nidulans*. *Fungal Biol* 115(4-5): 393-400.

Herrington PR, Craig JT, Chea CY, Sheridan JE. 1985. Inhibition of spore germination by volatiles from *Streptomyces griseoruber*. *Soil Biol Biochem* 17: 897-898.

Herrington PR, Craig JT, Sheridan JE. 1987. Methyl vinyl ketone - a volatile fungistatic inhibitor from *Streptomyces griseoruber*. *Soil Biol Biochem* 19: 509-512.

Holopainen JK, Blande JD. 2012. Molecular plant volatile communication. *Adv Exp Med Biol* 739: 17-31.

Hopwood DA. 2007. *Streptomyces in nature and medicine: the antibiotic makers.* New York: Oxford University Press.

Hora TS, Baker R. 1972. Extraction of a volatile factor from soil-inducing fungistasis. *Phytopathology* 62(12): 1475-1476.

Hornby D. 1983. Suppressive soils. *Annual Review of Phytopatology* 21: 65-85.

Hornby JM, Jensen EC, Lisec AD, Tasto JJ, Jahnke B, Shoemaker R. 2001. Quorum sensing in the dimorphic fungus *Candida albicans* is mediated by farnesol. *Appl Environ Microbiol* 67: 2982-2992.

Hornitschek P, Kohnen MV, Lorrain S, Rougemont J, Ljung K, Lopez-Vidriero I, Franco-Zorrilla JM, Solano R, Trevisan M, Pradervand S, et al. 2012. Phytochrome interacting factors 4 and 5 control seedling growth in changing light conditions by directly controlling auxin signaling. *Plant J* 71(5): 699-711.

Huang CJ, Tsay JF, Chang SY, Yang HP, Wu WS, Chen CY. 2012. Dimethyl disulfide is an induced systemic resistance elicitor produced by *Bacillus cereus* C1L. *Pest Manag Sci* 68(9): 1306-1310.

Huang Y, Niu B, Gao Y, Fu L, Li W. 2010. CD-HIT Suite: a web server for clustering and comparing biological sequences. *Bioinformatics* 26(5): 680-682.

Humphris SN, Bruce A, Buultjens E, Wheatley RE. 2002. The effects of volatile microbial secondary metabolites on protein synthesis in *Serpula lacrymans*. *FEMS Microbiol Lett* 210(2): 215-219.

Hung R, Lee S, Bennett JW. 2013. *Arabidopsis thaliana* as a model system for testing the effect of *Trichoderma* volatile organic compounds. *Fungal Ecol* 6(1): 19-26.

Hung R, Lee S, Bennett JW. 2015. Fungal volatile organic compounds and their role in ecosystems. *Appl Microbiol Biotechnol* 99(8): 3395-3405.

Hunziker L, Boenisch D, Groenhagen U, Bailly A, Schulz S, Weisskopf L. 2015. *Pseudomonas* strains naturally associated with potato plants produce volatiles with high potential for inhibition of *Phytophthora infestans*. *Appl Environ Microbiol* 81: 821-830.

Huot B, Yao J, Montgomery BL, He SY. 2014. Growth-defense tradeoffs in plants: a balancing act to optimize fitness. *Mol Plant* 7(8): 1267-1287.

Huson DH, Bryant D. 2006. Application of phylogenetic networks in evolutionary studies. *Mol Biol Evol* 23(2): 254-267.

Hynes J, Müller CT, Jones TH, Boddy L. 2007. Changes in volatile production during the course of fungal mycelial interactions between *Hypholoma fasciculare* and *Resinicium bicolor*. *J Chem Ecol* 33(1): 43-57.

I

Ilag L, Curtis RW. 1968. Production of ethylene by fungi. *Science* 159(3821): 1357-1358.

Insam H, Seewald MSA. 2010. Volatile organic compounds (VOCs) in soils. *Biol Fertil Soils* 46(3): 199-213.

J

Jauregui I, Aparicio-Tejo PM, Avila C, Rueda-Lopez M, Aranjuelo I. 2015. Root and shoot performance of *Arabidopsis thaliana* exposed to elevated CO2: A physiologic, metabolic and transcriptomic response. *J Plant Physiol* 189: 65-76.

Jensen SE, Goatcher LJ, Perley T, Kenefick S, Hrudey SE. 1994. Actinomycetes as a factor in odour problems affecting drinking water from the North Saskatchewan River. *Wat Res* 28: 1393-1401.

Jiang JY, He XF, Cane DE. 2007. Biosynthesis of the earthy odorant geosmin by a bifunctional *Streptomyces coelicolor* enzyme. *Nat Chem Biol* 3(11): 711-715.

Jones JDG, Dangl JL. 2006. The plant immune system. *Nature* 444(7117): 323-329.

Junker RR, Tholl D. 2013. Volatile organic compound mediated interactions at the plant-microbe interface. *J Chem Ecol* 39: 810–825.

K

Kai M, Effmert U, Berg G, Piechulla B. 2007. Volatiles of bacterial antagonists inhibit mycelial growth of the plant pathogen *Rhizoctonia solani*. *Arch Microbiol* 187(5): 351-360.

Kai M, Haustein M, Molina F, Petri A, Scholz B, Piechulla B. 2009. Bacterial volatiles and their action potential. *Appl Microbiol Biotechnol* 81(6): 1001-1012.

Kai M, Piechulla B. 2009. Plant growth promotion due to rhizobacterial volatiles - an effect of CO2? *FEBS Lett* 583(21): 3473-3477.

Karojet S, Kunz S, van Dongen JT. 2012. *Microbacterium yannicii* sp. nov., isolated from *Arabidopsis thaliana* roots. *Int J Syst Evol Microbiol* 62(Pt 4): 822-826.

Kazemi K. 2014. Composition of essential oil of *Artemisia indica*. *J Biol Environ Sci* 8(23): 95-97.

Kechid M, Desbrosses G, Rokhsi W, Varoquaux F, Djekoun A, Touraine B. 2013. The NRT2.5 and NRT2.6 genes are involved in growth promotion of *Arabidopsis* by the plant growth-promoting rhizobacterium (PGPR) strain *Phyllobacterium brassicacearum* STM196. *New Phytol* 198(2): 514-524.

Keeling CI, Bohlmann J. 2006. Genes, enzymes and chemicals of terpenoid diversity in the constitutive and induced defence of conifers against insects and pathogens. *New Phytol* 170(4): 657-675.

Kegge W, Pierik R. 2010. Biogenic volatile organic compounds and plant competition. *Trends Plant Sci* 15(3): 126-132.

Kertesz MA, Mirleau P. 2004. The role of soil microbes in plant sulphur nutrition. *J Exp Bot* 55(404): 1939-1945.

Kessler A, Baldwin IT. 2001. Defensive function of herbivore-induced plant volatile emissions in nature. *Science* 291: 2141-2144.

Kikuta Y, Ueda H, Nakayama K, Katsuda Y, Ozawa R, Takabayashi J, Hatanaka A, Matsuda K. 2011. Specific regulation of pyrethrin biosynthesis in *Chrysanthemum cinerariaefolium* by a blend of volatiles emitted from artificially damaged conspecific plants. *Plant Cell Physiol* 52(3): 588-596.

Kim EJ, Fathoni A, Jeong GT, Jeong HD, Nam TJ, Kong IS, Kim JK. 2013. *Microbacterium oxydans*, a novel alginate- and laminarin-degrading bacterium for the reutilization of brown-seaweed waste. *J Environ Manage* 130: 153-159.

Kim KK, Park HY, Park W, Kim IS, Lee ST. 2005. *Microbacterium xylanilyticum* sp. nov., a xylan-degrading bacterium isolated from a biofilm. *Int J Syst Evol Microbiol* 55(Pt 5): 2075-2079.

Kim KS, Lee S, Ryu CM. 2013. Interspecific bacterial sensing through airborne signals modulates locomotion and drug resistance. *Nat Commun* 4: 1809.

Kim TY, Lee SW, Oh MK. 2014. Biosynthesis of 2-phenylethanol from glucose with genetically engineered *Kluyveromyces marxianus*. *Enzyme Microb Technol* 61-62: 44-47.

Kim Y-C, Glick BR, Bashan Y, Ryu C-M 2012. Enhancement of plant drought tolerance by microbes. In: Aroca R ed. *Plant Responses to Drought Stress*. Berlin: Springer-Verlag Berlin Heidelberg, 383-413.

Kitaoka N, Matsubara T, Sato M, Takahashi K, Wakuta S, Kawaide H, Matsui H, Nabeta K, Matsuura H. 2011. A*rabidopsis CYP94B3* encodes jasmonyl-L-isoleucine 12-hydroxylase, a key enzyme in the oxidative catabolism of jasmonate. *Plant Cell Physiol* 52(10): 1757-1765.

Knudsen JT, Eriksson R, Gershenzon J, Stahl B. 2006. The chemistry diversity of floral scent. *The Botanical Review* 72(1): 1-120.

Komatsu M, Tsuda M, Ômura S, Oikawa H, Ikeda H. 2008. Identification and functional analysis of genes controlling biosynthesis of 2-methylisoborneol. *Proc Natl Acad Sci USA* 105(21): 7422-7427.

Koo AJ, Cooke TF, Howe GA. 2011. Cytochrome P450 CYP94B3 mediates catabolism and inactivation of the plant hormone jasmonoyl-L-isoleucine. *Proc Natl Acad Sci USA* 108(22): 9298-9303.

Korpi A, Jarnberg J, Pasanen AL. 2009. Microbial volatile organic compounds. *Crit Rev Toxicol* 39(2): 139-193.

Kottb M, Gigolashvili T, Grosskinsky DK, Piechulla B. 2015. *Trichoderma* volatiles effecting Arabidopsis: from inhibition to protection against phytopathogenic fungi. *Front Microbiol* 6: 995.

Kramer R, Abraham W-R. 2011. Volatile sesquiterpenes from fungi: what are they good for? *Phytochemistry Reviews* 11(1): 15-37.

Kruse C, Jost R, Lipschis M, Kopp B, Hartmann M, Hell R. 2007. Sulfur-enhanced defence: effects of sulfur metabolism, nitrogen supply, and pathogen lifestyle. *Plant Biol (Stuttg)* 9(5): 608-619.

Kudoyarova GR, Arkhipova TN, Melent'ev AI 2015. Role of bacterial phytohormones in plant growth regulation and their development. In: Maheshwari KD ed. *Bacterial Metabolites in Sustainable Agroecosystem*. Cham: Springer International Publishing, 69-86.

Kwon YS, Ryu CM, Lee S, Park HB, Han KS, Lee JH, Lee K, Chung WS, Jeong MJ, Kim HK, et al. 2010. Proteome analysis of *Arabidopsis* seedlings exposed to bacterial volatiles. *Planta* 232(6): 1355-1370.

L

Ladygina N, Dedyukhina EG, Vainshtein MB. 2006. A review on microbial synthesis of hydrocarbons. *Process Biochem* 41(5): 1001-1014.

Laffineur K, Avesani V, Cornu G, Charlier J, Janssens M, Wauters G, Delmée M. 2003. Bacteremia due to a novel *Microbacterium* species in a patient with leukemia and description of *Microbacterium paraoxydans* sp. nov. *J Clin Microbiol* 41(5): 2242-2246.

Lane DJ. 1991. *16S/23S rRNA sequencing*. Chichester, United Kingdom: John Wiley & Sons.

Langmead B, Salzberg SL. 2012. Fast gapped-read alignment with Bowtie 2. *Nat Meth* 9(4): 357-359.

Larsen TO, Frisvad JC. 1995. Comparison of different methods for collection of volatile chemical markers from fungi. *J Microbiol Methods* 24(2): 135-144.

Lee B, Farag MA, Park HB, Kloepper JW, Lee SH, Ryu CM. 2012. Induced resistance by a long-chain bacterial volatile: elicitation of plant systemic defense by a C13 volatile produced by *Paenibacillus polymyxa*. *PLoS One* 7(11): e48744.

Lee HH, Molla MN, Cantor CR, Collins JJ. 2010. Bacterial charity work leads to population-wide resistance. *Nature* 467: 82-U113.

Lee J-H, Lee J. 2010. Indole as an intercellular signal in microbial communities. *FEMS Microbiol Rev* 34(4): 426-444.

Lee JS, Lee KC, Park YH. 2006. *Microbacterium koreense* sp. nov., from sea water in the South Sea of Korea. *Int J Syst Evol Microbiol* 56(Pt 2): 423-427.

Lee LH, Azman AS, Zainal N, Eng SK, Ab Mutalib NS, Yin WF, Chan KG. 2014. *Microbacterium mangrovi* sp. nov., an amylolytic actinobacterium isolated from mangrove forest soil. *Int J Syst Evol Microbiol* 64(Pt 10): 3513-3519.

Lee S, Hung R, Yap M, Bennett JW. 2015. Age matters: the effects of volatile organic compounds emitted by Trichoderma atroviride on plant growth. *Arch Microbiol* 197(5): 723-727.

Lemanceau P, Corberand T, Gardan L, Latour X, Laguerre G, Boeufgras J, Alabouvette C. 1995. Effect of two plant species, flax (*Linum usitatissinum* L.) and tomato (*Lycopersicon esculentum* Mill.), on the diversity of soilborne populations of fluorescent pseudomonads. *Appl Environ Microbiol* 61(3): 1004-1012.

Lemfack MC, Nickel J, Dunkel M, Preissner R, Piechulla B. 2014. mVOC: a database of microbial volatiles. *Nucleic Acids Res* 42(Database issue): D744-748.

Létoffé S, Audrain B, Bernier SP, Delepierre M, Ghigo JM. 2014. Aerial exposure to the bacterial volatile compound trimethylamine modifies antibiotic resistance of physically separated bacteria by raising culture medium pH. *MBio* 5: e00944-00913.

Letunic I, Bork P. 2011. Interactive Tree Of Life v2: online annotation and display of phylogenetic trees made easy. *Nucleic Acids Res* 39(Web Server issue): W475-478.

Letunic I, Bork P. 2016. Interactive tree of life (iTOL) v3: an online tool for the display and annotation of phylogenetic and other trees. *Nucleic Acids Research* 44(W1): W242-W245.

Letunic I, Yamada T, Kanehisa M, Bork P. 2008. iPath: interactive exploration of biochemical pathways and networks. *Trends Biochem Sci* 33(3): 101-103.

Li B, Dewey CN. 2011. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinform* 12(1): 1-16.

Lin L, Xu X. 2013. Indole-3-acetic acid production by endophytic *Streptomyces* sp. En-1 isolated from medicinal plants. *Curr Microbiol* 67(2): 209-217.

Little DY, Rao H, Oliva S, Daniel-Vedele F, Krapp A, Malamy JE. 2005. The putative high-affinity nitrate transporter NRT2.1 represses lateral root initiation in response to nutritional cues. *Proc Natl Acad Sci USA* 102(38): 13693-13698.

Lockwood JL. 1977. Fungistasis in soils. *Biol. Rev.* 52: 1-43.

Lommen A, Kools HJ. 2012. MetAlign 3.0: performance enhancement by efficient use of advances in computer hardware. *Metabolomics* 8(4): 719-726.

Lorrain S, Allen T, Duek PD, Whitelam GC, Fankhauser C. 2008. Phytochrome-mediated inhibition of shade avoidance involves degradation of growth-promoting bHLH transcription factors. *Plant J* 53(2): 312-323.

Love MI, Huber W, Anders S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology* 15(12): 1-21.

Lupetti A, Danesi R, Campa M, Del Tacca M, Kelly S. 2002. Molecular basis of resistance to azole antifungals. *Trends Mol Med* 8: 76-81.

Lushchak VI. 2001. Oxidative stress and mechanisms of protection against it in bacteria. *Biochemistry (Mosc)* 66: 476-489.

M

Mackie AE, Wheatley RE. 1999. Effects and incidence of volatile organic compound interactions between soil bacterial and fungal isolates. *Soil Biol Biochem* 31(3): 375-385.

Madhaiyan M, Poonguzhali S, Lee JS, Lee KC, Saravanan VS, Santhanakrishnan P. 2010. *Microbacterium azadirachtae* sp. nov., a plant-growth-promoting actinobacterium isolated from the rhizoplane of neem seedlings. *Int J Syst Evol Microbiol* 60(Pt 7): 1687-1692.

Mandakovic D, Cabrera P, Pulgar R, Maldonado J, Aravena P, Latorre M, Cambiazo V, Gonzalez M. 2015. Complete genome sequence of *Microbacterium* sp. CGR1, bacterium tolerant to wide abiotic conditions isolated from the Atacama Desert. *J Biotechnol* 216: 149-150.

Marilley L, Casey MG. 2004. Flavours of cheese products: metabolic pathways, analytical tools and identification of producing strains. *Int J Food Microbiol* 90(2): 139-159.

Marquez-Villavicencio P, Weber B, Witherell RA, Willis DK, Charkowski AO. 2011. The 3-hydroxy-2-butanone pathway is required for *Pectobacterium carotovorum* pathogenesis. *PLoS One* 6: e22974.

Martins M, Henriques M, Azeredo J, Rocha SM, Coimbra MA, Oliveira R. 2007. Morphogenesis control in *Candida albicans* and *Candida dubliniensis* through signaling molecules produced by planktonic and biofilm cells. *Eukaryot Cell* 6: 2429-2436.

Mauriello G, Marino R, D'Auria M, Cerone G, Rana GL. 2004. Determination of volatile organic compounds from truffles via SPME-GC-MS. *J Chromatogr Sci* 42: 299-305.

Mazzola M. 2002. Mechanisms of natural soil suppressiveness to soilborne diseases. *Antonie Van Leeuwenhoek*(81): 557–564.

McDonald D, Price MN, Goodrich J, Nawrocki EP, DeSantis TZ, Probst A, Andersen GL, Knight R, Hugenholtz P. 2012. An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *ISME J* 6(3): 610-618.

McNeal KS, Herbert BE. 2009. Volatile organic metabolites as indicators of soil microbial activity and community composition shifts. *Soil Sci Am J* 73(2): 579.

McNerney R, Mallard K, Okolo PI, Turner C. 2012. Production of volatile organic compounds by mycobacteria. *FEMS Microbiol Lett* 328(2): 150-156.

Medema MH, Blin K, Cimermancic P, de Jager V, Zakrzewski P, Fischbach MA, Weber T, Takano E, Breitling R. 2011. antiSMASH: rapid identification, annotation and analysis of secondary metabolite biosynthesis gene clusters in bacterial and fungal genome sequences. *Nucleic Acids Research* 39(Web Server issue): W339-W346.

Meldau DG, Meldau S, Hoang LH, Underberg S, Wunsche H, Baldwin IT. 2013. Dimethyl disulfide produced by the naturally associated bacterium *Bacillus* sp. B55 promotes *Nicotiana attenuata* growth by enhancing sulfur nutrition. *Plant Cell* 25(7): 2731-2747.

Mendes R, Garbeva P, Raaijmakers JM. 2013. The rhizosphere microbiome: significance of plant beneficial, plant pathogenic, and human pathogenic microorganisms. *FEMS Microbiology Reviews* 37(5): 634-663.

Mendes R, Kruijt M, de Bruijn I, Dekkers E, van der Voort M, Schneider JH, Piceno YM, DeSantis TZ, Andersen GL, Bakker PA, et al. 2011. Deciphering the rhizosphere microbiome for disease-suppressive bacteria. *Science* 332(6033): 1097-1100.

Messina FJ, Durham SL, Richards JH, McArthur DE. 2002. Trade-off between plant growth and defense? A comparison of sagebrush populations. *Oecologia* 131(1): 43-51.

Midha S, Bansal K, Sharma S, Kumar N, Patil PP, Chaudhry V, Patil PB. 2015. Genomic resource of rice seed associated bacteria. *Front Microbiol* 6: 1551.

Minerdi D, Bossi S, Gullino ML, Garibaldi A. 2009. Volatile organic compounds: a potential direct long-distance mechanism for antagonistic action of *Fusarium oxysporum* strain MSA 35. *Environ Microbiol* 11: 844-854.

Minerdi D, Moretti M, Gilardi G, Barberio C, Gullino ML, Garibaldi A. 2008. Bacterial ectosymbionts and virulence silencing in a *Fusarium oxysporum* strain. *Environ Microbiol* 10: 1725-1741.

Mitchell AM, Strobel GA, Moore E, Robison R, Sears J. 2010. Volatile antimicrobials from *Muscodor crispans*, a novel endophytic fungus. *Microbiology* 156: 270-277.

Miyamoto K, Murakami T, Kakumyan P, Keller NP, Matsui K. 2014. Formation of 1-octen-3-ol from *Aspergillus flavus* conidia is accelerated after disruption of cells independently of Ppo oxygenases, and is not a main cause of inhibition of germination. *PeerJ* 2: e395.

Moldrup P, Olesen T, Gamst J, Schjønning P, Yamaguchi T, Rolston DE. 2000. Predicting the gas diffusion coefficient in repacked soil water-induced linear reduction model. *Soil Sci Am J* 64(5): 1588-1594.

Molina-Santiago C, Daddaoua A, Fillet S, Duque E, Ramos JL. 2014. Interspecies signalling: *Pseudomonas putida* efflux pump TtgGHI is activated by indole to increase antibiotic resistance. *Environ Microbiol* 16(5): 1267-1281.

Moody SC, Zhao B, Lei L, Nelson DR, Mullins JG, Waterman MR. 2012a. Investigating conservation of the albaflavenone biosynthetic pathway and CYP170 bifunctionality in streptomycetes. *FEBS J* 279: 1640-1649.

Moody SC, Zhao B, Lei L, Nelson DR, Mullins JG, Waterman MR, Kelly SL, Lamb DC. 2012b. Investigating conservation of the albaflavenone biosynthetic pathway and CYP170 bifunctionality in streptomycetes. *FEBS J* 279(9): 1640-1649.

Moore-Landecker E, Stotzky G. 1973. Morphological abnormalities of fungi induced by volatile microbial metabolites. *Mycologia* 65(3): 519-530.

Morath SU, Hung R, Bennett JW. 2012. Fungal volatile organic compounds: A review with emphasis on their biotechnological potential. *Fungal Biol Rev* 26(2-3): 73-83.

Morohoshi T, Wang WZ, Someya N, Ikeda T. 2011. Genome sequence of *Microbacterium testaceum* StLB037, an N-acylhomoserine lactone-degrading bacterium isolated from potato leaves. *J Bacteriol* 193(8): 2072-2073.

Muller A, Faubert P, Hagen M, Zu Castell W, Polle A, Schnitzler JP, Rosenkranz M. 2013. Volatile profiles of fungi - chemotyping of species and ecological functions. *Fungal Genet Biol* 54: 25-33.

Mumm R, Dicke M. 2010. Variation in natural plant products and the attraction of bodyguards involved in indirect plant defense. *Can J Zool* 88(7): 628-667.

Murashige T, Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Phys Plant* 15: 473-497.

N

Negi S, Ivanchenko MG, Muday GK. 2008. Ethylene regulates lateral root formation and auxin transport in *Arabidopsis thaliana*. *Plant J* 55(2): 175-187.

Nemcovic M, Jakubikova L, Viden I, Farkas V. 2008. Induction of conidiation by endogenous volatile compounds in *Trichoderma* spp. *FEMS Microbiol Lett* 284(2): 231-236.

Newton WA, Snell EE. 1965. Formation and interrelationships of tryptophanase and tryptophan synthetases in *Escherichia coli*. *J Bacteriol* 89(2): 355-364.

Niinemets U, Kannaste A, Copolovici L. 2013. Quantitative patterns between plant volatile emissions induced by biotic stresses and the degree of damage. *Front Plant Sci* 4: 262.

Noh B, Lee SH, Kim HJ, Yi G, Shin EA, Lee M, Jung KJ, Doyle MR, Amasino RM, Noh YS. 2004. Divergent roles of a pair of homologous jumonji/zinc-finger-class transcription factor proteins in the regulation of Arabidopsis flowering time. *Plant Cell* 16(10): 2601-2613.

Nurnberger T, Brunner F. 2002. Innate immunity in plants and animals: emerging parallels between the recognition of general elicitors and pathogen-associated molecular patterns. *Curr Opin Plant Biol* 5(4): 318-324.

Nutricati E, Miceli A, Blando F, De Bellis L. 2006. Characterization of two *Arabidopsis thaliana glutathione* S-transferases. *Plant Cell Rep* 25(9): 997-1005.

O

Ohta M, Matsui K, Hiratsu K, Shinshi H, Ohme-Takagi M. 2001. Repression domains of class II ERF transcriptional repressors share an essential motif for active repression. *Plant Cell* 13(8): 1959-1968.

Ohtsubo Y, Nagata Y, Numata M, Tsuchikane K, Hosoyama A, Yamazoe A, Tsuda M, Fujita N, Kawai F. 2015. Complete genome sequence of a polypropylene glycol-degrading strain, *Microbacterium* sp. no. 7. *Genome Announc* 3(6).

Ottmann C, Luberacki B, Küfner I, Koch W, Brunner F, Weyand M, Mattinen L, Pirhonen M, Anderluh G, Seitz HU, et al. 2009. A common toxin fold mediates microbial attack and plant defense. *Proc Natl Acad Sci USA* 106(25): 10359-10364.

P

Paré PW, Tumlinson JH. 1999. Plant volatiles as a defense against insect herbivores. *Plant Physiol* 121: 325-331.

Park MJ, Kim MK, Kim HB, Im WT, Yi TH, Kim SY, Soung NK, Yang DC. 2008. *Microbacterium ginsengisoli* sp. nov., a beta-glucosidase-producing bacterium isolated from soil of a ginseng field. *Int J Syst Evol Microbiol* 58(Pt 2): 429-433.

Park YS, Dutta S, Ann M, Raaijmakers JM, Park K. 2015. Promotion of plant growth by *Pseudomonas fluorescens* strain SS101 via novel volatile organic compounds. *Biochem Biophys Res Commun* 461(2): 361-365.

Parker JE. 2003. Plant recognition of microbial patterns. *Trends Plant Sci* 8(6): 245-247.

Pel MJC, Pieterse CMJ. 2012. Microbial recognition and evasion of host immunity. *J Exp Bot*.

Peng M, Xie Q, Hu H, Hong K, Todd JD, Johnston AW, Li Y. 2012. Phylogenetic diversity of the dddP gene for dimethylsulfoniopropionate-dependent dimethyl sulfide synthesis in mangrove soils. *Can J Microbiol* 58(4): 523-530.

Peñuelas J, Asensio D, Tholl D, Wenke K, Rosenkranz M, Piechulla B, Schnitzler JP. 2014. Biogenic volatile emissions from the soil. *Plant Cell Environ* 37(8): 1866-1891.

Pessi G, Haas D. 2000. Transcriptional control of the hydrogen cyanide biosynthetic genes hcnABC by the anaerobic regulator ANR and the quorum-sensing regulators LasR and RhlR in *Pseudomonas aeruginosa*. *J Bacteriol* 182: 6940-6949.

Phillips M, Cataneo RN, Cummin AR, Gagliardi AJ, Gleeson K, Greenberg J, Maxfield RA, Rom WN. 2003. Detection of lung cancer with volatile markers in the breath. *Chest* 123(6): 2115-2123.

Piccoli P, Bottini R 2013. Terpene production by bacteria and its involvement in plant growth promotion, stress alleviation, and yield increase. *Molecular Microbial Ecology of the Rhizosphere*: John Wiley & Sons, Inc., 335-343.

Pichersky E, Noel JP, Dudareva N. 2006. Biosynthesis of plant volatiles: nature's diversity and ingenuity. *Science* 311(5762): 808-811.

Piechulla B, Degenhardt J. 2014. The emerging importance of microbial volatile organic compounds. *Plant Cell Environ* 37(4): 811-812.

Pierik R, Djakovic-Petrovic T, Keuskamp DH, de Wit M, Voesenek LA. 2009. Auxin and ethylene regulate elongation responses to neighbor proximity signals independent of gibberellin and della proteins in *Arabidopsis*. *Plant Physiol* 149(4): 1701-1712.

Pieterse CM, Dicke M. 2007. Plant interactions with microbes and insects: from molecular mechanisms to ecology. *Trends Plant Sci* 12(12): 564-569.

Pittard J. 1996. The various strategies within the TyrR regulon of *Escherichia coli* to modulate gene expression. *Genes to Cells* 1(8): 717-725.

Plett JM, Daguerre Y, Wittulsky S, Vayssieres A, Deveau A, Melton SJ, Kohler A, Morrell-Falvey JL, Brun A, Veneault-Fourrey C, et al. 2014. Effector MiSSP7 of the mutualistic fungus *Laccaria bicolor* stabilizes the *Populus* JAZ6 protein and represses jasmonic acid (JA) responsive genes. *Proc Natl Acad Sci USA* 111(22): 8299-8304.

Polizzi V, Adams A, Malysheva SV, De Saeger S, Van Peteghem C, Moretti A, Picco AM, De Kimpe N. 2012. Identification of volatile markers for indoor fungal growth and chemotaxonomic classification of *Aspergillus* species. *Fungal Biology* 116(9): 941-953.

Poorter H, Navas LM. 2003. Plant growth and competition at elevated CO_2: on winners, losers and functional groups. *New Phytol* 157: 175–198.

Q

Qian F, An L, Wang M, Li C, Li X. 2007. Isolation and characterization of a xanthan-degrading *Microbacterium* sp. strain XT11 from garden soil. *J Appl Microbiol* 102(5): 1362-1371.

R

Raaijmakers JM, Mazzola M. 2016. Soil immune responses. *Science* 352(6292): 1392-1393.

Raaijmakers JM, Paulitz TC, Steinberg C, Alabouvette C, Moënne-Loccoz Y. 2009. The rhizosphere: a playground and battlefield for soilborne pathogens and beneficial microorganisms. *Plant and Soil* 321(1): 341-361.

Rajini KS, Aparna P, Sasikala C, Ramana CV. 2011. Microbial metabolism of pyrazines. *Crit Rev Microbiol* 37: 99-112.

Ramachandran S, Christensen HEM, Ishimaru Y, Dong C-H, Chao-Ming W, Cleary AL, Chua N-H. 2000. Profilin plays a role in cell elongation, cell shape maintenance, and flowering in *Arabidopsis*. *Plant Physiol* 124(4): 1637-1647.

Rasmann S, Kollner TG, Degenhardt J, Hiltpold I, Toepfer S, Kuhlmann U, Gershenzon J, Turlings TC. 2005. Recruitment of entomopathogenic nematodes by insect-damaged maize roots. *Nature* 434(7034): 732-737.

Robles L, Stepanova A, Alonso J. 2013. Molecular mechanisms of ethylene–auxin interaction. *Mol Plant* 6(6): 1734-1737.

Romoli R, Papaleo MC, De Pascale D, Tutino ML, Michaud L, LoGiudice. 2014. GC-MS volatolomic approach to study the antimicrobial activity of the antarctic bacterium *Pseudoalteromonas* sp. TB41. *Metabolomics* 10: 42-51.

Roze LV, Beaudry RM, Linz JE. 2012. Analysis of volatile compounds emitted by filamentous fungi using solid-phase microextraction-gas chromatography/mass spectrometry. *Methods Mol Biol* 944: 133-142.

Roze LV, Chanda A, Laivenieks M, Beaudry RM, Artymovich KA, Koptina AV, Awad DW, Valeeva D, Jones AD, Linz JE. 2010. Volatile profiling reveals intracellular metabolic changes in *Aspergillus parasiticus*: veA regulates branched chain amino acid and ethanol metabolism. *BMC Biochem* 11: 33.

Runyon JB, Mescher MC, De Moraes CM. 2006. Volatile chemical cues guide host location and host selection by parasitic plants. *Science* 313(5795): 1964-1967.

Ruperti B, Cattivelli L, Pagni S, Ramina A. 2002. Ethylene-responsive genes are differentially regulated during abscission, organ senescence and wounding in peach (*Prunus persica*). *J Exp Bot* 53(368): 429-437.

Růžička K, Ljung K, Vanneste S, Podhorská R, Beeckman T, Friml J, Benková E. 2007. Ethylene regulates root growth through effects on auxin biosynthesis and transport-dependent auxin distribution. *The Plant Cell* 19(7): 2197-2212.

Ryu C-M 2015. Bacterial volatiles as airborne signals for plants and bacteria. In: Lugtenberg B ed. *Principles of Plant-Microbe Interactions: Microbes for Sustainable Agriculture*. Cham: Springer International Publishing, 53-61.

Ryu CM, Farag MA, Hu CH, Reddy MS, Kloepper JW, Pare PW. 2004. Bacterial volatiles induce systemic resistance in *Arabidopsis*. *Plant Physiol* 134(3): 1017-1026.

Ryu CM, Farag MA, Hu CH, Reddy MS, Wei HX, Pare PW, Kloepper JW. 2003. Bacterial volatiles promote growth in *Arabidopsis*. *Proc Natl Acad Sci USA* 100(8): 4927-4932.

S

Saitou N, Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4(4): 406-425.

Sanchez-Lopez AM, Baslam M, De Diego N, Munoz FJ, Bahaji A, Almagro G, Ricarte-Bermejo A, Garcia-Gomez P, Li J, Humplik JF, et al. 2016. Volatile compounds emitted by diverse phytopathogenic microorganisms

promote plant growth and flowering through cytokinin action. *Plant Cell Environ* [Epub ahead of print]. doi: 10.1111/pce.12759.

Scheible WR, Gonzalez-Fontes A, Lauerer M, Muller-Rober B, Caboche M, Stitt M. 1997. Nitrate acts as a signal to induce organic acid metabolism and repress starch metabolism in tobacco. *The Plant Cell* 9(5): 783-798.

Schenkel D, Lemfack MC, Piechulla B, Splivallo R. 2015. A meta-analysis approach for assessing the diversity and specificity of belowground root and microbial volatiles. *Front Plant Sci* 6: 707.

Schloss PD, Handelsman J. 2006. Toward a census of bacteria in soil. *PLoS Comput Biol* 2(7): e92.

Schmidt R, Cordovez V, de Boer W, Raaijmakers J, Garbeva P. 2015a. Volatile affairs in microbial interactions. *ISME J* 9(11): 2329-2335.

Schmidt R, Etalo DW, de Jager V, Gerards S, Zweers H, de Boer W, Garbeva P. 2015b. Microbial small talk: volatiles in fungal-bacterial interactions. *Front Microbiol* 6: 1495.

Schnürer J, Olsson J, Börjesson T. 1999. Fungal volatiles as indicators of food and feeds spoilage. *Fungal Genet Biol* 27(2): 209-217.

Schulz S, Dickschat JS. 2007. Bacterial volatiles: the smell of small organisms. *Nat Prod Rep* 24(4): 814-842.

Schulz S, Dickschat JS, Kunze B, Wagner-Dobler I, Diestel R, Sasse F. 2010. Biological activity of volatiles from marine and terrestrial bacteria. *Mar Drugs* 8: 2976-2987.

Seemann T. 2014. Prokka: rapid prokaryotic genome annotation. *Bioinformatics*.

Sethi S, Nanda R, Chakraborty T. 2013. Clinical application of volatile organic compound analysis for detecting infectious diseases. *Clin Microbiol Rev* 26(3): 462-475.

Seyedmousavi S, Mouton JW, Melchers WJG, Bruggemann RJM, Verweij PE. 2014. The role of azoles in the management of azole-resistant aspergillosis: from the bench to the bedside. *Drug Resist Updat* 17: 37-50.

Shao J, Xu Z, Zhang N, Shen Q, Zhang R. 2014. Contribution of indole-3-acetic acid in the plant growth promotion by the rhizospheric strain *Bacillus amyloliquefaciens* SQR9. *Biol Fertil soils* 51(3): 321-330.

Sharma P, Diene SM, Thibeaut S, Bittar F, Roux V, Gomez C, Reynaud-Gaubert M, Rolain JM. 2013. Phenotypic and genotypic properties of *Microbacterium yannicii*, a recently described multidrug resistant bacterium isolated from a lung transplanted patient with cystic fibrosis in France. *BMC Microbiol* 13: 97.

Shatalin K, Shatalina E, Mironov A, Nudler E. 2011. H2S: a universal defense against antibiotics in bacteria. *Science* 334(6058): 986-990.

Sheng XF, He LY, Zhou L, Shen YY. 2009. Characterization of *Microbacterium* sp. F10a and its role in polycyclic aromatic hydrocarbon removal in low-temperature soil. *Can J Microbiol* 55(5): 529-535.

Shimono M, Koga H, Akagi A, Hayashi N, Goto S, Sawada M, Kurihara T, Matsushita A, Sugano S, Jiang CJ, et al. 2012. Rice WRKY45 plays important roles in fungal and bacterial disease resistance. *Mol Plant Pathol* 13(1): 83-94.

Simms EL 1992. The evolution of plant resistance and correlated characters. In: Menken SBJ, Visser JH, Harrewijn P eds. *Proceedings of the 8th International Symposium on Insect-Plant Relationships*. Dordrecht: Springer Netherlands, 15-25.

Snel B, Bork P, Huynen MA. 2002. The identification of functional modules from the genomic association of genes. *Proc Natl Acad Sci USA* 99(9): 5890-5895.

Snelders E, Veld RA, Rijs A, Kema GHJ, Melchers WJG, Verweij PE. 2009. Possible environmental origin of resistance of *Aspergillus fumigatus* to medical triazoles. *Appl Environ Microbiol* 75: 4053-4057.

Song C, Schmidt R, de Jager V, Krzyzanowska D, Jongedijk E, Cankar K, Beekwilder J, van Veen A, de Boer W, van Veen JA, et al. 2015. Exploring the genomic traits of fungus-feeding bacterial genus *Collimonas*. *BMC Genomics* 16: 1103.

Song GC, Ryu C-M. 2013. Two volatile organic compounds trigger plant self-defense against a bacterial pathogen and a sucking insect in cucumber under open field conditions. *Int J Mol Sci* 14(5): 9803-9819.

Soto-Rodriguez SA, Cabanillas-Ramos J, Alcaraz U, Gomez-Gil B, Romalde JL. 2013. Identification and virulence of *Aeromonas dhakensis*, *Pseudomonas mosselii* and *Microbacterium paraoxydans* isolated from Nile tilapia, Oreochromis niloticus, cultivated in Mexico. *J Appl Microbiol* 115(3): 654-662.

Soylu EM, Yigitibas H, Tok FM, Soylu S, Kurt S, Baysal O, Kaya AD. 2005. Chemical composition and antifungal activity of the essential oil of *Artemisia annua* L. against foliar and soil-borne fungal pathogens. *J Plant Dis Protect* 112(3): 229–239.

Spadone JC, Takeoka G, Liardon R. 1990. Analytical investigation of Rio off-flavor in green coffee. *J Agric Food Chem* 38: 226-233.

Splivallo R, Ottonello S, Mello A, Karlovsky P. 2011. Truffle volatiles: from chemical ecology to aroma biosynthesis. *New Phytol* 189(3): 688-699.

Stackebrandt E, Rainey FA, Ward-Rainey NL. 1997. Proposal for a new hierarchic classification system, Actinobacteria classis nov. *Int J Syst Evol Microbiol* 47(2): 479-491.

Stamatakis A. 2014. RAxML Version 8: A tool for Phylogenetic Analysis and Post-Analysis of Large Phylogenies. *Bioinformatics*.

Stappen I, Wanner J, Tabanca N, Wedge DE, Ali A, Khan IA, Kaul VK, Lal B, Jaitak V, Gochev V, et al. 2014. Chemical composition and biological effects of *Artemisia maritima* and *Artemisia nilagirica* essential oils from wild plants of western Himalaya. *Planta Med* 80(13): 1079-1087.

Stotzky G, Schenck S. 1976. Volatile organic compounds and microorganisms. *CRC Crit Rev Microbiol* 4: 333-382.

Strehmel N, Hummel J, Erban A, Strassburg K, Kopka J. 2008. Retention index thresholds for compound matching in GC-MS metabolite profiling. *J Chromatogr B Analyt Technol Biomed Life Sci* 871(2): 182-190.

Strobel GA, Dirkse E, Sears J, Markworth C. 2001. Volatile antimicrobials from *Muscodor albus*, a novel endophytic fungus. *Microbiology* 147(Pt 11): 2943-2950.

T

Takamatsu S, Lin X, Nara A, Komatsu M, Cane DE, Ikeda H. 2011. Characterization of a silent sesquiterpenoid biosynthetic pathway in *Streptomyces avermitilis* controlling epi-isozizaene albaflavenone biosynthesis and isolation of a new oxidized epi-isozizaene metabolite. *Microb Biotechnol* 4: 184-191.

Tamura K, Nei M. 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol Biol Evol* 10(5): 1073-1095.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol Biol Evol* 30(12): 2725-2729.

Thines B, Katsir L, Melotto M, Niu Y, Mandaokar A, Liu G, Nomura K, He SY, Howe GA, Browse J. 2007. JAZ repressor proteins are targets of the SCFCOI1 complex during jasmonate signalling. *Nature* 448(7154): 661-665.

Tholl D. 2006. Terpene synthases and the regulation, diversity and biological roles of terpene metabolism. *Curr Opin Plant Biol* 9(3): 297-304.

Tikunov YM, Laptenok S, Hall RD, Bovy A, de Vos RC. 2012. MSClust: a tool for unsupervised mass spectra extraction of chromatography-mass spectrometry ion-wise aligned data. *Metabolomics* 8(4): 714-718.

Todd JD, Curson AR, Dupont CL, Nicholson P, Johnston AW. 2009. The dddP gene, encoding a novel enzyme that converts dimethylsulfoniopropionate into dimethyl sulfide, is widespread in ocean metagenomes and marine bacteria and also occurs in some Ascomycete fungi. *Environ Microbiol* 11(6): 1376-1385.

Todd JD, Curson AR, Kirkwood M, Sullivan MJ, Green RT, Johnston AW. 2011. DddQ, a novel, cupin-containing, dimethylsulfoniopropionate lyase in marine roseobacters and in uncultured marine bacteria. *Environ Microbiol* 13(2): 427-438.

Todd JD, Kirkwood M, Newton-Payne S, Johnston AW. 2012. DddW, a third DMSP lyase in a model *Roseobacter* marine bacterium, *Ruegeria pomeroyi* DSS-3. *ISME J* 6(1): 223-226.

Todd JD, Rogers R, Li YG, Wexler M, Bond PL, Sun L, Curson AR, Malin G, Steinke M, Johnston AW. 2007. Structural and regulatory genes required to make the gas dimethyl sulfide in bacteria. *Science* 315(5812): 666-669.

Trefz P, Koehler H, Klepik K, Moebius P, Reinhold P, Schubert JK, Miekisch W. 2013. Volatile emissions from *Mycobacterium avium* subsp. paratuberculosis mirror bacterial growth and enable distinction of different strains. *PLoS One* 8(10): e76868.

Trombetta D, Castelli F, Sarpietro MG, Venuti V, Cristani M, Daniele C. 2005. Mechanisms of antibacterial action of three monoterpenes. *Antimicrob Agents Chemother* 49: 2474-2478.

Tsitsigiannis DI, Keller NP. 2007. Oxylipins as developmental and host-fungal communication signals. *Trends Microbiol* 15(3): 109-118.

V

van Agtmaal M, van Os GJ, Hol WHG, Hundscheid MPJ, Runia WT, Hordijk CA, de Boer W. 2015. Legacy effects of anaerobic soil disinfestation on soil bacterial community composition and production of pathogen-suppressing volatiles. *Front Microbiol* 6: 701.

van de Mortel JE, de Vos RC, Dekkers E, Pineda A, Guillod L, Bouwmeester K, van Loon JJ, Dicke M, Raaijmakers JM. 2012. Metabolic and transcriptomic changes induced in *Arabidopsis* by the rhizobacterium *Pseudomonas fluorescens* SS101. *Plant Physiol* 160(4): 2173-2188.

van der Honing HS, Kieft H, Emons AM, Ketelaar T. 2012. *Arabidopsis* VILLIN2 and VILLIN3 are required for the generation of thick actin filament bundles and for directional organ growth. *Plant Physiol* 158(3): 1426-1438.

Venkataraman A, Rosenbaum MA, Werner JJ, Winans SC, Angenent LT. 2014. Metabolite transfer with the fermentation product 2,3-butanediol enhances virulence by *Pseudomonas aeruginosa*. *ISME J* 8: 1210-1220.

Verhulst NO, Beijleveld H, Knols BG, Takken W, Schraa G, Bouwmeester HJ, Smallegange RC. 2009. Cultured skin microbiota attracts malaria mosquitoes. *Malar J* 8: 302.

Vespermann A, Kai M, Piechulla B. 2007. Rhizobacterial volatiles affect the growth of fungi and *Arabidopsis thaliana*. *Appl Environ Microbiol* 73(17): 5639-5641.

W

Wagner U, Edwards R, Dixon DP, Mauch F. 2002. Probing the diversity of the arabidopsis glutathione S-transferase gene family. *Plant Molecular Biology* 49(5): 515-532.

Wan M, Li G, Zhang J, Jiang D, Huang H-C. 2008. Effect of volatile substances of *Streptomyces platensis* F-1 on control of plant fungal diseases. *Biol Control* 46(3): 552-559.

Wang Z, Wang C, Li F, Li Z, Chen M, Wang Y, Qiao X, Zhang H. 2013. Fumigant activity of volatiles from *Streptomyces alboflavus* TD-1 against *Fusarium moniliforme* Sheldon. *J Microbiol* 51(4): 477-483.

War AR, Paulraj MG, Ahmad T, Buhroo AA, Hussain B, Ignacimuthu S, Sharma HC. 2012. Mechanisms of plant defense against insect herbivores. *Plant Signal Behav* 7(10): 1306-1320.

Watve MG, Tickoo R, Jog MM, Bhole BD. 2001. How many antibiotics are produced by the genus *Streptomyces*? *Arch Microbiol* 176(5): 386-390.

Weber T, Blin K, Duddela S, Krug D, Kim HU, Bruccoleri R, Lee SY, Fischbach MA, Müller R, Wohlleben W, et al. 2015. antiSMASH 3.0 - a comprehensive resource for the genome mining of biosynthetic gene clusters. *Nucleic Acids Res* 43(Web Server issue): W237-W243.

Weise T, Kai M, Gummesson A, Troeger A, von Reuss S, Piepenborn S. 2012. Volatile organic compounds produced by the phytopathogenic bacterium *Xanthomonas campestris* pv. *vesicatoria* 85-10. *Beilstein J Org Chem* 8: 579-596.

Weisskopf L. 2013. *The potential of bacterial volatiles for crop protection against phytopathogenic fungi*: Badajoz: Formatex Research Center.

Weller DM, Raaijmakers JM, Gardener BB, Thomashow LS. 2002. Microbial populations responsible for specific soil suppressiveness to plant pathogens. *Annu Rev Phytopathol* 40: 309-348.

Wheatley RE. 2002. The consequences of volatile organic compound mediated bacterial and fungal interactions. *Antonie Van Leeuwenhoek* 81(1-4): 357-364.

Wheatley RE 2008. The role of soil microbial volatile products in community functional interactions. In: Karlovsky P ed. *Secondary Metabolites in Soil Ecology*. Berlin, Heidelberg: Springer Berlin Heidelberg, 269-288.

Wheatley RE, Millar SE, Griffiths DW. 1996. The production of volatile organic compounds during nitrogen transformations in soils. *Plant and Soil* 181(1): 163-167.

Wilkins K, Scholler C. 2009. Volatalie organic metabolites from selected *Streptomyces* strains. *Actinomycetologica* 23: 27-33.

Wu M, Eisen JA. 2008. A simple, fast, and accurate method of phylogenomic inference. *Genome Biol* 9(10): 1-11.

Wu Y, Yuan J, E Y, Raza W, Shen Q, Huang Q. 2015. Effects of volatile organic compounds from *Streptomyces albulus* NJZJSA2 on growth of two fungal pathogens. *J Basic Microbiol* 54: 1-14.

Y

Yamada Y, Kuzuyama T, Komatsu M, Shin-Ya K, Omura S, Cane DE, Ikeda H. 2015. Terpene synthases are widely distributed in bacteria. *Proc Natl Acad Sci USA* 112(3): 857-862.

Yang F, Yang L, Guo X, Wang X, Li L, Liu Z, Wang W, Li X. 2014. Production and purification of a novel xanthan lyase from a xanthan-degrading *Microbacterium* sp. strain XT11. *The Scientific World J* 2014: 8.

Young IM, Crawford JW, Nunan N, Otten W, Spiers A 2008. Microbial distribution in soils: physics and scaling. 81-121.

Z

Zagotta MT, Hicks KA, Jacobs CI, Young JC, Hangarter RP, Meeks-Wagner DR. 1996. The *Arabidopsis ELF3* gene regulates vegetative photomorphogenesis and the photoperiodic induction of flowering. *Plant J* 10(4): 691-702.

Zhang H, Kim MS, Krishnamachari V, Payton P, Sun Y, Grimson M, Farag MA, Ryu CM, Allen R, Melo IS, et al. 2007. Rhizobacterial volatile emissions regulate auxin homeostasis and cell expansion in *Arabidopsis*. *Planta* 226(4): 839-851.

Zhang J. 1990. *Microbial Taxonomy*. Shanghai: Press of Fudan University.

Zoller HF, Clark WM. 1921. The production of volatile fatty acids by bacteria of the dysentery group. *J Gen Physiol* 3: 325-330.

Summary

Plants and microorganisms are constantly engaged in highly dynamic interactions both above- and belowground. Several of these interactions are mediated by volatile organic compounds (VOCs), small carbon-based compounds with high vapor pressure at ambient temperature. In the rhizosphere, VOCs have an advantage in intra- and interorganismal signaling since they can diffuse through soil pores over longer distances than other metabolites and are not dependent on water availability. The research described in this PhD thesis explored how beneficial and pathogenic microorganisms that live in the rhizosphere and endosphere modulate plant growth, development and resistance *via* the production of VOCs. *In vitro* and *in vivo* bioassays as well as different 'omic' approaches, such as volatomics, transcriptomics and genomics, were employed to investigate underlying mechanisms of VOC-mediated microbe-microbe and microbe-plant interactions.

To investigate the diversity and functions of microbial VOCs, a disease-suppressive soil was used as the source of the VOC-producing microorganisms. Previous metagenomics studies reported Actinobacteria, in particular *Streptomyces* and *Microbacterium* species, as the most abundant bacterial genera found in a soil naturally suppressive to the fungal root pathogen *Rhizoctonia solani*. VOCs of several *Streptomyces* isolates inhibited hyphal growth of *R. solani* and in addition, promoted plant growth. Coupling the *Streptomyces* VOC profiles with their effects on fungal growth pinpointed methyl 2-methylpentanoate and 1,3,5-trichloro-2-methoxy benzene as antifungal VOCs. Also *Microbacterium* isolates showed VOC-mediated antifungal activity and plant growth promotion. VOC profiling of *Microbacterium* sp. EC8 revealed several sulfur-containing compounds and ketones such as dimethyl disulfide, trimethyl trisulfide and 3,3,6-trimethylhepta-1,5-dien-4-one (also known as Artemisia ketone). Genome analysis of strain EC8 revealed genes involved in sulfur metabolism. Resolving the role of the identified compounds and genes in VOC-mediated plant growth promotion and induced resistance will be subject of future studies. VOC-mediated chemical warfare underground has been proposed as a key mechanism of natural disease-suppressive soils. The results presented in this thesis indeed point in that direction. However, to more conclusively determine the role of the identified Actinobacterial VOCs in soil suppressiveness to *R. solani*, it will be important to demonstrate that the fungicidal VOCs are actually produced *in situ* at the right place and at sufficient concentrations to suppress plant infection by the pathogenic fungus.

In agriculture, VOCs and VOC-producing microorganisms provide a potential alternative to the use of pesticides to protect plants and to improve crop production. In the past decades, several *in vitro* studies have described the effects of microbial VOCs on other

(micro)organisms. However, little is still known on the potential of VOCs in large-scale agriculture and horticulture. The results described in this thesis show that VOCs from *Microbacterium* sp. EC8 stimulate the growth of *Arabidopsis*, lettuce and tomato, but do not control damping-off disease of lettuce caused by *R. solani*. Significant biomass increases were also observed for plants exposed only shortly to the bacterial VOCs prior to transplantation of the seedlings to soil. These results indicate that VOCs from strain EC8 can prime plants for growth promotion without direct contact and prolonged colonization. Furthermore, the induction of the plant growth-promoting effects appeared to be plant tissue specific. Root exposure to the bacterial VOCs led to a significant increase in plant biomass whereas shoot exposure did not result in significant biomass increase of lettuce and tomato seedlings. Genome-wide transcriptome analysis of *Arabidopsis* seedlings exposed to VOCs from this bacterium showed an up-regulation of genes involved in sulfur and nitrogen metabolism and in ethylene and jasmonic acid signaling. These results suggest that the blend of VOCs of strain EC8 favors, in part, the plant's assimilation of sulfate and nitrogen, essential nutrients for plant growth, development and also resistance.

Similar to beneficial microorganisms, plant pathogenic microorganisms have also evolved strategies to modulate growth and defense of their hosts. For instance, compounds secreted by pathogens may suppress or interfere with plant defense. In this thesis I show that *R. solani* produces an array of VOCs that promote growth, accelerate development, change VOC emission and reduce insect resistance of plants. Plant growth-promoting effects induced by the fungal VOCs were not transgenerational. Genome-wide transcriptome analysis of *Arabidopsis* seedlings revealed that exposure to fungal VOCs caused up-regulation of genes involved in auxin signaling, but down-regulation of genes involved in ethylene and jasmonic acid signaling. These findings suggest that this soil-borne pathogen uses VOCs to predispose plants for infection by stimulating lateral root formation and enhancing root biomass while suppressing defense mechanisms. Alternatively, upon perception of VOCs from soil-borne pathogens, plants may invest in root biomass while minimizing investments in defense, a trade-off that helps them to speed up growth and reproduction and to survive pathogen attack.

In conclusion, the research presented in this thesis shows that both plants and microorganisms engage *via* VOCs in long-distance interactions and that beneficial and pathogenic soil microorganisms can alter plant physiology in different ways. Here, I provided a first step in identifying microbial genes involved in the regulation of biologically active VOCs as well as candidate plant genes involved in VOC perception and signal

transduction. How plants sense and differentiate among VOCs from beneficial and pathogenic soil microorganisms will be an intriguing subject for future studies.

Samenvatting

Tussen planten en micro-organismen vinden continu uiterst dynamische interacties plaats, zowel boven- als ondergronds. Diverse interacties worden gereguleerd door vluchtige organische stoffen ('volatile organic compounds' of VOCs), kleine koolstofverbindingen met hoge dampdruk bij kamertemperatuur. Voor het afgeven van signalen in de rhizosfeer tussen organismen, hebben VOCs het voordeel dat ze zich over langere afstanden kunnen verspreiden dan andere metabolieten, terwijl ze niet afhankelijk zijn van de aanwezigheid van water. In dit proefschrift is onderzocht hoe nuttige en ziekteverwekkende micro-organismen die in de rhizosfeer en endosfeer leven, plantengroei, -ontwikkeling en resistentie kunnen moduleren *via* de productie van VOCs. Om onderliggende mechanismen van VOC-gemoduleerde interacties tussen microben en planten en tussen microben onderling te kunnen onderzoeken, zijn naast *in vitro* en *in vivo* bioassays, verschillende 'omica' benaderingen gebruikt zoals volatomica, transcriptomica en genomica.

Om de verscheidenheid en functies van microbiële VOCs te bestuderen, is een ziektewerende grond gebruikt als bron van de VOC-producerende micro-organismen. Uit eerdere studies was gebleken dat Actinobacteria, met name de *Streptomyces* en *Microbacterium* soorten, het meest talrijk waren in een bodem met een natuurlijke onderdrukkende werking tegen de ziekteverwekker *Rhizoctonia solani,* een schimmel die plantenwortels aantast. VOCs van verschillende *Streptomyces* isolaten remden de groei van de hyfen van *R. solani* en stimuleerden daarnaast ook plantengroei. Het combineren van de *Streptomyces* VOC profielen met hun effect op schimmelgroei resulteerde in de identificatie van methyl 2-methylvaleraat en 1,3,5-trichloor-2-methoxy benzeen als VOCs met anti-schimmel activiteit. Ook *Microbacterium* isolaten stimuleerden plantengroei en remden schimmelgroei via VOCs. VOC-profilering van *Microbacterium* sp. EC8 onthulde verschillende zwavelhoudende stoffen en ketonen zoals dimethyl disulfaat, trimethyl trisulfaat en 3,3,6-trimethylhepta-1,5-dien-4-one (ook bekend als Artemisia keton). Genoomanalyse van de EC8 stam onthulde de genen die een rol spelen in zwavel metabolisme. Toekomstig onderzoek zal moeten uitwijzen welke rol de geïdentificeerde stoffen en genen spelen bij VOC-gemoduleerde plantgroeistimulatie en geïnduceerde resistentie. VOC-gereguleerde chemische oorlogsvoering ondergronds wordt gezien als een sleutelmechanisme van natuurlijk ziektewerende gronden. De resultaten die in dit proefschrift worden gepresenteerd, wijzen inderdaad in die richting. Echter, in hoeverre de geïdentificeerde VOCs daadwerkelijk een sleutelrol spelen in de ziektewerende eigenschappen van gronden tegen *R. solani* vereist vervolgonderzoek waarin aangetoond moet worden dat de VOCs die *in vitro* schimmelgroei remmen inderdaad ook *in situ* op de

juiste plek en in voldoende concentratie worden geproduceerd om infectie van de plant door de ziekteverwekkende schimmel te onderdrukken.

In de landbouw bieden VOCs en VOC-producerende micro-organismen een potentieel alternatief voor het gebruik van pesticiden om planten te beschermen en gewasproductie te verbeteren. In de afgelopen decennia hebben verschillende studies de *in vitro* effecten van microbiële VOCs op andere (micro)organismen beschreven. Echter, tot nu toe is er weinig bekend over het potentieel van VOCs in de grootschalige land- en tuinbouw. De onderzoeksresultaten die in dit proefschrift staan beschreven, laten zien dat VOCs van *Microbacterium* sp. EC8 de groei van *Arabidopsis*, sla en tomaat stimuleren, maar de door *R. solani* veroorzaakte 'damping-off' ziekte (omvalziekte) in sla niet kunnen voorkomen. Ook bij planten die als zaailing voor aanplant in de bodem slechts kort waren blootgesteld aan de bacteriële VOCs, werd een significante toename in plantengroei waargenomen. Deze resultaten wijzen er op dat tijdens een korte blootstelling aan VOCs van *Microbacterium* sp. EC8 de groeistimulatie reeds geïnitieerd wordt en dat direct contact en langdurige kolonisatie door de VOCs-producerende bacteriën niet nodig is. Daarnaast bleken de stimulerende effecten op plantengroei te variëren tussen specifieke typen plantenweefsel. De blootstelling van het wortelstelsel aan de bacteriële VOCs leidde tot een hogere plant biomassa, terwijl bij blootstelling van de bladeren de biomassa van sla en tomaat zaadlingen niet significant hoger was. Genoom-brede transcriptomica analyse van *Arabidopsis* zaailingen die aan bacteriële VOCs waren blootgesteld, liet een verhoogde expressie zien van genen betrokken bij zwavel en stikstof metabolisme, en bij de signaaltransductie van ethyleen en jasmonzuur. Deze resultaten suggereren dat de mix van VOCs geproduceerd door *Microbacterium* EC8 gedeeltelijk ten goede komt aan sulfaat en stikstof metabolisme in de plant en daarmee een effect heeft op plantengroei, -ontwikkeling en–resistentie.

De resultaten beschreven in dit proefschrift laten zien dat ook plantenziekte verwekkende micro-organismen strategieën hebben ontwikkeld om de groei en verdediging van hun gastheren te moduleren. Stoffen die door pathogenen worden uitgescheiden kunnen bijvoorbeeld de verdediging van de plant onderdrukken of beïnvloeden. In dit proefschrift werd aangetoond dat de bodemschimmel *R solani* een scala aan VOCs produceert die plantengroei stimuleren, ontwikkeling van de plant versnellen, VOC afgifte door de plant zelf veranderen en ook weerstand van de plant tegen insectenvraat verminderen. Deze plantengroeistmulerende effecten waren niet transgenerationeel. Uit genoom-brede transcriptomica analyse van Arabidopsis zaailingen bleek dat blootstelling aan de schimmel-VOCs leidde tot verhoogde expressie van genen die betrokken zijn bij de signaaltransductie van auxine, maar de expressie van genen die

betrokken zijn bij signaaltransductie van ethyleen en jasmonzuur verlaagde. Deze bevindingen wijzen er op dat deze plantpathogene bodemschimmel VOCs gebruikt om planten meer vatbaar te maken voor infectie door de vorming van zijwortels te stimuleren en de wortelbiomassa te vergroten, terwijl de afweermechanismen worden onderdrukt. Het is evenwel ook mogelijk dat planten, wanneer ze blootgesteld worden aan VOCs van bodemgebonden pathogenen, meer investeren in wortelbiomassa terwijl ze de verdediging minimaliseren; dit is een uitruil ('trade-off') die hen helpt groei en voortplanting te versnellen om zo een aanval van pathogenen voor te zijn of te overleven.

Concluderend laat het onderzoek beschreven in dit proefschrift zien dat zowel planten als micro-organismen langeafstandsinteracties onderhouden *via* VOCs, en dat nuttige en ziekteverwekkende bodemmicro-organismen de fysiologie van de plant op verschillende wijzen kunnen veranderen. Mijn onderzoek is een eerste stap in de identificatie van enerzijds microbiële genen die een rol spelen bij de regulatie van biologisch actieve VOCs en anderzijds van kandidaat plantengenen die een rol spelen bij de perceptie van VOCs en de daaropvolgende signaaltransductie. Hoe planten VOCs waarnemen en of zij onderscheid kunnen maken tussen VOCs van nuttige en ziekteverwekkende bodemmicro-organismen wordt een intrigerend onderwerp voor toekomstig onderzoek.

Resumo

Plantas e microrganismos interagem dinamicamente, tanto acima quanto abaixo do solo. Várias destas interações são mediadas por compostos orgânicos voláteis (COVs), pequenos compostos à base de carbono com alta pressão de vapor sob temperatura ambiente. Na rizosfera, COVs têm uma vantagem na sinalização intra e inter organismos, uma vez que podem se difundir através dos poros do solo sobre distâncias maiores do que outros metabólitos e não dependem da disponibilidade de água para se difundir. A pesquisa descrita nesta tese de doutorado explorou como microrganismos benéficos e patogênicos que vivem na rizosfera e endosfera modulam o crescimento, o desenvolvimento e a resistência das plantas através da produção de COVs. Experimentos *in vitro* e *in vivo*, assim como diferentes abordagens ômicas, tais como volatômica, transcriptômica e genômica, foram empregadas para investigar os mecanismos envolvidos nas interações mediadas por COVs entre microrganismos e entre microrganismos e plantas.

Para investigar a diversidade e as funções dos COVs produzidos por microrganismos, um solo supressivo foi usado como fonte dos microrganismos produtores de COVs. Estudos metagenômicos anteriores relataram actinobactérias e, em particular, *Streptomyces* e *Microbacterium*, como os gêneros de bactérias mais abundantes encontrados em um solo naturalmente supressivo ao fungo patogênico *Rhizoctonia solani*. COVs produzidos por vários isolados de *Streptomyces* inibiram o crescimento de hifas de *R. solani*, assim como promoveram o crescimento das plantas. A associação do perfil de COVs produzidos pelos isolados de *Streptomyces* aos seus efeitos no crescimento do fungo identificaram metil 2-metilpentanoato e 1,3,5-tricloro-2-metoxi benzeno como COVs com ação antifungíca. Isolados de *Microbacterium* também promoveram o crescimento de plantas e apresentaram atividade antifúngica através de COVs. O perfil de COVs produzidos por *Microbacterium* sp. EC8 revelou vários compostos contendo enxofre e cetonas, como dimetil dissulfido, dimetil trissulfido e 3,3,6-trimetil-1,5-dien-4-ona (conhecido também como Artemísia cetona). A análise do genoma do isolado EC8 revelou vários genes envolvidos no metabolismo de enxofre. A resolução do papel dos compostos e genes identificados na promoção de crescimento e resistência de plantas mediados por COVs será objeto de estudos futuros. Uma “guerra química subterrânea” mediada por COVs tem sido proposta como um mecanismo chave na supressão de doenças em solos. Os resultados apresentados nesta tese, de fato, apontam nessa direção. No entanto, as funções dos COVs produzidos pelos isolados de actinobactérias em solo supressivo ao fungo *R. solani* ainda são desconhecidas. Será importante demonstrar que os COVs fungicidas são, na verdade, produzidos no solo em concentrações suficientes para suprimir a infecção da planta pelo fungo patogênico.

Na agricultura, COVs e microrganismos produtores de COVs fornecem uma alternativa para o uso de pesticidas para proteger plantas e para melhorar a produção agrícola. Nas últimas décadas, vários estudos *in vitro* descreveram os efeitos de COVs produzidos por microrganismos em outros macro- e microrganismos. No entanto, até agora, pouco se sabe sobre o potencial dos COVs para agricultura e horticultura em grande escala. Os resultados descritos nesta tese demonstram que COVs produzidos por *Microbacterium* sp. EC8 estimulam o crescimento de *Arabidopsis*, alface e tomate. No entanto, esses COVs não reduzem os sintomas da doença de tombamento ('damping-off') de plântulas de alface causada por *R. solani*. Aumentos significativos da biomassa também foram observados após uma prévia exposição de plântulas aos COVs produzidos pela bactéria antes do transplante para o solo. Estes resultados indicam que COVs produzidos pelo isolado EC8 podem preparar as plantas para a promoção do crescimento, sem contato direto e colonização prolongada. Além disso, a indução dos efeitos promotores do crescimento de plantas parece ser específica ao tecido vegetal. Raízes de alface e tomate expostas aos COVs produzidos pela bactéria apresentaram um aumento significativo da biomassa, ao contrário das partes aéreas. A análise do transcriptoma de plântulas de *Arabidopsis* expostas a COVs da bactéria mostrou um aumento na expressão de genes envolvidos no metabolismo do enxofre e nitrogênio e na sinalização de etileno e ácido jasmônico. Estes resultados sugerem que COVs produzidos pelo isolado EC8 favorecem, em parte, a assimilação de sulfato e nitrogênio, nutrientes essenciais para o crescimento, desenvolvimento e também resistência de plantas.

Assim como para microrganismos benéficos, as estratégias de microrganismos patogênicos de plantas também evoluíram para modular o crescimento e a defesa dos seus hospedeiros. Por exemplo, os compostos secretados por patógenos podem suprimir ou interferir com a defesa da planta. Mostra-se nesta tese que *R. solani* produz uma variedade de COVs que promovem o crescimento, aceleram o desenvolvimento, alteram a emissão de COVs e reduzem a resistência a insetos de plantas. Efeitos promotores do crescimento induzidos por COVs de fungos não foram transmitidos para a próxima geração de plantas. A análise de transcriptoma de plântulas de *Arabidopsis* revelou que a exposição a COVs fúngicos causou um aumento na expressão de genes envolvidos na sinalização de auxina, mas uma diminuição na expressão de genes envolvidos na sinalização de etileno e ácido jasmônico. Estas descobertas sugerem que este patógeno de solo usa COVs para predispor plantas à infecção, estimulando a formação de raízes laterais e aumentando a biomassa radicular, enquanto suprime os mecanismos de defesa. Alternativamente, após a percepção de COVs de patógenos de solo, as plantas podem investir em biomassa radicular, minimizando investimentos em mecanismos de defesa,

uma troca (‘trade off’) que favorece o aceleramento do crescimento e reprodução de plantas, facilitando a sobrevivência ao ataque de patógenos.

Em conclusão, a pesquisa apresentada nesta tese mostra que ambos plantas e microrganismos interagem a longa distância através de COVs e que microrganismos benéficos e patogênicos de solo podem alterar a fisiologia da planta de diferentes maneiras. Nesta tese, foi apresentado um primeiro passo na identificação de genes microbianos envolvidos na regulação de COVs biologicamente ativos, assim como genes de plantas envolvidos na percepção e transdução de sinal de COVs. O mecanismo pelo qual as plantas detectam e diferenciam os COVs de microrganismos benéficos daqueles de microrganismos patogênicos será um tema intrigante para pesquisas futuras.

Acknowledgments

In the past 5 years I have been supported, encouraged and inspired by a number of people. My thanks go to all of you for being part of my PhD journey and for contributing not only to my academic but also personal development.

I would like to start by thanking my promotors and co-promotor. Jos, thank you very much for being so open and enthusiastic when we first talked about writing my PhD project proposal for the EPS Talent Programme. Thanks for your guidance, advices and optimism even with the most challenging experiments. During the past 5 years, I have learned a lot from you and I'm happy we can continue working together for the next 3 years. *Francine*, thank you for your support and especially for the quick feedback on the final versions of this thesis. I very much enjoyed reviewing the thesis chapters and discussing the propositions with you. *Victor*, thanks for your help with experiments, data analyses and manuscripts. I'm very happy for being your first PhD student. We made a great team despite our different work strategies. Gracias por estar siempre ahí!

A number of people contributed to my PhD journey even before it started. I would like to express my sincere appreciation to *Gert Kema*, *Ioannis Stergiopoulos* and *Pierre de Wit* for encouraging me to join the EPS Talent Programme. I am also thankful to *Ton Bisseling* for guiding and motivating me to stay in the programme. Thanks for bringing me into contact with Jos. I really appreciate that!

My special thanks go to my dear paranymphs and friends, *Ruth* and *Chunxu*. *C.*, you are one of the most constant figures in my history in Wageningen, where so many people come and go. I'm very happy you decided to extend your stay in the low lands. I'm also looking forward to our collaboration in the Back to the Roots project. *Ruthinha*, although we only started interacting halfway my PhD when we moved to NIOO, it feels like we have always been together on this journey. Thanks for sharing not only an office, coffee breaks, sports, moving, shopping, and the 'Japan' passion, but especially for sharing the PhD experiences. Muchas gracias! Soon it will be your turn to enjoy the feeling of such an accomplishment. Como siempre te digo: fuerza!

I also would like to thank my external supervisor *Gilles van Wezel*. *Gilles*, thank you for introducing me to the smelly and colorful world of actinomycetes. Also thank you for your help and suggestions in the beginning of my PhD project and for taking the time to discuss paths for my future career at the end of my PhD. Thanks also to *Hua Zhu* for showing me how to isolate actinomycetes. I still remember us walking back from the lab late in the evening after a whole day isolating bacteria. I was so happy to go back to Wageningen with my first isolates! I hope we will meet again soon.

I'm very grateful to all the collaborators I had during my PhD project. The experimental work presented in this thesis would not have been possible without their

support. *Liesje*, thank you for your help with designing and setting up the experiments for the chapter on fungal VOCs. I enjoyed working with you and I really appreciate your support and encouragement after so many trials with the y-tube experiments, which unfortunately did not make it to this thesis.

Collection of VOCs was new and somewhat challenging but I'm lucky to have had the help of several people. Special thanks go to my 'volatile girls' Dani and Kay. *Dani*, my friend and collaborator, thank you for taking the time to show me how to collect and analyze plant VOCs. I will never forget all your effort and enthusiasm even when I came to you with tiny amounts of *Arabidopsis* for the VOC collection or when the machine stopped working. Kay, my student, my collaborator and my friend, I'm very happy you were part of my project and I'm even happier that you have decided to continue with your PhD with part of my project. Thanks for helping with the VOC collection and the insect assays. Thanks also to *Desalegn*, *Teris van Beek*, *Hans Zweers*, *Kees Hordijk*, *Roland Mumm* and *Paolina Garbeva* for the assistance with the GC-MS and VOC analyses.

Thanks to *Victor de Jager* for helping with the genome, transcriptome and other bioinformatics-related analyses. Thanks to *Ronald Pierik*, for helping with the ethylene measurements and for providing the *Arabidopsis* mutant seeds.

Part of my PhD project was carried out in close collaboration with industry, which I enjoyed very much. Thanks to *Inge Hanssen, Filip Coppens, Hans Jacobs*, and *Hervé Dupré* for being enthusiastic about my research and for providing the opportunity to explore a more applied side of my PhD project.

Thanks to *Saskia*, *Paulien*, *Maria*, *Roos*, *Roel*, *Tanja*, *Gregor* and *Eke* for the assistance in the labs and in the greenhouses.

My PhD journey has been shared between two nice departments at WUR and at NIOO, where I interacted with many people. Thanks to *Menno*, *Judith*, *Xu*, *Valerie*, *Bilal*, *Daniela*, *Ester*, *Ali* and to all the other members of the Laboratory of Phytopathology. I have learned a lot from you from the start of my MSc to the end of my PhD. My special thanks go to my (current and former) office mates at NIOO. Thank you for the support and patience during the last (stressful) steps of my PhD. *Ruth G.*, *Juan*, *Ruth S.*, *Paolo*, *Julia*, *Yiying*, *Késia*, *Manoeli*, *Leonardo*, *Márcio,* and *Ronny*, I really enjoyed the nice atmosphere in our office and I could not have asked for better company! Thanks also to *Irene*, *Desalegn*, *Olaf*, *Thiago*, *Ohana*, *Nurmi*, *Afnan*, *Maurício*, *Adam*, *Nori*, *Kristin*, *Adrian*, *Lucas*, *Matheus*, *Marcelo*, *Sang Yoon* and the other ME members for all the fun during coffee breaks, borrels and dinners. Thanks to my MSc and BSc students, *Mariana*, *Kay* and *Sharella* for making part of my project.

A big thank you goes to *Frank, Kay*, *Chunxu*, *Nurmi*, *Je-Seung*, *Ruth G.*, *Ohana* and *Afnan* for helping me with proofreading this thesis.

Thanks to my dear 'cabrones' who shared not only my professional but also my personal development through these years. *Ruth*, *Chunxu* and *Vic*, thank you so much for all the fun we had together! From the WUR to the NIOO days, we have shared so many stories, trips, dinners, and movie shootings! You certainly made my days more colorful here in the Netherlands. I'm also thankful to *Je-Seung*, *Kay*, *Wei*, *Cynara*, *Rafa*, *Mariana*, *Andrielli*, *Pieter*, *Loan*, *Anna G.* and *Tania*! Your friendship made it easier to feel at home in Wageningen. Some of you already left or will leave Wageningen soon but I hope we will meet again in the future.

Finally, I would like to thank my Brazilian and Dutch families, for their never ending love, support and understanding. *Mãe*, *pai* e *Day*, obrigada pelo apoio e torcida à 10.000 km de distância. Esta conquista também pertence a vocês! Lieve *Frank*, heel erg bedankt! I can't thank you enough for all your support and guidance even before I arrived in the Netherlands. Thank you for introducing me the city of Life Sciences and for encouraging me with my academic journey. Most of all, thanks for all the care and patience with my long hours in the lab and work during our weekends at home. *Marion*, bedankt voor je voor constante zorg en ondersteuning. *Hans*, dankjewel voor het maken van mijn thesis omslag! Eerder ontwierp je mijn BSc en MSc, en nu ook mijn PhD omslag. Ik ben erg dankbaar!

Thank you!

About the author

Publications

Viviane Cordovez da Cunha was born on 10th December 1983 in Presidente Prudente (SP), Brazil. In 2008 she concluded her BSc degree in Biology at the State University of Maringá (UEM). After a short intermezzo in Italy, where she obtained the Italian nationality, she moved to the Netherlands. In the beginning of 2009 she started her MSc studies in Plant Biotechnology at Wageningen University. During her MSc thesis at the Laboratory of Phytopathology, she studied the molecular aspects of the interaction between tomato plants and the fungal pathogen *Cladosporium fulvum* as well as between banana plants and *Mycosphaerella fijiensis,* under the supervision of Prof. Dr Pierre de Wit and Dr Ioannis Stergiopoulos. After that, she concluded an internship at Plant Research International (PRI) under the supervision of Dr Gert Kema, where she studied the effects of mutations in the promoter region of *Mycosphaerella fijiensis* and *Aspergillus fumigatus* on the resistance to fungicides. At the end of her MSc studies she joined the MSc Talent Programme sponsored by the Graduate School Experimental Plant Sciences (EPS) and the Netherlands Organization for Scientific Research (NWO). In 2011 she was awarded a personal grant to carry out her PhD research under the supervision of Prof. Dr Jos Raaijmakers (Netherlands Institute of Ecology, NIOO-KNAW) and Prof. Dr Francine Govers (Wageningen University). The findings of this research project are described in this thesis. Since August 2016 she has been working as a postdoctoral researcher in the STW project 'Back to the Roots' together with Prof. Dr Jos Raaijmakers and Dr Victor Carrion at the Department of Microbial Ecology at the Netherlands Institute of Ecology.

Cordovez V, Schop S, Carrion, VJ, Jacobs H, Coppens F, Hanssen I, Raaijmakers JM. Exploring volatile organic compounds of *Microbacterium* for plant growth promotion and biocontrol. *(Chapter 5, to be submitted)*

Cordovez V, Carrion VJ, de Jager V, Raaijmakers JM. Genomic and functional analyses of rhizospheric and endophytic *Microbacterium* species. *(Chapter 4, to be submitted)*

Diaz-Trujillo C*, Chong P*, Cordovez V, Guzman M, de Wit PJ, Stergiopoulos I, Meijer HJG, Arango Isaza RE, Scalliet G, Sierotzki H, Peralta EL, Kema GHJ. A new resistance mechanism to azole fungicides in the fungal banana black Sigatoka pathogen *Pseudocercospora fijiensis* is driven by increased expression of *Pfcyp51* through multiple promotor repeats. *(submitted)*

Cordovez V, Mommer L, Moisan K, Lucas-Barbosa D, Pierik R, Mumm R, Carrion VJ, Raaijmakers JM. Volatiles from a soil-borne pathogenic fungus modulate the trade-off between plant growth and insect resistance. *(Chapter 6, submitted)*

Cheng X, Cordovez V, Etalo DW, van der Voort M, Raaijmakers JM. Role of the GacS/GacA two-component system in the regulation of volatile production by plant growth-promoting *Pseudomonas fluorescens* SBW25. *(submitted)*

Mesarich CH, Stergiopoulos I, Beenen HG, Cordovez V, Guo Y, Karimi Jashni M, Bradshaw RE, de Wit PJ. 2016. A conserved proline residue in Dothideomycete Avr4 effector proteins is required to trigger a Cf-4-dependent hypersensitive response. *Mol Plant Pathol* 17 (1): 84-95.

Cordovez V, Carrion VJ, Etalo DW, Mumm R, Zhu H, van Wezel GP, Raaijmakers JM. 2015. Diversity and functions of volatile organic compounds produced by *Streptomyces* from a disease-suppressive soil. *Front Microbiol* 6 (1081).

Schmidt R*, Cordovez V*, de Boer W, Raaijmakers J, Garbeva P. 2015. Volatile affairs in microbial interactions. *ISME J* 9: 2329-2335.

Stergiopoulos I, Cordovez V, Ökmen B, Beenen HG, Kema GHJ, de Wit. PJ. 2014. Positive selection and intragenic recombination contribute to high allelic diversity in effector genes of *Mycosphaerella fijiensis*, causal agent of the black leaf streak disease of banana. *Mol Plant Pathol* 15 (5): 447-460.

Leal AA, Mangolin CA, do Amaral Júnior AT, Gonçalves LSA, Scapim CA, Mott AS, Eloi IBO, Cordovez V, da Silva MFP. 2010. Efficiency of RAPD versus SSR markers for determining genetic diversity among popcorn lines. *Genet Mol Res* 9 (1): 9-18.

**first shared authorship*

Education statement

Education Statement of the Graduate School Experimental Plant Sciences

Issued to: Viviane Cordovez da Cunha
Date: 4 November 2016
Group: Laboratory of Phytopathology
University: Wageningen University & Research

1) Start-up phase	*date*
► First presentation of your project	
Title: The role of Actinobacteria in plant defense against fungal pathogens	May 04, 2012
► Writing or rewriting a project proposal	
Proposal for EPS MSc Talent Program, *title:* The role of Actinobacteria in plant defense against fungal pathogens	May-Jun 2011
► Writing a review or book chapter	
Title: Volatile affairs in microbial interactions, The ISME Journal (2015), 1-7. DOI: 10.1038/ismej.2015.42	Sep-Dec 2014
► MSc courses	
► Laboratory use of isotopes	
Subtotal Start-up Phase	*6.5 credits**

2) Scientific Exposure	*date*
► EPS PhD student days	
4th European Plant Science Retreat for PhD Students - John Innes Centre, Norwich, UK	Aug 15-17, 2012
EPS PhD student days - Amsterdam, NL	Nov 30, 2012
EPS Get2gether - Soest, NL	Jan 29-30, 2015
► EPS theme symposia	
EPS theme 2 symposium “Interactions between plants and biotic agents” - Wageningen University, NL	Feb 10, 2012
EPS theme 2 symposium “Interactions between plants and biotic agents” - Amsterdam University, NL	Feb 25, 2014
EPS Theme 3 Symposium: "Metabolism and Adaptation", Wageningen University, NL	Mar 11, 2014
► Lunteren days and other National Platforms	
Meeting 'Molecular Genetics' - Lunteren, NL	Oct 07, 2011
Meeting 'Molecular Genetics' - Lunteren, NL	Oct 04-05, 2012
Annual Meeting 'Experimental Plant Sciences' - Lunteren, NL	Apr 22-23, 2013
KKNVM/NVMM Scientific Spring Meeting - Arnhem, NL	Apr 15-16, 2014

► Seminars (series), workshops and symposia	
Invited seminars 2012 (Gilles van Wezel, Birgit Piechulla, Gabriele Berg)	2012
Invited seminars 2013 (Bertus Beaumont, Andrew Sugden, David Berry, Kathrin Riedel, Pieter Dorrestein, David M. Weller, Ross Mann)	2013
Invited seminars 2014 (Cristine Barreto, Gilles van Wezel, Isabelle Benoit, Laure Weiskopf, Hans Clevers, Eoin Brodie, Marco Kai)	2014
Invited seminars 2015 (Jeroen Jansen, Kevin Foster, Harald Gross, Laure Weiskopf, Lionel Dupuy, Jeroen S. Dickschat)	2015
Workshop on Microbial Volatiles - NIOO-KNAW Wageningen, NL	Apr 14, 2014
Farewell Symposium Prof. J.A. van Veen - Perspectives in Microbial Ecology - Wageningen, NL	Jun 27, 2014
Farewell Symposium Prof. Pierre de Wit	Jun 05, 2014
Inaugural speech Prof. Jos Raaijmakers	Nov 13, 2015
Inaugural speech Prof. Liesje Mommer	Feb 25, 2016
► Seminar plus	
► International symposia and congresses	
Actinobacteria whithin soils: capacities for mutualism, symbiosis and pathogenesis - Munster, Germany	Oct 25-28, 2012
ISME15 International Society of Microbial Ecology - Seoul, South Korea	Aug 24-29, 2014
10th International PGPR Workshop Liège, Belgium	Jun 16-19, 2015
► Presentations	
Poster: 4th European Plant Science Retreat for PhD Students - John Innes Centre, Norwich, UK	Aug 15, 2012
Oral: KNVM/NVMM Scientific Spring Meeting - Arnhem, NL	Apr 15, 2014
Oral: EPS Spring School Host-Microbe Interactomics, Wageningen, NL	Jun 03, 2014
Oral: ISME15 International Society of Microbial Ecology - Seoul, South Korea	Aug 28, 2014
Oral: Korea Research Institute of Bioscience & Biotechnology - Daejeon, South Korea	Aug 29, 2014
Oral: 10th International PGPR Workshop Liège, Belgium	Jun 17, 2015
► IAB interview	
► Excursions	
Korea Research Institute of Bioscience & Biotechnology - Daejeon, South Korea	Aug 29, 2014
DCM Fertilizer Company, Grobbendonk, Belgium	Jan 27, 2016
Subtotal Scientific Exposure	*18.0 credits**

3) In-Depth Studies	*date*
► **EPS courses or other PhD courses**	
Genomics Training Workshop - Institute for Genome Sciences, University of Maryland, Baltimore, USA University of Maryland School of Medicine	Mar 19-23, 2012
EPS Spring School Host-Microbe Interactomics, Wageningen, NL	Jun 02-04, 2014
The power of RNAseq, Wageningen, NL	Feb 10-12, 2016
► **Journal club**	
Literature discussion, Bacterial Ecology & Genomics Group of Phytopathology, WUR	2011-2014
Literature discussion, Microbial Ecology Department, NIOO-KNAW	2015-2016
► **Individual research training**	
Isolation of actinomycetes at Leiden University	2011-2012
Analysis of volatile compounds by GC-MS at Organic Chemistry Department, WUR	2014
Subtotal In-Depth Studies	*9.0 credits**

4) Personal development	*date*
► **Skill training courses**	
Dutch course	2011-2012
PhD Competence Assessment	Apr 16-18, 2013
Postdoc Career Development Initiative (PCDI) Retreat- Life Sciences	Mar 25-27, 2015
Last Stretch of the PhD Programme, Wageningen, NL	Dec 11, 2016
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
PhD representative at the Department of Microbial Ecology, NIOO-KNAW	2014-2016
PhD lunch meetings at the Department of Microbial Ecology, NIOO-KNAW	2014-2016
Subtotal Personal Development	*4.6 credits**

TOTAL NUMBER OF CREDIT POINTS*	38.1

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

** A credit represents a normative study load of 28 hours of study.*

The research described in this thesis was performed in the Laboratory of Phytopathology of Wageningen University (WUR) and the Department of Microbial Ecology at the Netherlands Institute of Ecology (NIOO-KNAW). This research was financially supported by the Netherlands Organization for Scientific Research (NWO).

This is NIOO-thesis number 133.

Cover and layout design by Hans Keeris and Viviane Cordovez
Printed by Uitgeverij BOXpress | proefscriftmaken.nl